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The role of CCL3/macrophage inflammatory protein-1α in experimental colitis

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Abstract

CCL3/macrophage inflammatory protein (MIP-1)-1α is elevated in the rectal biopsies of patients with active inflammatory bowel diseases, but its role remains undefined. The present study examined the role of CCL3/MIP-1α during trinitrobenzene sulfonic acid (TNBS)-induced colitis in the rat. Colonic CCL3/MIP-1α levels were elevated (>20-fold above control) within 24 h and remained elevated to day 7 of colitis induction by TNBS administration. In addition, significant increases in colonic neutrophil accumulation were observed within 24 h to day 7 of TNBS treatment. Pre-treatment of rats with a single dose of CCL3/MIP-1α antibody significantly reduced (47%) colonic neutrophil accumulation during the early (24 h) phase of TNBS-induced colitis. In contrast, chronic (repeated) administration of CCL3/MIP-1α antibody did not attenuate colonic neutrophil accumulation during the late phase (day 7) of TNBS-induced colitis. These results suggest a role for CCL3/MIP-1α in promoting colonic neutrophil accumulation during the early (24 h) phase of TNBS-induced colitis.

Keywords: Neutrophil; CC chemokine; Colitis; Inflammation; TNBS

1. Introduction

There is much evidence to suggest an essential role for neutrophils in tissue ulceration and inflammation during inflammatory bowel disease including Crohn's disease and ulcerative colitis (Anezaki et al., 1998; Izzo et al., 1992; Panes and Granger, 1998). For instance, a number of clinical studies have reported increased neutrophil accumulation in rectal biopsies of patients with active inflammatory bowel disease compared to healthy controls (Anezaki et al., 1998; Izzo et al., 1992; Panes and Granger, 1998). Furthermore, a series of animal studies have associated colonic neutrophilia with acute colitis (Galvez et al., 2000; McCafferty et al., 1994). However, in the last couple of years, a number of studies have also provided evidence suggesting that colonic neutrophil accumulation does not contribute to the progression of colonic injury during acute colitis (Buell and Berin, 1994; Wallace et al., 1998; Yamada et al., 1991). As a result, in recent years, much effort has been focussed on characterising the role played by endogenous mediators in promoting neutrophil recruitment during active inflammatory bowel disease.

Chemokines constitute a family of small (~8–15 kDa) structurally related proteins that are widely regarded as one of the most important regulators of leukocyte trafficking and activation during the inflammatory process (Baggiolini, 1998; Luster, 1998). The CC chemokine CCL3/macrophage inflammatory protein (MIP-1)-1α is a potent neutrophil chemoattractant in vivo, with increases in its expression associated with the progression of tissue injury and inflammation during enteritis (Morteau et al., 2002), allergic inflammation (Das et al., 1999), sepsis (Standiford et al., 1995), hepatitis (Ajuebor et al., 2004a,b) and lung injury (Smith et al., 1994). The notion that CCL3/MIP-1α could contribute to the pathogenesis of inflammatory bowel disease stems from a number of clinical studies in which

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rectal biopsies of patients with active ulcerative colitis or Crohn's disease were observed to highly express CCL3/MIP-1α relative to controls (Banks et al., 2003; Vainer et al., 1998). Furthermore, studies by Banks et al. (2003) and Grimm et al. (1996) showed that the expression of CCL3/MIP-1α correlated with the severity of colonic inflammation. However, the contribution of CCL3/MIP-1α to the pathogenesis of inflammatory bowel disease remains undefined (Sun et al., 2001).

The present study assessed the role of CCL3/MIP-1α during trinitrobenzene sulfonic acid-induced colitis in rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (175±20 g) were purchased from Charles River Breeding Farms Limited (Montreal, Canada). The animals were fed a standard chow pellet diet, had free access to water and were maintained on a 12-h light/dark cycle. All procedures were approved by the Animal Care Committee of the University of Calgary and were performed in compliance with the guidelines of the Canadian Council on Animal Care.

