CCL2 (pM Levels) as a Therapeutic Agent in Inflammatory Bowel Disease Models in Mice

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Background: Chemokines regulate the pathways that restrict homing of specific subsets of immune cells, and thereby fine tune the immune response at specific lymphoid and peripheral tissues. CCL2 is a chemokine that induces migration of monocytes, memory T cells, and dendritic cells. Previously, we demonstrated that pM levels of CCL2 dramatically inhibit migration of T cells. The aim was to test whether subphysiological doses of CCL2 can ameliorate murine colitis and inflammation-induced colorectal cancer.

Methods: TNBS (2,4,6 trinitrobenzene sulfonic acid) colitis and dextran sodium sulfate (DSS) colitis were induced in Balb/c and C57BL/6 mice, respectively. Mice were treated daily with intraperitoneal CCL2 injections. Disease activity was assessed clinically, histologically, and by measuring inflammatory cytokine levels. In addition, an inflammatory cancer model was induced by azoxymethane-DSS (AOM-DSS) in Balb/c mice. Mice were treated daily with CCL2 for 11 weeks and then assessed for number of tumors in the colons.

Results: Daily administration of CCL2 (60–120 ng) significantly decreased the development of TNBS- and DSS-induced colitis. In a DSS-AOM model, CCL2-treated mice developed significantly fewer tumors ($P < 0.005$) at 11 weeks. Chronic inflammation in the CCL2-treated mice was significantly less pronounced as compared to phosphate-buffered saline-treated mice.

Conclusions: Administration of pM levels of CCL2 significantly inhibits migration of T cells in amelioration of TNBS and DSS colitis and inhibits development of colorectal cancer in an AOM-DSS colitis model in mice. Thus, pM levels of CCL2 may be clinically beneficial as an antiinflammatory agent in IBD.

Key Words: CCL2, inflammatory bowel disease, murine colitis models, colorectal cancer

Inflammatory bowel disease (IBD) is a generic term for a group of inflammatory disorders of the gastrointestinal tract characterized by intestinal inflammation and mucosal damage. Crohn’s disease (CD) and ulcerative colitis (UC) are the two major forms of IBD. UC primarily affects the mucosal lining of the colon and rectum, whereas CD primarily affects all intestinal wall layers and may potentially extend to any part of the gastrointestinal tract.

A disregulated activation of the intestinal immune system plays a pivotal role in the pathogenesis of IBD. It has been established that inflammatory mediators such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interferon-$\gamma$ (IFN-$\gamma$), and interleukin (IL)-12 produced by infiltrating CD4+ T cells and macrophages have a key role in the pathogenesis of disease exacerbation. However, the etiologies of both CD and UC still remain largely unclear, and probably result from an aberrant immune response to an environmental trigger in a genetically susceptible host.

IBDs pose a major therapeutic challenge, as their course is chronic-relapsing with significant damage to the quality of life of patients. None of the available therapies result in complete remission in all patients. Moreover, IBD colitis significantly increases the risk of colorectal cancer (CRC) compared to the general population. A better understanding of the pathophysiology of these diseases and the development of new therapeutic options can favorably affect a sizable portion of the population suffering from IBD.

None of the existing IBD models constitutes a faithful reproduction of the human diseases. Therefore, it is essential to evaluate the effect of any drug or treatment in several animal IBD models. One of the widely used animal models is dextran sodium sulfate (DSS) colitis, induced by DSS administration through drinking water, leading to...
many of the events presumed to initiate and sustain human IBD. This model allows generation of variable diseases of acute or chronic nature, depending on the mouse strain and the dose and frequency of DSS administration. It is generally believed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier.

Another model of colitis is achieved by intrarectal instillation of the haptenizing substance 2,4,6 trinitrobenzene sulfonic acid (TNBS) in ethanol. Ethanol is required to break the mucosal barrier, whereas TNBS is believed to haptenize colonic autologous or microbial proteins, rendering them immunogenic to the host immune system. It is thought that this model resembles CD because of the resulting of Th1 response but it was also shown to comprise a Th2 component.7

The intensity of the inflammatory response in IBD is determined both by the local expression of growth factors and proinflammatory cytokines within the mucosa, and by a coordinated mechanisms of cellular recruitment, involving the upregulation of both vascular adhesion molecules and chemokine expression.8 Chemokines play a major role in the maintenance of inflammatory processes, and the final composition of leukocytes present in the inflamed intestine is most likely due to both secreted chemokines and the relative expression of specific chemokine cell surface receptors on different cell types. The production of chemokines within the intestine establishes a chemotactic gradient capable of increasing the migration of monocytes/macrophages, granulocytes, and lymphocytes from the bloodstream into the submucosa during chronic IBD.

