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Evaluation of chemical mediators and cellular response during acute and chronic gut inflammatory response induced by dextran sodium sulfate in mice☆

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ABSTRACT

Inflammatory bowel disease (IBD) affects millions of people worldwide but its pathophysiology remains unclear. Therefore, experimental models of colitis have contributed crucially for the understanding of IBD, and also in the investigations for effective therapies. Herein we investigated the kinetics of inflammatory mediator production and cell infiltration during acute and chronic dextran sodium sulfate (DSS)-induced colitis. The induction phases with DSS were characterized by severe disease activity with massive colonic polymorphonuclear infiltration and increased levels of tumor necrosis factor-α (TNF-α), keratinocyte-derived chemokine (CXCL1/KC), interleukin (IL)-17 and vascular adhesion molecule-1 (VCAM-1). Interestingly, in the recovery periods, we found marked increase of anti-inflammatory mediators IL-10, IL-4, transforming growth factor-β (TGF-β) and cyclooxygenase 2 (COX-2) that seems be essential for the resolution of intestinal inflammation. Furthermore, nuclear factor κB (NFκB) and regulatory T cell marker forkhead box P3 (FoxP3) were increased gradually during experimental colitis, demonstrating a discrepant profile response and evident immune disbalance in the chronic phase of intestinal mucosal inflammation. Taken together, these results provide valuable information for studies on DSS-induced colitis and especially for the identification of biomarkers that predict disease course and possible therapeutic interventions.

1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD), and are characterized by an abnormal cell influx to the intestinal tissues and massive release of pro-inflammatory mediators [1]. These events are crucial for intestinal inflammation and may induce an atypical signaling pathway leading to de-regulation of intestinal homeostasis [2,3]. Besides the altered immune response driven by microbial factors in the enteric environment, a combination of many other factors such as genetic susceptibility and environmental conditions are thought to be necessary for clinical expression of the disease [2,4,5].

Animal models have greatly contributed to our understanding about the factors that influence IBD development and they also provide helpful tools for emergent novel therapeutic strategies [6]. One of the most common IBD-related models is the dextran sulfate sodium (DSS)-induced colitis. The administration of DSS polymers in drinking water induces acute colitis [7] and is useful for studying the involvement of innate immune mechanisms. Indeed, the great majority of published papers have used animal models in acute periods, but CD and UC are known as chronic pathologies. Therefore an important feature of this model is that the administration of repetitive cycles of DSS leads to chronic intestinal inflammation, which permits important observations about the adaptive immune system [8] and allows studies of mediators and cell Influx involved in the chronification process of IBD.

The inflammatory mediators responsible for the pathogenesis of DSS-induced colitis are of a heterogeneous nature, and several research groups have reported contrasting results, particularly for cell influx and protein expression. Such discrepant findings may at least in part be explained by the specific methodology used, different time points at which analyses were performed, mice strain susceptibility and/or DSS solution concentration. Furthermore, to the best of our knowledge very few studies aimed to clarify the kinetics of inflammatory events mediating acute and chronic phases of DSS-induced colitis.

In this context, the present study aimed to assess cellular influx and inflammatory markers during the acute and chronic phases of

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DSS-induced colitis in mice. Our results showed very interesting differences between the induction phase and the recovery period which may be valuable for future evolution of the DSS colitis model and, particularly, may provide strong evidence for the identification of biomarkers relevant for the prediction of disease course and the design of possible therapeutic interventions.

2. Materials and methods

2.1. Animals

Male BALB/c mice (eight to 10 weeks of age) were obtained from the Laboratório de Farmacologia Experimental (LAFEX), Universidade Federal de Santa Catarina (UFSC, Florianópolis, SC, Brazil), and housed in collective cages at 22 ± 1 °C under a 12-h light/dark cycle (lights on at 07:00 h) with free access to laboratory chow and tap water. Experiments were performed during the light phase of the cycle. The experimental procedures were previously approved by the UFSC's Committee on the Ethical Use of Animals (CEUA, protocol PP00568), where the study was carried out, and were conducted in accordance with Brazilian regulations on animal welfare.

2.2. DSS-induced colitis

Male BALB/c mice (n = 6–8 per group) were provided with a solution of filtered water containing 2% dextran sodium sulfate (DSS) (MW 36,000–50,000) (MP Biomedicals, Solon, OH, USA) ad libitum over a five-day period. Every other day, the total of 200 ml of 2% DSS solution was replenished. The total volume of DSS solution consumed per mouse was proximally 4.2 ml/day. No differences between experimental groups were observed. Following the first five-day period (cycle 1), DSS was replaced with normal drinking water for 10 days and at the end of the fifteenth day the animals were provided with a new cycle of 2% DSS for a five-day period (cycle 2). At the end of DSS cycle 2, the solution was replaced with normal drinking water for a further 10 days and then the animals were euthanized on the thirtieth day (see scheme in Fig. 1). Control mice received only drinking water. All animals were examined once a day and the disease activity index (DAI) was assessed as previously described [9,10]. DAI was the combined score of weight loss, stool consistency and bleeding. Scores were defined as follows: stool consistency was graded zero for no diarrhea, two for loose stool that did not stick to the anus, and four for liquid stool that did stick to the anus. The Feca-cult kit (INLAB, São Paulo, SP, Brazil) was used for detect the presence of fecal blood and the data was graded zero for none, two for moderate and four for gross bleeding. For weight loss, a value of zero was assigned if body weight remained within 1% of baseline or higher, one for greater than 1–5% loss, two for greater than 5–10% loss, three for greater than 10–15% loss and four for weight loss greater than 15%. At the end of each period (fifth, fifteenth, twentieth and thirtieth day), the colons were removed for length measurement and then frozen at −70 °C for future analysis.

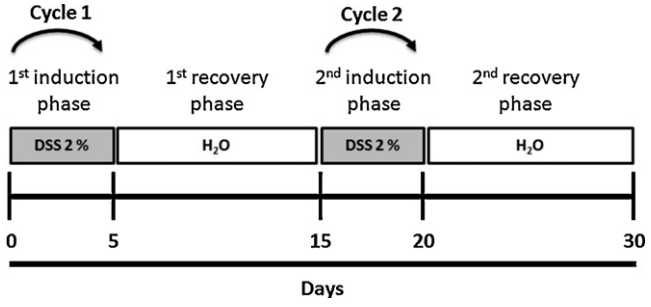

Fig. 1. Acute and chronic phases of DSS-induced colitis. Male Balb/c mice received DSS (2%) for five consecutive days in drinking water. On day 5 DSS was removed and replaced by filtered water, which was supplied for 10 days. After this period, animals were conditioned to a new DSS 2% cycle for five more days, followed by 10 days of filtered water, resulting in a total of 30 days of experimental protocol. The DSS regime phase was designated as the "induction phase," whereas the filtered water offer periods were called the "recovery phase.".

2.3. Myeloperoxidase, eosinophil peroxidase and N-acetylglucosaminidade assays

Neutrophil, eosinophil and macrophage infiltration in the colon were assessed indirectly by measuring the myeloperoxidase (MPO), eosinophil peroxidase (EPO) and *N*-acetylglucosaminidase (NAG) activities. MPO, EPO and NAG were performed as previously described [11]. Colon tissue segments were homogenized in 5% EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 × g for 15 min at 4 °C. The pellet was resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4) and the samples were frozen in liquid nitrogen and thawed three times. After thawing, the samples were similarly centrifuged and 25 μl of the supernatant was used for the MPO, EPO and NAG assays. The MPO enzymatic reaction was assessed by addition of 1.6 mM tetramethylbenzidine (TMB), 80 mM $NaPO_4$, and 0.3 mM hydrogen peroxide (H_2O_2); the EPO by 1.5 mM O-phenylenediamine (OPD), 0.075 mM Tris-HC, and 6.6 mM H_2O_2; and the NAG activity by 2.25 mM *p*-nitrophenil-2-acetamide-D-glucopiranoside, and 50 mM citrate buffer (pH 4.5). The absorbance was measured spectrophotometrically at 690, 490 and 405 nm for MPO, EPO and NAG respectively, and the results expressed in optical density per milligram of tissue.