2.2. Induction of colitis

Rats were lightly anaesthetized with halothane and the hapten TNBS (60 mg/ml in 0.5 ml of 50% ethanol, Sigma; Ajuebor et al., 2000, 2001) was administered into the distal colon via a cannula. At selected times thereafter (24 h, day 3, day 7), rats were sacrificed. The colon was excised, and examined for macroscopic damage by an observer unaware of the treatments. This involved scoring the damage on a visual scale of 0–10 according to criteria outlined in Table 1 (Ajuebor et al., 2001). After examination, colonic samples were taken for histological evaluation, measurement of colonic CCL3/MIP-1α levels and tissue myeloperoxidase (MPO) activity. MPO activity served as an index of neutrophil infiltration into the colon (Ajuebor et al., 2000, 2001; Galvez et al., 2000; McCafferty et al., 1992). Age-matched, untreated rats served as healthy controls.

Table 1
Criteria for macroscopic scoring of colonic damage

Feature	Score
Normal appearance	0
Focal hyperemia, no ulcers	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
Ulceration/inflammation at two or more sites	4
Major sites of damage extending >1 cm along the length of the colon	5
When an area of damage extended >2 cm along the length of the colon, the score was increased by 1 for each additional cm of involvement	6–10

2.3. Histology and cytochemistry

Colonic tissues were fixed overnight in 10% neutral buffered formalin, dehydrated in ethanol, then embedded in paraffin and sectioned at 5 μm thick. Histological sections were stained with hematoxylin/eosin (H and E) according to standard protocols. Another set of colonic sections were stained with a chloroacetate esterase stain (Leder Stain; Sigma), to quantify colonic neutrophil accumulation (Ajuebor et al., 2004a,b; Bonder et al., 2004; Nakamura et al., 2001; Zaiss et al., 2002). Data are presented as the mean number of positive-stained neutrophil esterase of 10 high power field (hpf).

2.4. CCL3/MIP-1α enzyme-linked immunosorbent assay (ELISA)

Rat CCL3/MIP-1α levels in distal colonic tissue homogenates were determined by a specific ELISA as described (Ajuebor et al., 2004a,b; Blease et al., 2001; Schuh et al., 2002; Standiford et al., 1995), and the total protein concentration was determined by colorimetric protein assay (Bio-Rad Laboratories).

2.5. Effects of anti-MIP-1α serum

For the acute study, rats received a single injection of anti-MIP-1α serum (0.5 ml/rat; i.p., Standiford et al., 1995) or control serum 2 h prior to TNBS administration. All rats were sacrificed 24 h following intracolonic administration of TNBS for assessment of both colonic neutrophil accumulation and colonic injury. For the chronic study, groups of rats received either anti-MIP-1α serum (0.5 ml/rat; i.p.) or normal rabbit serum 2 h prior to TNBS administration and every 24 h thereafter for 6 days. All rats were killed 7 days after TNBS administration for assessment of colonic neutrophil accumulation and colonic injury evaluation. The efficacy of this dose has been previously demonstrated (Standiford et al., 1995).

2.6. Statistical analysis

All data are shown as mean±standard error of the mean (S.E.M.). Comparisons between two experimental groups of data were performed using the Student's unpaired *t*-test. Comparison among three or more experimental groups was performed using a one-way analysis of variance followed by either a Dunnett's multiple comparison test or Newman–Keuls post hoc test. Values of probability less than 5% ($P \leq 0.05$) were considered significant.

3. Results

3.1. Time course of neutrophil accumulation

In agreement with previous reports, intracolonic administration of a single dose of TNBS caused significant increases in neutrophil accumulation in the colon as demonstrated by a striking increase in MPO activity at 24 h (18-fold increase above control; $P<0.01$), day 3 (22-fold increase above control; $P<0.01$) and day 7 (10-fold increase above control; $P<0.05$) post-TNBS administration (Fig. 1A). It is noteworthy that the MPO activity at day 7 post-TNBS treatment was significantly ($P<0.05$) reduced relative to MPO activities at days 1 and 3. Furthermore, consistent with previous reports (Ajuebor et al., 2000, 2001; Galvez et al., 2000; Sans et al., 2001; Zingarelli et al., 1999) intracolonic administration of TNBS was associated with severe ulceration and damage to the colon of the rat as shown by visual macroscopic colonic damage score (Fig. 1B).