Surveillance of the body for foreign antigens is a critical function of the immune system. Lymphocytes migrate from the blood into tissues and secondary lymphoid organs and return to the blood via lymphatic vessels and the thoracic duct. The majority of lymphocytes are capable of tissue selective trafficking (homing), recognizing organ-specific adhesion molecules on specialized endothelial cells. We were the first to characterize the pathway that negatively regulate homing of lymphocytes to the lymph nodes. We demonstrated that pM levels of CCL2 exert strong inhibition of T- and B-cell migration and their homing to lymph nodes.9,10

In this study we examined whether administration of pM levels of CCL2 to mice can ameliorate development of TNBS and DSS colitis, and further evaluated whether CCL2 may inhibit development inflammation-induced CRC in mice.

**MATERIALS AND METHODS**

**Mice**

Specific pathogen-free Balb/c and C57BL/6 male mice, age 8 weeks, were purchased from Harlan (Indianapolis, IN), weighing 22–25 g; mice were maintained in standard wire cages and allowed free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee.

**Induction of TNBS Colitis**

TNBS colitis was induced in Balb/c mice as previously described.11 In brief, mice were anesthetized. Next, 100 µL of TNBS (55% volume of 50% ethanol mixed with 45% volume of TNBS solution (trinitrobenzene sulfonic acid; Sigma-Aldrich, St. Louis, MO) was infused into the colonic lumen via a 1-mL syringe attached to a feeding needle.

**Assessment of Colitis**

In all mice body weight, rectal bleeding, and survival were monitored daily.

**Macroscopic Assessment of Colitis**

A person blinded to the identity of the groups performed the scoring of the severity of disease. After the mice were sacrificed the colon was examined under ×5 magnification to evaluate the macroscopic lesions according to the Wallace criteria. The Wallace score ranks macroscopic colon lesions on a scale from 0 to 16 based on criteria reflecting inflammation, such as hyperemia, thickening of the bowel, and the extent of ulceration.12

**Induction of DSS Colitis**

DSS (MP Biomedicals, Solon, OH) colitis was induced in C57BL/6 mice as previously described.13 In brief, DSS 2% was mixed with the drinking water for 5 days. Thereafter, mice were rested for another 5 days with access to DSS-free water. Mice were sacrificed on day 10 of the experiment.

**Treatment with CCL2**

The mice were divided into six groups with 10 mice in each CCL2 group. Each group was intraperitoneal (i.p.) injected with different CCL2 concentrations in 200 µL of PBS from day 0 (immediately after TNBS or DSS induction) to day 6 (TNBS) or 9 (DSS), daily.

**Histological Assessment of DSS Colitis Inflammation Score**

Colons were fixed in 4% paraformaldehyde for histology with hematoxylin and eosin (H&E). The degree of histological damage and inflammation was graded in a blinded fashion. The following manifestations were included in the evaluation: distribution of lesions (0–4), extent of epithelial damage (0–4), and layers involved (0–4). The overall histological score represented the sum of the three manifestations (maximal score of 12).1
Azoxymethane-DSS CRC Model

Balb/c mice weighing 18–20 g at 7 weeks of age were injected i.p. with a single dose (7.4 mg/kg) of azoxymethane (AOM) followed by 3% DSS in drinking water for 1 week, then 2 weeks of drinking water without DSS. On the fourth week mice were again treated with a 1-week course of 1.5% DSS in their water.

The mice were divided into two groups. Each group was treated daily with i.p. injections of either 200 µL phosphate-buffered saline (PBS) (control) or CCL2.

On week 11 mice were sacrificed and each colon was cut longitudinally, cleansed with PBS, and the distal half of each colon was rinsed by methylene blue; thereafter, tumors were counted and measured.