2.4. Determination of cytokine levels

Briefly, colon segments were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 UI aprotinin A. The homogenate was centrifuged at 3000 × g for 10 min, and the supernatants were stored at −70 °C for further analysis. Tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-12, IL-10, IL-4, keratinocyte-derived chemokine (CXCL1/KC) and transforming growth factor β (TGF-β) levels were evaluated with enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems according to the manufacturer's recommendations. The amount of protein in each sample was measured with the Bradford method [12].

2.5. RNA extraction and real-time PCR

Total RNA from colons was extracted with the SV Total RNA Isolation System Z3100 (Promega, Madison, WI, USA) according to the manufacturer's recommendations and its concentration was determined with a NanoDrop 1100 (NanoDrop Technologies, Wilmington, DE, USA). An amount of 100 ng of total RNA was used for cDNA synthesis. A reverse transcription assay was performed as described in the M-MLV Reverse Transcriptase protocol according to the manufacturer's instructions. cDNA was amplified in duplicate with the TaqMan® Universal PCR Master Mix Kit with specific TaqMan Gene Expression target genes, the 3′ quencher MGB and FAM-labeled probes for mouse IL-17 (Mm00439618_m1), formyl-peptide receptor 2 (FPR2/ALX) (Mm00484464_s1), forkhead box P3 (FoxP3) (Mm00475165_m1), interleukin-2 receptor (CD25/IL-2R) (Mm01340213), intercellular adhesion molecule 1 (ICAM-1) (Mm005616024_g1), vascular cell adhesion molecule 1 (VCAM-1) (Mm01320970_m1), cyclooxygenase 2 (COX-2) (Mm01307334_g1) and GAPDH (NM_008084.2) that was used as an endogenous control

for normalization. The PCR reactions were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, USA). The thermocycler parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression of the target genes was calibrated against conditions found in control animals (i.e. animals that received vehicle).

2.6. Flow cytometric analysis

Mesenteric lymph nodes (MLN) obtained from each animal group were macerated in RPMI 1640 medium and filtered through a 220-μm filter. The resulting suspension was centrifuged at 1500 × *g* for 7 min, the supernatant was discarded, and the cell pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 20 mM HEPES, 3×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated with the following antibodies for 20 min at 4 °C: anti-CD4-PerCP, anti-CD8a-APC and anti-CD25-PE. The data were collected with FACSCanto II (BD Biosciences) and analyzed by means of FlowJo (version 7.5) software.

2.7. Preparation of cytosolic and nuclear fractions

The colonic tissue was removed five, 15, 20 and 30 days after the beginning of DSS administration and homogenized in ice-cold lysis buffer containing 10 mM HEPES, pH 7.4; 2 mM MgCl2; 10 mM KCl; 1 mM PMSF; 1 μg/mL leupeptin; 1 μg/mL aprotinin, 1 mM sodium orthovanadate; 10 mM β-glycerophosphate; 50 mM sodium fluoride and 1 mM dithiothreitol (DTT). The homogenates were chilled on ice, centrifuged at 14,000 × *g* for 45 min and the supernatant containing the cytosolic fraction was collected for posterior protein concentration determination. The pellet was reconstituted with lysis buffer and 0.1% Triton X. After centrifugation (14,000 × *g* for 45 min), the supernatant containing the membrane-rich fraction was dispensed and the pellet was reconstituted again with lysis buffer and 25% glycerol. After centrifugation, the supernatant containing the nuclear fraction was collected and the protein concentration was determined by the standard Bradford method. The supernatants containing the cytosolic and nuclear-rich fractions were stored at −70 °C until analysis.

2.8. Western blot analysis

Equivalent amounts of protein were mixed in a buffer containing 200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM; β-mercaptoethanol and 0.04% bromophenol blue and boiled for 5 min. Proteins were resolved in a 10% SDS gel by electrophoresis. After transfer to a polyvinylidene fluoride membrane, the blots were assembled directly into a Snap i.d. Protein Detection System (Millipore Corporation, Billerica, MA, USA) blot holder for immunodetection The filters were blocked with 1% bovine serum albumin – TBS-T buffer (Tris-buffered saline solution – 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20 surfactant). Filters were then probed with specific primary antibodies anti-p-IκBα, anti-p-p65 and anti-lamin A/C (1:200), for 10 min at room temperature. The blots were washed three times with TBS-T buffer. After washing, membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG secondary antibody (1:800) or horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG secondary antibody (1:800) for 10 min at room temperature. The membranes were washed again with TBS-T and then the immunocomplexes were visualized with the ECL chemiluminescence detection system. Band density measurements were obtained with Scion Image Software (Scion Corporation, Frederick, MD, USA).

2.9. Drugs and reagents

DSS was obtained from MP Biomedicals (Solon, Ohio, USA). Monoclonal mouse anti-phospho-p65 NF-κB, monoclonal mouse anti-IκBα and anti-lamin A/C were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG secondary antibody was acquired from Promega Corporation (Madison, WI, USA) and horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The ECL chemiluminescence detection system was provided by GE Healthcare (São Paulo, SP, Brazil). Hydrogen peroxide, Tween 20, EDTA, aprotinin, phosphate-buffered saline, tetramethylbenzidine (TMB), *O*-phenylenediamine (OPD), *p*-nitrophenil-2-acetamide-D-glucopiranoside (NAG), HTAB, PMSF, benzethonium chloride, sodium orthovanadate, β-glycerophosphate and sodium fluoride were purchased from Sigma Chemical Co. (St Louis, MI, USA). Sodium chloride was obtained from Cromadine (São Paulo, SP, Brazil). $NaPO_4$ and $MgCl_2$ were purchased from Synth (Diadema, SP, Brazil). Bradford reagent and Tris–HCl was obtained from BioAgency (São Paulo, SP, Brazil). Sodium citrate and Triton X was purchased from Vetec (Duque de Caxias, RJ, Brazil). Fetal bovine serum, RPMI, HEPES, 2-mercaptoethanol, penicillin, streptomycin and dithiothreitol (DTT) were obtained from Gibco (Grand Island, NY, USA). Potassium chloride was purchased from Merck (Rio de Janeiro, RJ, Brazil). Mouse TNF-α, IL-1β, IL-12, IL-10, IL-4, CXCL1/KC and TGF-β DuoSet kits were obtained from R&D Systems (Minneapolis, MN, USA). SV Total RNA Isolation System, M-MLV reverse transcriptase and glycerol were purchased from Promega (Madison, WI, USA). Primers and probes for mouse TNF-α, IL-1β, CXCL1/KC, IL-17, IL-10, TGF-β, FPR2/ALX, FoxP3, CD25/IL-2R, ICAM-1, VCAM-1, COX-2, GAPDH and TaqMan® Universal PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, CA, USA). Antibodies for CD4-PerCP and anti-CD8a-APC were purchased from Caltag Laboratories (Burlingame, CA, USA) and anti-CD25-PE from BD Pharmingen™ (San Jose, CA, USA).