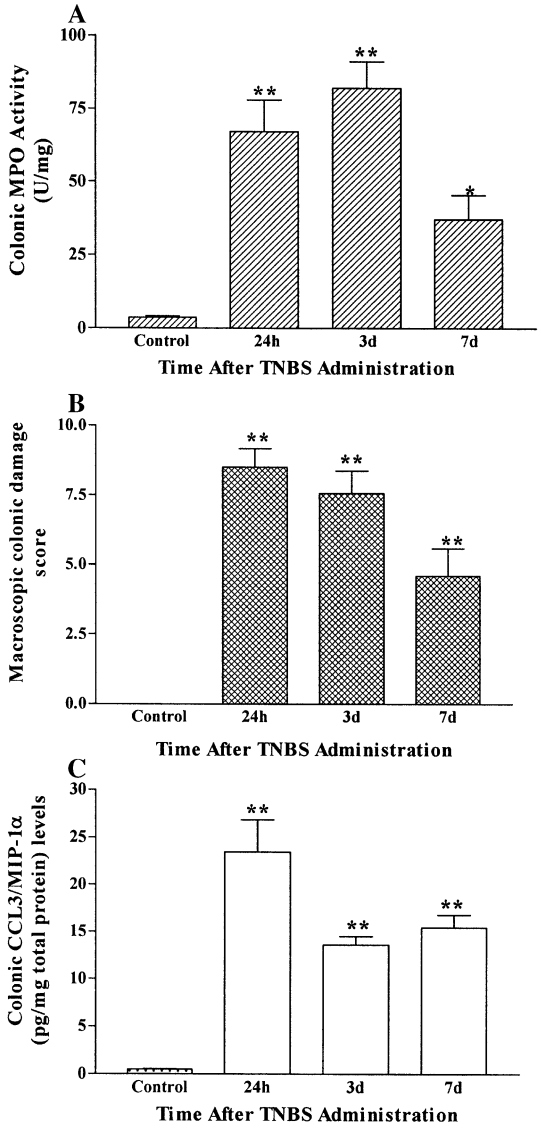

Fig. 1. Time course of (A) colonic myeloperoxidase activity (index of neutrophil infiltration), (B) colonic macroscopic colonic damage score, (C) colonic CCL3/MIP-1α levels from 24 h to 7 days after intracolonic administration of TNBS. Results are expressed as mean±S.E.M., with five to seven rats per group. *$P<0.05$; **$P<0.01$ vs. healthy controls (rats without colitis).

3.2. CCL3/MIP-1α levels

We next determined the time course of the changes in CCL3/MIP-1α levels in the colon after TNBS administration. By ELISA, we observed significant increases in colonic levels of CCL3/MIP-1α at 24 h (48-fold increase above control; $P<0.01$; Fig. 1C), day 3 (28-fold increase above control; $P<0.01$) and day 7 (31-fold increase above control; $P<0.01$) following TNBS administration (Fig. 1C). Based on this, we next determined the biological role of CCL3/MIP-1α in colonic neutrophil recruitment and colonic damage during the early (24 h) and late (day 7) phase of TNBS-induced colitis.

3.3. Effects of acute CCL3/MIP-1α blockade on neutrophil recruitment and colonic injury

We initially investigated the effect of a single dose administration of anti-MIP-1α serum on colonic neutrophil recruitment during the early phase (i.e. 24 h) of TNBS-induced acute colitis. Pre-treatment of rats with a single dose of anti-MIP-1α serum caused a significant reduction (47%) in neutrophil accumulation in the colon at 24 h after TNBS administration when compared to control group as shown by reduced MPO activity (Fig. 2A). Histological examination of H and E-stained colonic sections of colitic rats treated with anti-MIP-1α serum showed mild neutrophil accumulation at 24 h after TNBS instillation whereas colonic sections from colitic rats given control serum exhibited marked neutrophil infiltration following TNBS administration (Fig. 2B). We further confirmed our finding of reduced accumulation of neutrophils in the colon of anti-MIP-1α serum-treated colitic rats by cytochemistry using a neutrophil esterase stain which stains neutrophil positive cells pink. As shown in Fig. 2C and D, the colon of colitic rats treated with control serum contained significantly more neutrophils when compared with colitic rats given anti-MIP-1α serum. Surprisingly, despite the reduction in colonic neutrophil accumulation at 24 h post-colitis induction following CCL3/MIP-1α neutralization, the visual colonic damage score and histological injury score were unaltered by anti-MIP-1α serum treatment (Fig. 2E and F).