2.10. Data analysis

All data are expressed as the mean ± S.E.M. For non-parametric data, a Kruskal–Wallis test followed by a Dunn's test was used. For parametric data, the statistical differences between groups were determined by one-way ANOVA followed by a Student Newman–Keuls test. Statistical analyses were performed with GraphPad Prism® 4 software (GraphPad Software Inc., San Diego, CA). A *P*-value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

3.1. Disease activity and colonic myeloid cellular infiltration in acute and chronic DSS-induced colitis

In order to characterize acute and chronic colonic inflammation, mice were subjected to two separate cycles of five-day DSS administration, followed by normal drinking water for 10 days (recovery phase), the disease activity index (DAI) and body weight change were analyzed every other day for 30 days and a number of inflammatory parameters were observed at the end of each DSS cycle and at the end of the recovery phases (day 5, day 15, day 20 and day 30). In this study we opted by use a solution of 2% DSS, since previous data of our lab showed that this concentration caused a moderate colitis that not interfere with mice survival in the acute period, allowing the conduction of a new cycle of DSS.

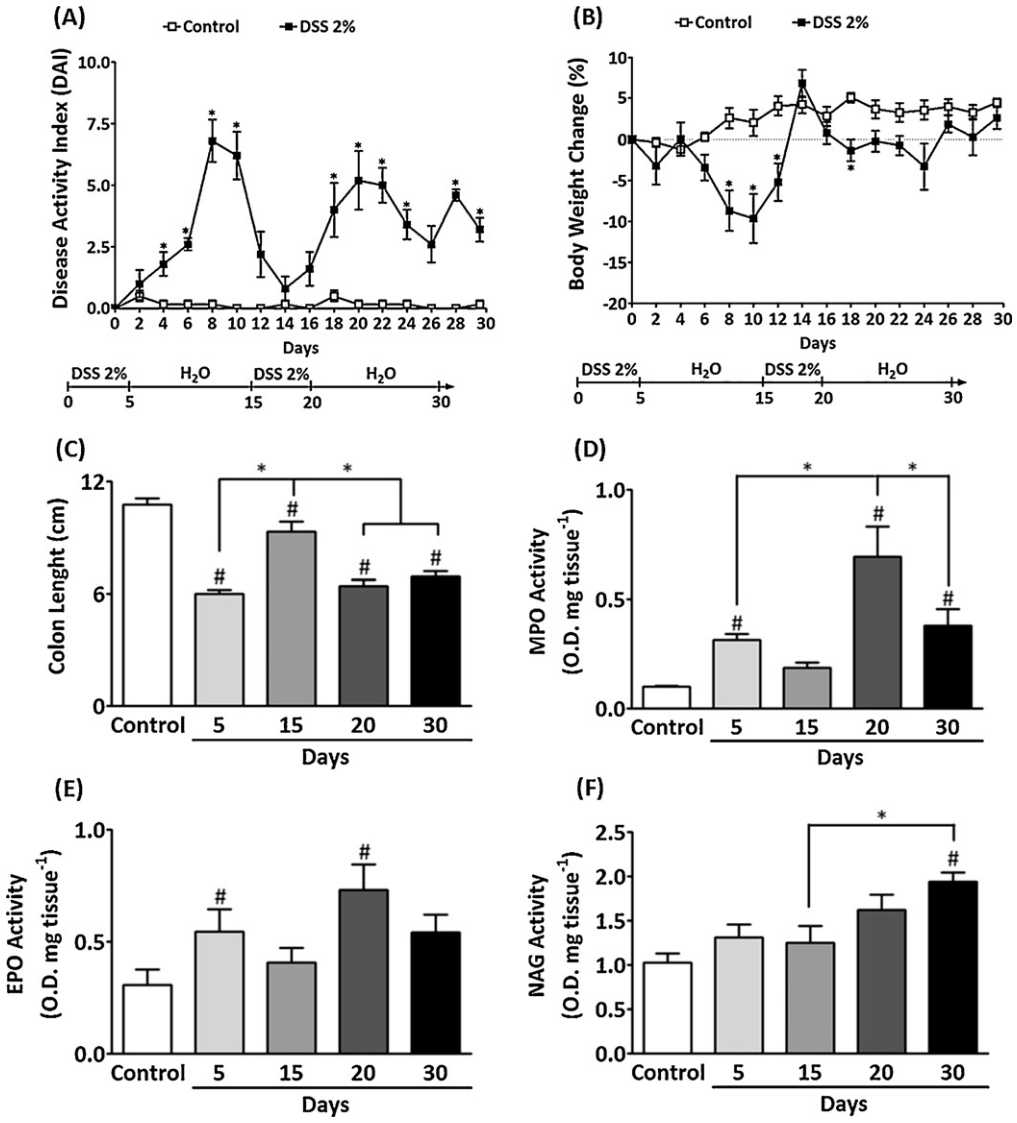

Fig. 2. Disease activity index and myeloid cellular migration during DSS-induced colitis. The animals were subjected to two cycles of 2% DSS, from day zero to day 5 (cycle 1), and from day 15 to day 20 (cycle 2). The recovery periods correspond to treatment with filtered water (H_2O), from day 5 to day 15 and from day 20 to day 30. (A) Two percent DSS solution in drink water increased the Disease Activity index (DAI) in the first cycle of DSS, which was attenuated after the first phase of recovery with filtered water. Likewise, after the second cycle of DSS, DAI was significantly increased, and remained significantly elevated at the end of the second phase of recovery (day 30). (B) Similarly to the results in panel A, DSS solution caused weight loss in the first cycle of DSS, which returned to baseline after the recovery period. After the second cycle of DSS weight loss was also observed. (C) In the first cycle of DSS the colon was significantly shortened compared to the control group; while at the end of fifteenth day the colon length was significantly restored. After the second cycle of DSS colon was again shortened until day 30. Furthermore, the infiltration of neutrophils, eosinophils and macrophages into the tissue of the colon were evaluated indirectly by measuring the MPO, EPO and NAG activities, respectively. (D) MPO activity increased after the first and second DSS cycle, and still increased 30 days after the beginning of the experimental protocol. (E) At the end of cycles 1 and 2 (five and 20 days, respectively), but not in recovery phases, colonic tissue showed high levels of EPO. (F) The NAG activity showed an increase only 30 days after initiation of treatment with DSS. Data are reported as means ± S.E.M. of six to eight mice per group. $^{\#}P < 0.05$ versus control group; $^{*}P < 0.05$ versus selected groups.

Following the first cycle of DSS, animals exhibited a marked increase in DAI score, characterized by body weight loss, stool consistency alterations and bleeding, which peaked at day 8 and decreased thereafter. At the end of the first recovery phase (fifteenth day), the DAI score was reduced to control group levels (Fig. 2A). During the second cycle of DSS, a faster new increase in DAI score was observed, but it did not decrease after the recovery phase to the basal level until day 30, indicating a possible chronification of intestinal inflammation (Fig. 2A). As observed in Fig. 2B, body weight loss peaked at day 10 following the first DSS cycle, but at the end of the recovery phase, on day 15, body mass was significantly regained, presenting the same course as the DAI score. After the second cycle of DSS, the animals lost weight faster, but not in the same intensity as observed in the first phase and, differently from the DAI score, the body weight was retrieved at the end of the second recovery phase (Fig. 2B). Furthermore, in the first cycle of DSS the colon was significantly shortened compared to the control group; while at the end of fifteenth day the colon length was significantly restored (Fig. 2C). However, after the second cycle of DSS colon was again shortened until day 30 (Fig. 2C), suggesting chronic colonic inflammation.

The MPO and EPO activities associated with DAI score and body weight loss showed a significant increase when assessed five days after the beginning of DSS treatment (fifth day), compared with the control group. Similarly, at the end of the first recovery phase (fifteenth day), MPO and EPO levels were reduced to control group levels (Fig. 2D and E). The second cycle of DSS increased EPO activity when compared with the control group, but induced a greater increase in MPO activity, indicating possible aggravation of

neutrophils infiltration in colonic tissue. Following the second period with drinking water (thirtieth day), MPO activity was significantly decreased (compared with day 20), but nonetheless its level was still greater than that of MPO activity observed in the control group and even higher than the levels observed in the acute phase, after the first cycle of DSS (Fig. 2D), suggesting the involvement of neutrophils in chronic intestinal inflammation as well. EPO activity levels decreased after the second recovery phase, and although levels were still greater than those of the control group, this enhancement did not reach statistical significance (Fig. 2E).

Interestingly, no significant increase was observed in the NAG activity at the end of the first DSS cycle, and no differences were observed between DSS cycle 1 and the first recovery phase. NAG levels showed an increase only at the end of the second recovery phase (thirtieth day) (Fig. 2F). These data suggest that mononuclear cells play a significant role mainly in chronic stages of DSS-induced colitis.

3.2. $CD4^+$ and $CD8^+$ T lymphocytes in mesenteric lymph node and colonic regulatory T cell markers during DSS-induced colitis

To gain further insight into indirect measurement of colonic cell infiltration of myeloid lineage, we performed the $CD4^+$ and $CD8^+$ quantification of T cells in mesenteric lymph nodes by flow cytometry during the different phases of DSS-induced colitis. Animals were euthanized on days 5, 15, 20 or 30, as previously described, and the mesenteric lymph nodes were removed for analysis. The results for $CD4^+$ and $CD8^+$ T cells quantification showed that only at the thirtieth day after the experimental protocol initiation was cell population significantly increased (Fig. 3A–C), suggesting the involvement of these inflammatory cells during the chronic phases of intestinal inflammation.

Next we assessed the activation of $CD4^+$ and $CD8^+$ T lymphocytes by performing the co-expression of CD25 cellular marker through the double staining of $CD4^+CD25^+$ and $CD8^+CD25^+$. The population of $CD4^+CD25^+$ T lymphocytes was significantly increased at the end of the second recovery phase (thirtieth day), exactly when the $CD4^+$ cells population was already elevated in the mesenteric lymph nodes (Fig. 4A and B). The $CD8^+CD25^+$ cell population did not increase in any period compared with the control group (Fig. 4C and D).

There is a considerable amount of evidence indicating that regulatory T lymphocytes (T_{reg}) play a critical role in IBD, owing to their effects on the regulation and maintenance of intestinal mucosal homeostasis. Thus, we also performed colonic mRNA quantification for Treg characteristic markers FoxP3 and CD25 in colonic tissue. The quantification of both FoxP3 and CD25 was

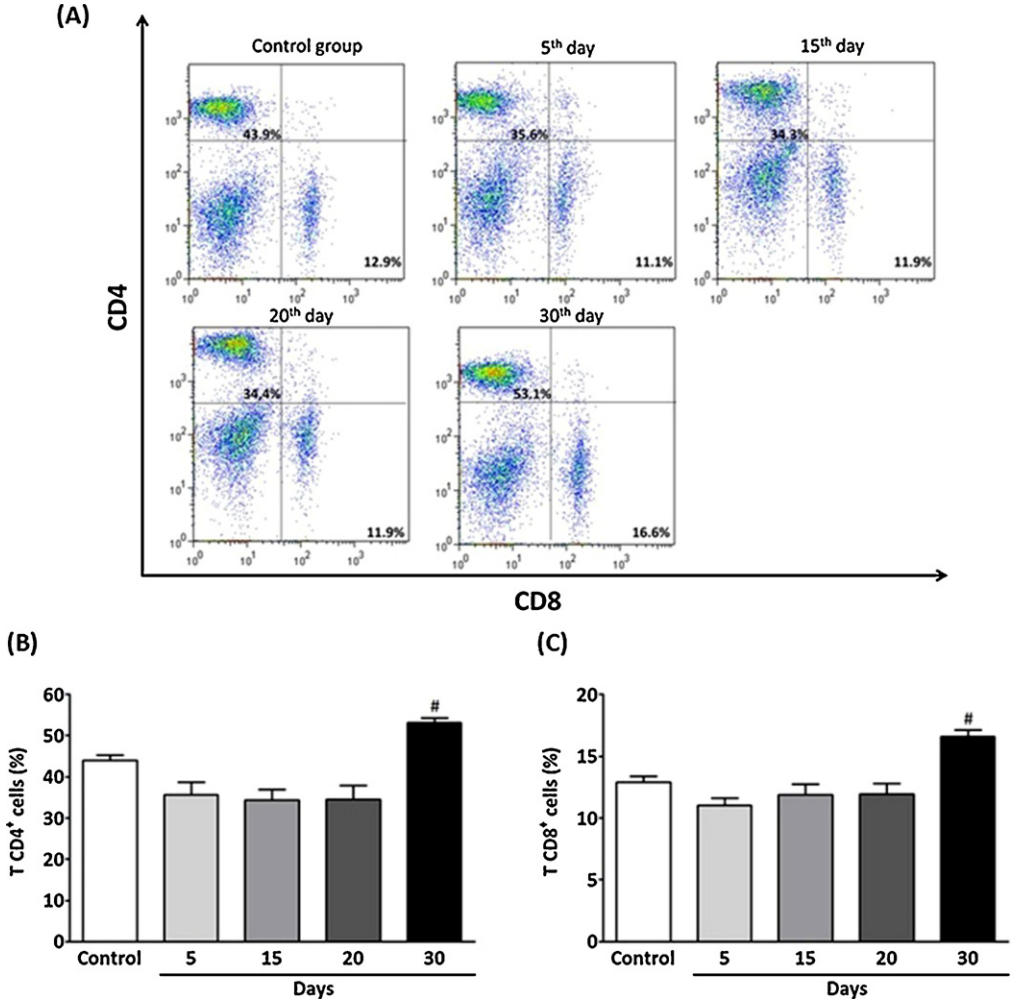

Fig. 3. Population of $CD4^+$ and $CD8^+$ cells in mesenteric lymph node during DSS-induced colitis. The mesenteric lymph nodes from mice were removed at the end of 5, 15, 20 and 30 days, according to the experimental protocol set out in the Materials and Methods section. (A) Representative dot plot from cells after DSS-induced colitis. Population of $CD4^+$ (*y*-axis) and $CD8^+$ (*x*-axis). (B) Quantification of $CD4^+$ T cells in the different time periods, demonstrating a significant increase in T $CD4^+$ cell population 30 days after the initiation of the experimental protocol. (C) Quantification of $CD8^+$ T cells in the different time periods that showed a significant increase in T $CD8^+$ cell population 30 days after the initiation of the experimental protocol. Data are reported as means ± S.E.M. of six to eight mice per group. $^{\#}P < 0.05$ versus control group.