3.4. Effects of chronic CCL3/MIP-1α blockade on neutrophil recruitment and colonic injury

We next examined the effects of chronic (repeated) anti-MIP-1α serum administration on neutrophil recruitment and

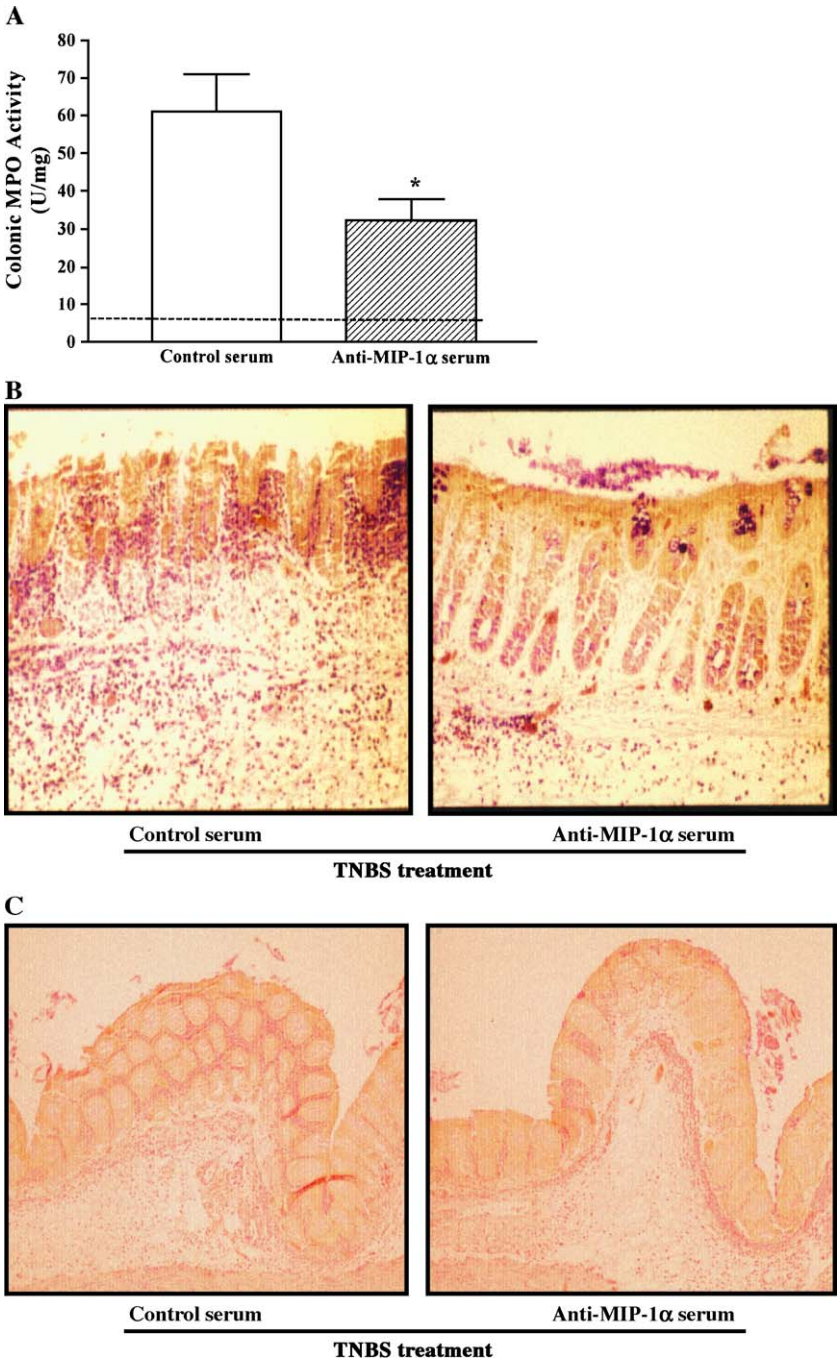

Fig. 2. Effects of anti-MIP-1α serum treatment on MPO activity and colonic injury score 24 h after induction of colitis by intracolonic administration of TNBS. A single dose of anti-MIP-1α serum (0.5 ml/rat, i.p.) or control serum was given to rats 2 h before the induction of colitis. The dotted line denotes the mean level of MPO activity in rats without colitis (i.e. naïve rats). (A) Data are presented as mean±S.E.M., with five rats per group. *P<0.05 vs. control serum treated-group. (B) Representative hematoxylin and eosin-stained sections from colitic rats that received control serum (left panel) or anti-MIP-1α serum (right panel); rats receiving control serum exhibited extensive neutrophil infiltration in mucosa and submucosa 24 h after induction of colitis whereas a significant reduction in neutrophil infiltrates can be seen in colitic rats that received anti-MIP-1α serum. (C) A representative pictorial of Leder (esterase) stain of colonic sections from colitic rats given control serum (left panel) or anti-MIP-1α serum (right panel). Increased neutrophils counts (which are stained pink) can