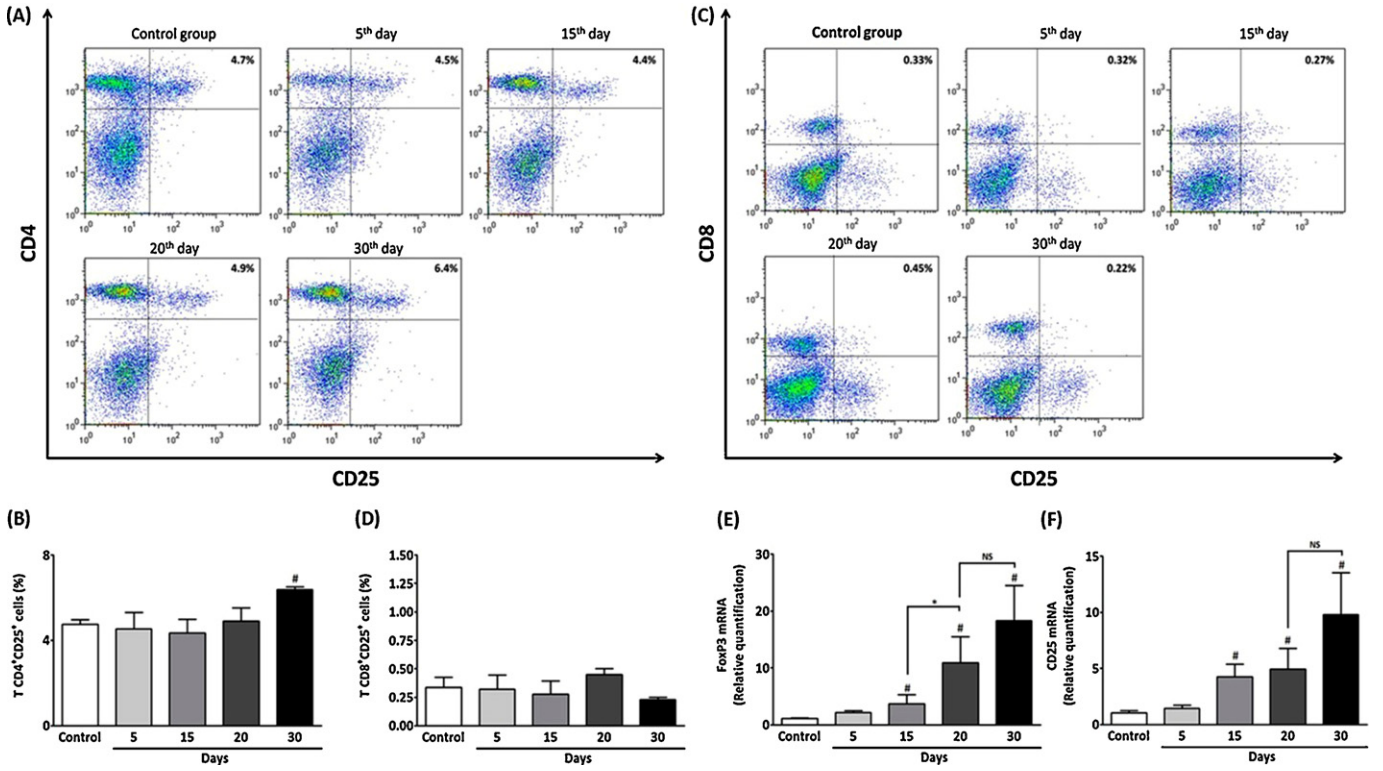

Fig. 4. Population of $CD4^+CD25^+$ and $CD8^+CD25^+$ cells in mesenteric lymph node and colonic T regulatory cell markers during DSS-induced colitis. The mesenteric lymph nodes from mice were removed at day 5, 15, 20 and 30 following the protocol initiation, as described in the Materials and Methods section. (A) Representative dot plot from cells after DSS-induced colitis. Population of $CD4^+$ (*y*-axis) and $CD25^+$ (*x*-axis) that was used as an activation marker of T cells. (B) Quantification of $CD4^+$ $CD25^+$ in the different time periods showed a significant increase in $CD4^+$ $CD25^+$ T cell population 30 days after the initiation of the experimental protocol. (C) Representative dot plot from cells after DSS-induced colitis. Population of $CD8^+$ (*y*-axis) and $CD25^+$ (*x*-axis) that was used as an activation marker of T cells. (D) Quantification of $CD8^+$ $CD25^+$ in the different time periods did not show any difference in $CD8^+$ $CD25^+$ T cell population in the analyzed periods. In another set of experiments, colon tissue was collected and processed for real-time PCR quantification. (E) FoxP3 mRNA was gradually increased after the end of the first recovery phase (fifteenth day), until 30 days after the beginning of the DSS protocol. (F) mRNA for CD25 marker also increased after the end of the first recovery phase (fifteenth day), until 30 days after the beginning of the DSS treatment. Data are reported as means ± S.E.M. of six to eight mice per group. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus selected groups. NS = not significant.

significantly increased after the first recovery phase (fifteenth), and showed a gradual increase until the end of the experimental protocol (thirtieth day) (Fig. 4E and F). These data also suggest the presence of regulatory T cells, especially in chronic phases of DSS-induced colitis.

3.3. Cytokine protein levels in colonic tissue in acute and chronic DSS-induced colitis

In order to analyze some of the inflammatory mediators involved in colonic inflammation during DSS-induced colitis, colons were removed at the end of day 5, 15, 20 and 30 after DSS administration and further processed for cytokine production by ELISA assay, as described in the Materials and Methods section. Our results demonstrated a significant increase in TNF levels in colon tissue in all analyzed periods, compared with the control group, especially in the acute phase of experimental colitis (fifth day) (Fig. 5A). Moreover, the colonic level of pro-inflammatory cytokine IL-1β was significantly increased after DSS cycle 2, and remained elevated until the end of the second recovery phase (thirtieth day), suggesting its possible role in chronic intestinal inflammation (Fig. 5B). In addition, the levels of the pro-inflammatory cytokine IL-12 and the chemokine CXCL1/KC were also measured as mentioned above. The results showed that IL-12 levels were significantly increased only five days after the beginning of DSS administration, compared with the control animals (Fig. 5C). CXCL1/KC levels, however, showed a marked increase at the end of both DSS induction phases (fifth and twentieth days), but not in the recovery periods (Fig. 5D).

The tissue levels of the anti-inflammatory mediators IL-10, IL-4 and TGF-β were also evaluated in the colon alongside DSS-induced colitis. Differently from the pro-inflammatory cytokine results, a significant increase in IL-10 production was observed at the end of both recovery phases (fifteenth and thirtieth days), and no change was observed during the induction phase compared with the control group (Fig. 5E). In addition, IL-4 quantification presented a significant increase fifteen days after the beginning of the DSS regimen (first recovery phase), but not in other analyzed periods (Fig. 5F). Finally, the quantification of TGF-β protein demonstrated that its level was enhanced after the latest recovery phase (day 30) compared with the control group (Fig. 5G). Notably, no significant differences were found for other periods analyzed for TGF-β levels in comparison with control animals.

3.4. Investigation of mRNA expression for inflammatory mediators and adhesion molecules during DSS-induced colitis

Since COX-2 plays a crucial role in the production of many lipid mediators involved in intestinal inflammation and since lipoxin A_4 receptor (ALX/FPR2) plays an important role in the resolution of inflammation [13,14], we performed mRNA expression for these proteins in colon tissue during acute and chronic phases of DSS-induced colitis. Interestingly, the COX-2 mRNA expression was significantly increased in all analyzed time points, but showed a stronger increase in the recovery phases of intestinal inflammation (fifteenth and thirtieth days) (Fig. 6A). On the other hand, ALX/FPR2 mRNA expression showed a significant increase only in the DSS second induction phase (Fig. 6B).