be seen in the mucosa and submucosa of control serum-treated rats (left panel) whereas lower counts of neutrophils could be seen in the mucosa and submucosa after anti-MIP-1α serum treatment (right panel) at the 24-h time-point after TNBS administration. (D) denotes colonic neutrophil counts from control serum and anti-MIP-1a serum treated rats at 24 h after TNBS administration. Data are presented as mean±S.E.M., with five rats per group. *P<0.05 vs. control serum treated-group. Dotted line is the mean number of positive stained-neutrophils/hpf±S.E.M. in naïve rats. The effects of anti-MIP-1α serum treatment on visual colonic damage score (E) and histological score (F) at 24 h after induction of colitis by intracolonic administration of TNBS. Results are presented as mean±S.E.M., with five to six rats per group.

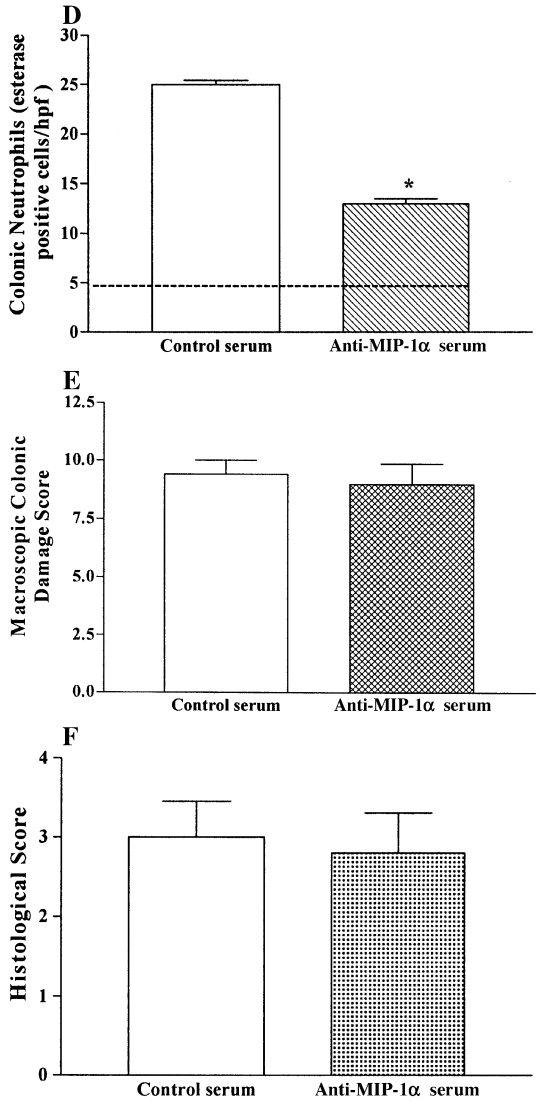

Fig. 2 (*continued*).

colonic damage during the late phase (i.e. day 7) of TNBS-induced acute colitis. We observed that chronic (i.e. repeated) administration of anti-MIP-1α serum for 6 days to colitic rats caused a small (17%) but significant reduction in visual colonic tissue damage score at day 7 after TNBS administration (Fig. 3A), but histological colonic score was not reduced (Fig. 3B). In addition, colonic neutrophil accumulation was not reduced as shown by MPO activity (Fig. 3C).