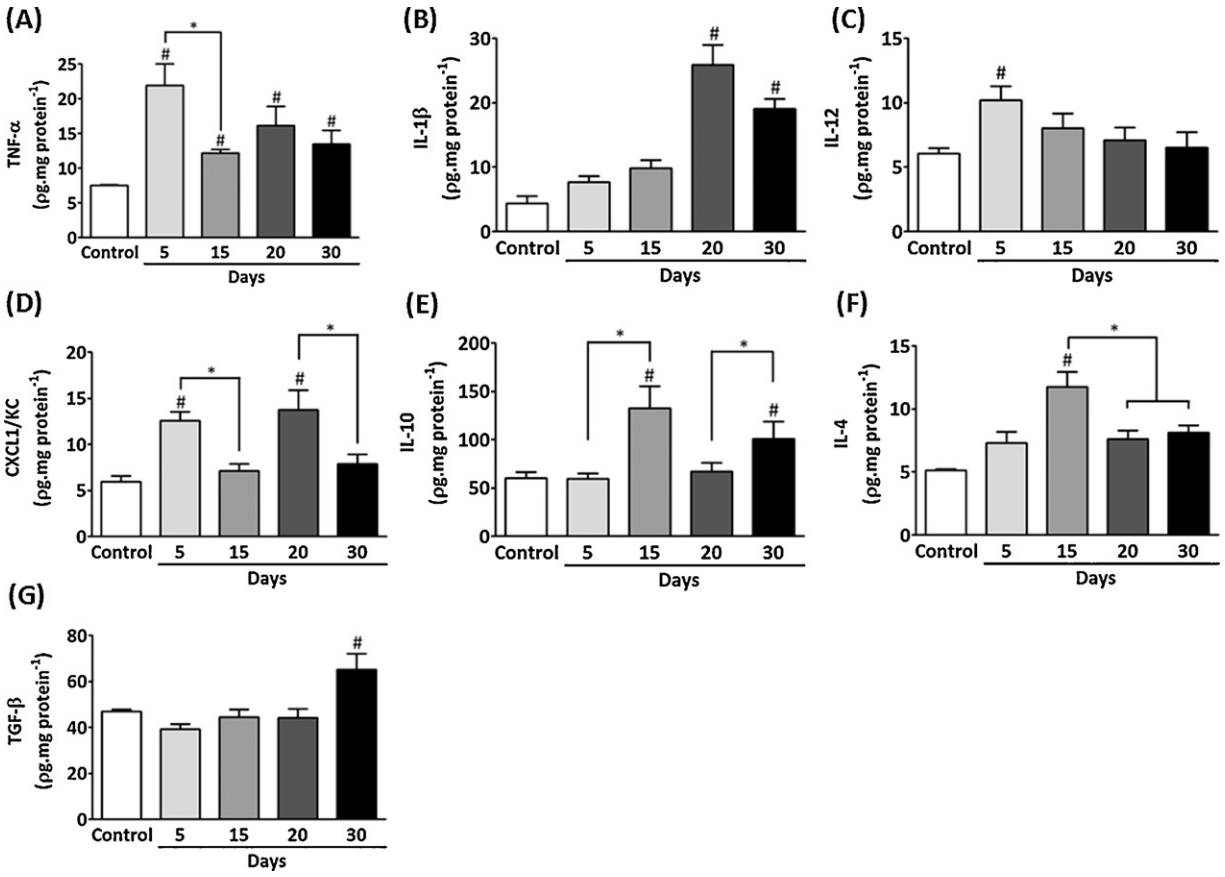

Fig. 5. Determination of cytokines in colon tissue during experimental colitis induced by DSS. At day 5, 15, 20 and 30 following colitis induction, colon tissue was collected and processed for cytokine levels by ELISA assay. (A) The tissue levels of TNF-α increased significantly in all periods evaluated, particularly five days after the beginning of the DSS treatment. (B) Quantification of IL-1β in the colon showed a marked increase at the end of the second cycle of DSS (twentieth day), which remained elevated until the thirtieth day. (C) The measurement of IL-12 increased just five days after the DSS treatment. (D) The levels of CXCL1/KC showed significant increase at the end of cycle 1 (fifth day) and cycle 2 (twentieth day) of DSS. (E) IL-10 levels were significantly increased at the end of the recovery phases (fifteenth and thirtieth days). (F) Quantification for IL-4 showed an increase only on the fifteenth day after the beginning of the DSS regimen. (G) TGF-β levels were significantly increased at the end of the second recovery phase (thirtieth day). Data are reported as means ± S.E.M. of six to eight mice per group. $^{\#}P < 0.05$ versus control group. $^{*}P < 0.05$ versus selected groups.

Because of the results previously described showing inflammatory cell infiltration in the colonic tissue, we next evaluated the mRNA expression for adhesion molecules VCAM-1 and ICAM-1, which are directly connected to cell transmigration in inflamed colonic tissue [15]. Our results revealed a significant increase of VCAM-1 mRNA expression on the fifth, twentieth and thirtieth days after the start of DSS treatment compared with control animals (Fig. 6C). Moreover, mRNA expression for ICAM-1 showed a significant increase only at the end of the second DSS cycle (twentieth day), indicating its possible role in the later stages of DSS-induced colitis (Fig. 6D). A pro-inflammatory cytokine IL-17 mRNA level was also measured in colonic tissue during experimental colitis. The results showed elevated expression of IL-17 mRNA in all analyzed periods, and an especially strong increase at the end of the DSS second cycle (Fig. 6E).

3.5. Activation of NFκB pathway in DSS-induced colitis

Many inflammatory mediators involved in the pathogenesis of inflammatory bowel diseases are transcribed by the NFκB signaling pathway. Therefore we evaluated the phosphorylated protein expression for IκB-α (p-IκB-α) and p65 NFκB (p-p65 NFκB) by western blot. Our results showed that p-IκB-α was increased after the first recovery phase (fifteenth day) compared with the control group (Fig. 7A). Interestingly, its expression increased gradually in the twentieth and thirtieth days after the beginning of the DSS regimen, indicating possible increase of IκB-α cleavage and consequent release of p65 subunit. Likewise, p-p65 NFκB is also increased gradually from the fifteenth until the thirtieth day after the DSS-colitis initiation (Fig. 7B).

4. Discussion

Over the past 20 years much research has highlighted the importance of understanding the pathogenesis of IBD for the development of efficient and safe pharmacological treatments. In this context, we investigated some of the immunological events that occur during acute and chronic phases as well as the remission period in the widely used experimental model of DSS-induced colitis. Our results showed that in the first recovery phase the endeavor of the body to resolve inflammation resulted in a complete decrease of the DAI, although some inflammatory mediators did not reach basal levels. Therefore after the second cycle with DSS, a greater increase and a different partner of inflammatory mediators were observed. Although during the second recovery phase a new effort of the body to compensate inflammation was observed, the clinical parameters were still present, demonstrating the chronification of the process.

Our data demonstrated that the first induction phase was characterized by the infiltration of neutrophils and eosinophils but not mononuclear cells to the colon. The relevance of eosinophils in gut inflammation has been described by several authors [11,16–18]. Nevertheless, our group and others have demonstrated a major role of neutrophils in colonic tissue damage [11]. In fact,

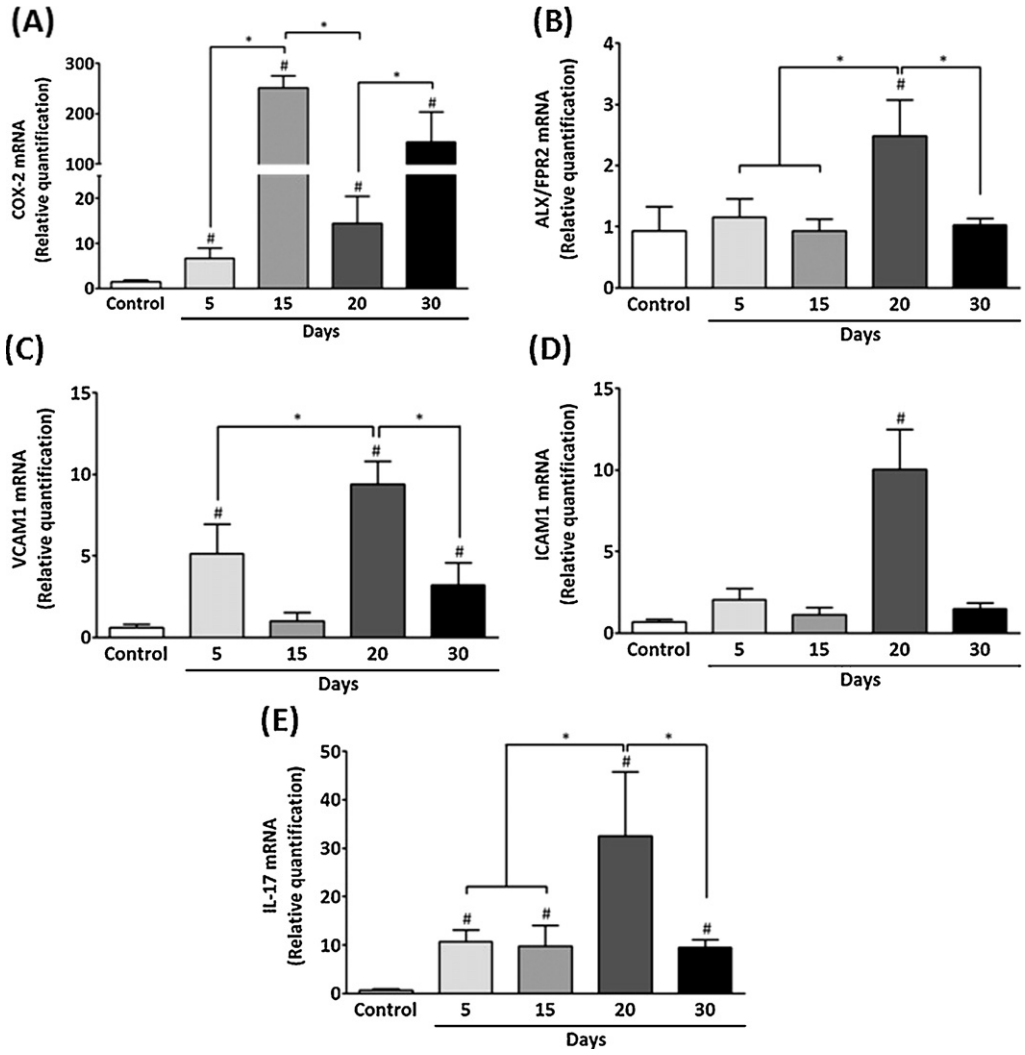

Fig. 6. Evaluation of mRNA for colonic proteins during DSS-induced colitis. On day 5, 15, 20 and 30, colon tissue was collected and processed for real-time PCR. (A) The mRNA levels for COX-2 increased significantly in all periods, especially in the recovery phases (fifteenth and thirtieth days). (B) ALX/FPR2 receptor mRNA levels increased only in the second DSS cycle (twentieth day). (C) mRNA quantification for adhesion molecule VCAM-1 increased five, 20 and 30 days after the beginning of the DSS regime. (D) ICAM-1 mRNA levels increased only in the second DSS cycle (twentieth day). (E) IL-17 levels increased in all analyzed periods, especially at the end of the DSS second cycle. Data are reported as means ± S.E.M. of six to eight mice per group. $^{\#}P < 0.05$ versus control group. $^{*}P < 0.05$ versus selected groups.

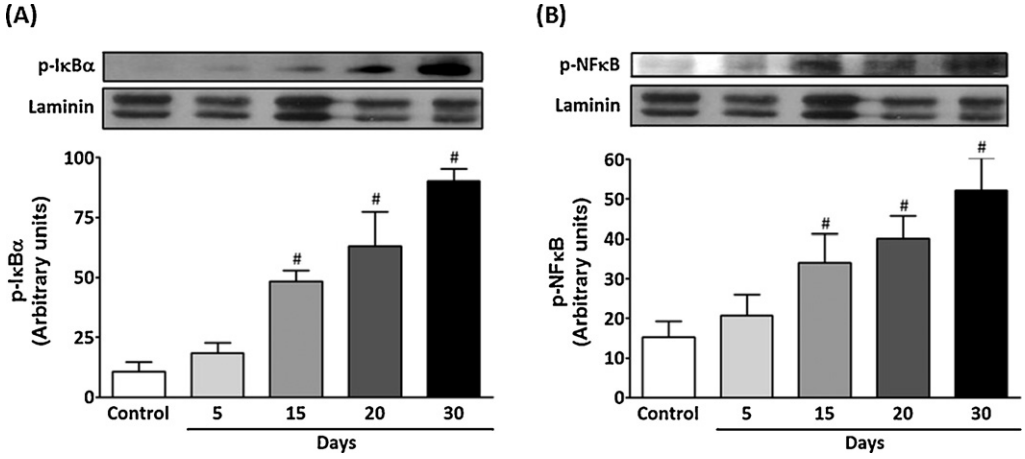

Fig. 7. Phosphorylated IκB-α and NFκB expression during DSS-induced colitis. Analysis p-IκB-α and p-NFκB p65 expression was performed in the colon tissue by the western blot method at different time points. (A) The protein expression of phosphorylated IκB-α was significantly higher after the fifteenth day, and gradually increased until the end of the experimental protocol. (B) Quantification of the activated p65 subunit of NFκB showed a similar result to the p-IκB-α expression, revealing an increase after the first recovery phase (fifteenth day), until day 30. Data are reported as means ± S.E.M. of six to eight mice per group. $^{\#}P < 0.05$ versus control group.

following the first induction phase, DSS was effective in inducing diarrhea, stool bleeding and significant weight loss, all known signals of colon damage. Polymorphonuclear cell infiltration was accompanied by the increase of the chemokine CXCL1/KC, which is essential for neutrophil migration to the injury focus, and VCAM-1 that has been described as important for leukocyte adhesion and infiltration during several inflammation conditions [15], e.g. DSS-induced colitis [14].

In the acute inflammatory phase a significant increase in levels of IL-12, IL-17 and especially TNF-α was observed. In fact, all these mediators are important for leukocyte activation and/or migration [19–21]. It was shown that TNF-α and IL-17 are produced by neutrophils and that TNF-α enhances IL-12 production [22,23]. Interestingly, in clinical practice, therapy with neutralizing antibodies against both IL-12 and TNF cytokines improved IBD [23,24]. Furthermore, knockout mice for IL-17A showed a reduction in DSS-induced colitis severity [25], pointing to the relevance of those cytokines for tissue damage.

At the end of the first recovery phase, when animals appeared to be healthy, the anti-inflammatory response could be observed by the increase of both IL-10 and IL-4 levels. It has been demonstrated that these cytokines act synergistically in the down-regulation of chemokine production and in the function of lamina propria mononuclear cells of IBD patients, leading a significant improvement of inflammatory symptoms [26,27]. This synergism seems to be evident principally in the first recovery phase, as we did not observe any significant increase of IL-4 colonic production in the second recovery period.