4. Discussion

The pathological association between leukocytes and inflammatory bowel diseases including Crohn's disease and ulcerative colitis has long been recognized. Neutrophils are known to play a crucial role in the pathogenesis of inflammatory bowel diseases (Panes and Granger, 1998). However, despite tremendous progress in our understanding of the pathophysiology of inflammatory bowel diseases, several key questions remain unresolved. First and foremost, the mechanisms governing neutrophil migration from the blood into tissues during inflammatory bowel diseases remain incompletely understood (Panes and Granger, 1998). Furthermore, the endogenous mediators that promote the recruitment of neutrophils to the colon during inflammatory bowel diseases have been poorly characterized. There is now a growing realization that chemokines may facilitate neutrophil recruitment and activation during inflammatory bowel diseases. Moreover, the last decade has seen the accumulation of a large

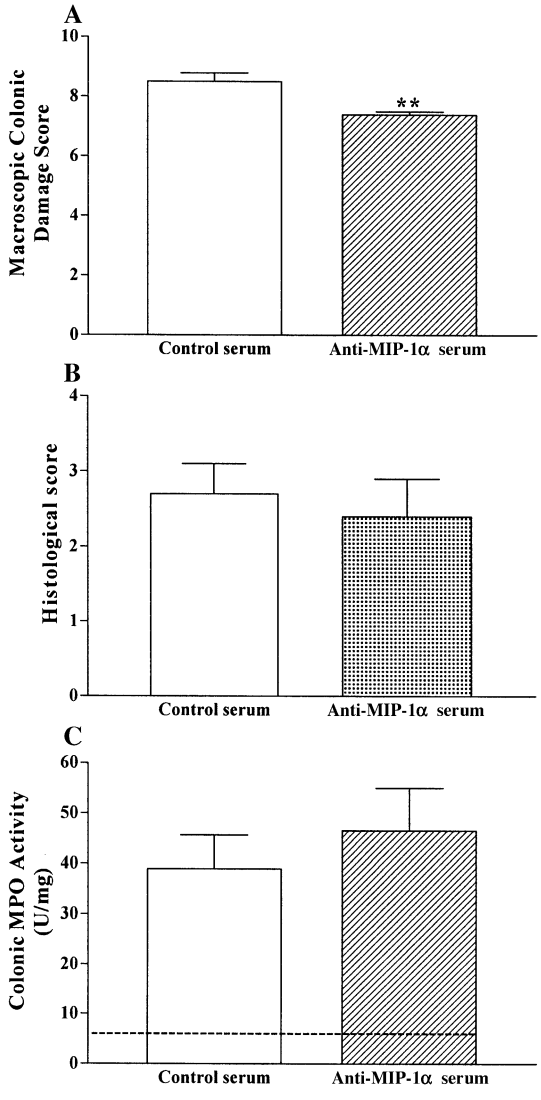

Fig. 3. Effects of chronic anti-MIP-1α serum treatment on visual colonic macroscopic damage score (A), histological score (B) and MPO activity (C) during TNBS-induced colitis. Anti-MIP-1α serum (0.5 ml/rat, i.p.) or control serum was administered to rats 2 h before induction of colitis and every 24 h thereafter for 6 days. All rats were sacrificed 7 days after colitis induction. Results are presented as mean±S.E.M., with five to six rats per group. $^{**}P<0.01$ vs. rats that received control serum.

volume of evidence supporting an important role for chemokines and their receptors in the recruitment of leukocytes during inflammatory bowel diseases (Ajuebor and Swain, 2002; Papadakis, 2004)). Data derived from animal studies demonstrates a pathogenic role for chemokines/chemokine receptors in the development of inflammatory bowel diseases. Specifically, blockade of the chemokine receptors CCR1, CCR2, CCR5 by chemokine receptor antagonist or through gene deficiency have all been shown to ameliorate colonic injury by modulating the recruitment of leukocytes to colonic tissue (Ajuebor et al., 2001; Andres et al., 2001). However, a recent study (Sasaki et al., 2002) reports that chemokines could also attenuate colonic injury independent of leukocyte recruitment. Specifically, Sasaki et al. (2002) demonstrated that neutralization of the chemokine CXCL10/IP-10 in the dextran sodium sulfate model of acute colitis protected mice from epithelial ulceration without modulating the recruitment of immune cells. In addition, a recent study also highlights the fact that antibody neutralization of the chemokine CXCL1/KC or its receptor CXCR2 impaired neutrophil recruitment to colonic tissue during TNBS-induced acute colitis without protecting rats from colonic damage (Ajuebor et al., 2004a,b). CCL3/MIP-1α is a CC chemokine that promotes the recruitment of various leukocyte subtypes, including neutrophils. However, the role played by CCL3/MIP-1α in modulating colonic inflammation and the mechanisms underlying this modulation in inflammatory bowel's diseases remain incompletely undefined. The present study delineates the role of CCL3/MIP-1α in the pathogenesis of acute colitis induced by TNBS administration in rats.