The recovery phase was also characterized by a huge enhancement of COX-2 expression. In fact, COX-2 expression has already significantly increased in the first inflammatory phase, where it might be important for pro-inflammatory eicosanoid synthesis [13]. Nonetheless, our data suggest that this enzyme is critical for inflammation resolution, probably because of the formation of pro-resolution lipid mediators, according to previous work [13]. It has been shown that COX-2 is associated with the increase of the anti-inflammatory prostaglandin D_2 (PGD_2) [28], which acts as a "stop signal," reducing granulocyte infiltration in experimental colitis [29].

In concert with the anti-inflammatory response and with mice amelioration, MPO and EPO activities as well as KC and VCAM-1 decreased to basal levels, reinforcing the influence of those cells on colitis aggravation and highlighting the efficacy of the body's anti-inflammatory response. IL-12 and TNF-α levels also decreased; however, the anti-inflammatory effect could not completely restore homeostasis since TNF and IL-17 remained elevated compared with control groups. The maintenance of those cytokine levels suggests that they are produced by cells other than polymorphonuclear ones. Although we could not observe any increase in lymphocyte influx at the acute stage, possible sources of IL-17 are the Paneth cells and resident T γδ cells [30,31]. The results identify both TNF-α and IL-17 as key cytokines involved in the process of chronification of intestinal inflammation.

After the second cycle with DSS we could observe a faster new increase in DAI score. Notably, the worsening of murine conditions was found in concert with a new wave of polymorphonuclear cell infiltration, CXCL1/KC and VCAM-1 expression. Indeed there was an increase in neutrophil migration and VCAM-1 expression compared with the first induction phase. Also, in the late inflammatory phase, the increase in the expression of ICAM-1, an adhesion molecule relevant for eosinophil recruitment [32], was detected. Interestingly, VCAM-1 is uniquely expressed by activated endothelium [33], whereas ICAM-1 is expressed by leukocytes and other cells being further up-regulated during inflammation [34]. Thus, the increase of ICAM-1 levels in the second induction phase may at least in part be owed to higher leukocyte infiltration. Interestingly, it has been suggested that in acute phases TNF-α is largely responsible for the early up-regulation of these adhesion molecules, whereas IL-1β enhances the expression of VCAM-1 and ICAM-1 in late disease [35].

Differently from the first inflammatory phase, in the second one, high levels of IL-1β were observed. The TNF-α level remained elevated although it did not suffer a second up-regulation. These pro-inflammatory cytokines probably contribute to the worsening of experimental colitis by enhancing neutrophil migration [36]. Also, a meaningful increase in IL-17 and decreased production of IL-12 were observed, corroborating other studies showing the inverse correlation between these cytokines [37]. Interestingly, although many studies have highlighted the pro-inflammatory action of IL-17 [25], others challenge this view [38]. Recently a population of regulatory T cells able to produce this cytokine was described [39]. Accordingly, even in this phase, we observed an increase in FoxP3 expression, the major transcription factor of regulatory T cells.

The receptor ALX/FRP2 is present in leukocytes and can be activated by pro-resolution lipid mediators, including LXA_4 and resolvin D1 (RvD1) [40], but also by microbial peptides, exerting a dual role in inflammation [14,40,41]. Notably, transcription of ALXR/FRP2 has been shown to be up-regulated by various cytokines, suggesting regulation of receptor expression in an inflammatory milieu [42,43] and corroborating our finding of increased ALX/FPR2 expression in the second DSS induction phase. However, further studies are required to verify this hypothesis.

In the second recovery phase, the infiltration of mononuclear cells and increased levels of FoxP3, IL-10, TGF-β but not IL-4 levels were detected in colon tissue. In addition, $CD4^+CD25^+$ lymphocytes were observed in mesenteric Lymph nodes. These results support the notion that Treg cells in mesenteric lymph node migrate to the inflamed colonic mucosa and release significant amounts of IL-10 and TGF-β. Although these components certainly account for the decrease of inflammation [44], the resolution was not completely achieved since TNF-α, IL1-β and IL-17 levels decreased but were still elevated compared with the control group. Interestingly, increased levels of IL-1β and TGF-β are known to enhance FoxP3 expression [45], The sustained level of pro-inflammatory cytokines is probably related to the maintenance of elevated MPO activity and therefore to tissue damage, since the DAI index was still elevated, demonstrating that the animals did not recover totally from the disease.

It is well established that the transcription of most pro-inflammatory mediators is controlled by NFκB [46], NFκB activation results from IκB protein phosphorylation by the IκB kinase (IKK) complex, which in turn results in IκB ubiquitylation following degradation [47]. Nevertheless, our data showed that both p-IκB and p-p65-NFκB gradually increased from the first recovery phase onwards, suggesting a critical role of the NFκB pathway in the up-regulation of cytokines, chemokines and adhesion molecules during intestinal inflammation.

In conclusion, our present data revealed different chemical inflammatory mediators and cell influx in the acute and chronic phases of intestinal inflammation caused by DSS. Although the predominance of pro-inflammatory response was observed in both induction phases, pro-resolution mediators were evident during the recovery phases. Interestingly, we identified TNF-α and IL-17 as key mediators that were still present after the first recovery phase and seemed to contribute to the greater inflammatory response observed in the second induction phase. In addition, our results revealed an imbalance between pro- and anti-inflammatory responses in the late recovery period which impaired the complete resolution of inflammation and tissue

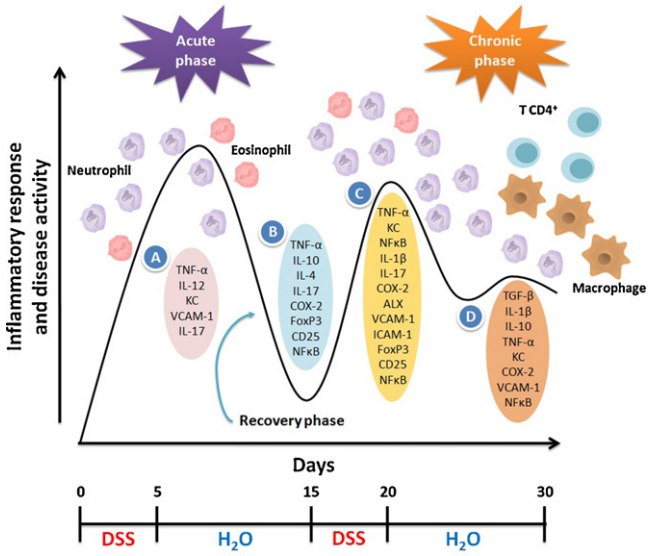

Fig. 8. Inflammatory events during acute and chronic DSS-induced colitis. An acute inflammatory response was initiated during the first induction phase with DSS (cycle 1), reflected in an increase in the disease activity index. (A) The increase of inflammatory response is accompanied by increased expression/production of certain inflammatory mediators and massive infiltration of leukocytes, such as neutrophils and eosinophils. (B) The reduction of the disease activity is accompanied by increased production of anti-inflammatory mediators, reduction of cellular infiltration and production/expression of pro-inflammatory mediators. (C) The DSS new cycle restarts the increase of inflammatory response and disease activity index, leading to a greater increase in neutrophil infiltration and production of several inflammatory mediators not observed in the acute phase. (D) In the second recovery phase, colonic tissues are full of a variety of pro-and anti-inflammatory mediators and mononuclear cells (macrophages and lymphocytes), which were not present in other phases.

repair (see scheme in Fig. 8). Thus, our study contributes to better understanding of the DSS colitis model and reinforces the relevance of understanding the underlying mechanisms associated with the pathogenesis of experimental colitis for development of relevant therapeutic interventions in human IBD.

Conflict of interest

The authors declared no conflict of interest.

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