The induction of acute colitis resulted in striking increases in colonic levels of CCL3/MIP-1α. Moreover, the increase in colonic levels of CCL3/MIP-1α was paralleled by marked increases in neutrophil accumulation. Therefore, we evaluated the role of colonic CCL3/MIP-1α during the early (i.e. 24 h) and late phase (i.e. day 7) of TNBS-induced acute colitis. Firstly, we observed that the administration of a single dose of anti-MIP-1α serum significantly decreased (47%) neutrophil accumulation in the colon during the early phase (i.e. 24 h) of TNBS-induced acute colitis. The effectiveness of anti-MIP-1α serum in attenuating colonic neutrophil accumulation at this time-point could suggest a role for colonic CCL3/MIP-1α in promoting the recruitment of neutrophils to the colon during the early (24 h) phase of TNBS-induced colitis. Despite, the reduction in colonic neutrophil accumulation documented above, colonic injury was not reduced by CCL3/MIP-1α antibody neutralization. At first glance, our data would seem to differ from previous studies in the TNBS-induced acute colitis model where reduced colonic neutrophil accumulation was associated with reduced colonic damage score during the early phase (<48 h) of acute colitis (Galvez et al., 2000; McCafferty et al., 1994). However, our finding is consistent with the study by Wallace et al., 1998 where treatment of colitic rats with anti-neutrophil serum strikingly reduced (95%) neutrophil accumulation without altering colonic damage score at 24 h after TNBS administration. It is noteworthy that similar observations have been reported in the acetic acid-induced acute colitis model (Buell and Berin, 1994; Yamada et al., 1991). However, our observation that neutrophil infiltrates in the colon was reduced by ~50% after CCL3/MIP-1α neutralization could suggest that other inflammatory mediators may account for the persistent colonic damage we have documented in this study after CCL3/MIP-1α antibody treatment.

Secondly, we found that repeated treatment of colitic rats with anti-MIP-1α serum for 6 days caused a small reduction (17%) in visual colonic damage score 7 days after TNBS administration however, neutrophil accumulation in the colon was not altered. The effect of chronic anti-MIP-1α serum treatment on visual colonic damage score was statistically significant. However when considered objectively, the miniscule reduction is neither biologically nor clinically significant since histological colonic injury was not reduced by CCL3/MIP-1α antibody neutralization. Presently, it is difficult to explain why and how CCL3/MIP-1α could promote colonic neutrophil accumulation during the early phase of TNBS-induced colitis but not at the late phase of the inflammatory process. The lack of effect of the anti-MIP-1α serum on neutrophil recruitment during the late phase of TNBS-mediated colonic inflammation could be related to the shift in inflammation from neutrophil-mediated to macrophage-mediated at day 7 post-TNBS administration (Tozawa et al., 2003). However, other mediators of colonic inflammation (such as interleukin-1, platelet activating factor, tumor necrosis factor-α and P-selectin) may play a more prominent role than CCL3/MIP-1α in promoting colonic neutrophil recruitment at this time-point (Galvez et al., 2000; McCafferty et al., 1992; Molla et al., 2001; Sans et al., 2001). Alternatively, the pro-inflammatory effect of CCL3/MIP-1α on colonic neutrophil recruitment we documented in this study could be an indirect effect of CCL3/MIP-1α deficiency.

In summary, present data support the concept of a CCL3/MIP-1α-dependent pathway promoting the initial neutrophil recruitment to the colon during the early phase (i.e. 24 h) of TNBS-induced acute colitis and a CCL3/MIP-1α-independent pathway mediating colonic neutrophil accumulation at the late phase (i.e. day 7) of the inflammatory process.

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