Colons were fixed in 4% paraformaldehyde for histology and stained with H&E. Specimens were cut longitudinally into five different sections and carefully assessed for number of tumors, adenomas, and microadenomas by a pathologist (E.B.) blinded to the treatment received.

Enzyme-linked Immunosorbent Assay (ELISA)

In each colitis experiment at least two colons were used for ELISA. For preparation of colon tissue samples, colon tissue samples in PBS containing a cocktail of protease inhibitors (1 µL to 20 mg of tissues according to the manufacturer’s protocol) were homogenized using a polytron homogenizer and centrifuged at 12,000 g for 30 minutes. The supernatants were subjected to ELISA. Tissue levels of the inflammatory cytokines, TNF-α, IL-12, and IFN-γ, were assessed using an ELISA kit (e-Bioscience, San Diego, CA) according to the manufacturer’s protocol.

Isolation of Lamina Propria Lymphocytes (LPLs)

LPLs were isolated using a modification of the method described previously. Colons were washed with PBS until all content was removed. Colons were then opened lengthwise. The gut epithelium was removed from the lamina propria by incubation with 1 mM DTT and 1 mM EDTA in PBS for 30 minutes. The remaining tissues were digested by collagenase type VIII (Sigma) and 5 U/mL DNase (Roche, Nutley, NJ) at 37°C for 2 hours in RPMI. In order to further purify LPL, cells were centrifuged on a discontinuous Percoll gradient for 20 minutes. Viable cells at the 40%/70% interface were collected and used in the LPL migration assay.

Isolation of BM Monocytes

BM cells were harvested from the femora and tibiae of C57BL/6 mice, enriched for mononuclear cells on a Ficoll density gradient, and then isolated by MACS enrichment using biotinylated anti-CD115 antibodies and streptavidin-coupled magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol.

Transwell Migration Assay

Lymphocytes

LPL cells were incubated with 1 ng/mL CCL2 for 30 minutes or left untreated. Chemotaxis toward CXCL12 (100 ng/mL) was assayed using Transwell chambers as previously described. Migrating cells retrieved from the lower chambers were counted by fluorescence activated cell sorting (FACS).

Monocytes

Monocytes were suspended in 0.5% bovine serum albumin (BSA) in RPMI and incubated with either CCL2 or left untreated for 30 minutes at 37°C. Chemotaxis was assayed using a Transwell chamber (Corning Inc, Corning, NY). Approximately 5 × 10⁶ mononuclear cells were placed in the upper chamber of the Transwell plate apparatus. Next, 600 µL of medium containing 0.5% BSA with or without 50 ng/mL mouse CXCL12 (R&D Systems, Minneapolis, MN) was placed in the bottom chamber. The migration toward the chemokine CXCL12, added to the lower part of the apparatus, was analyzed after 2.5 hours by FACS.

Murine Colonoscopy

A high-resolution mouse video endoscopic system (“Coloview system”) previously described for murine endoscopic procedures, was used. This system consists of a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump to achieve regulated inflation of the mouse peritoneal cavity (Karl Storz, Tuttingen, Germany). The endoscopic procedure was viewed on a color monitor and digitally recorded on tape. For methylene blue staining, 100 µL of 1:5 diluted methylene blue was intrarectally administered to anesthetized mice. After 6 minutes the colon was washed in tap water and colonoscopic examination was performed. Mice underwent murine colonoscopy on week 7 after disease induction.

Statistics

Data are presented as the mean ± standard error. The results were analyzed statistically using Student’s t-test.

RESULTS

Low Levels of CCL2 Inhibit the Inflammatory Response to TNBS Colitis

The powerful inhibitory effect of CCL2 on chemokine triggered migration and integrin-dependent adhesion of T-lymphocytes in vitro and in vivo suggested that this
chemokine might serve as an anti-inflammatory mediator at pM levels in IBD models.

We therefore evaluated the effect of various low dosages of CCL2 (30, 60, 120, 180 ng/mL) on development of TNBS colitis. In each group 10 mice were evaluated.

When we calculated the Wallace score for each concentration it resulted in a "U"-shaped graph (Fig. 1A). Dosages of 60 ng, 120 ng, and 180 ng significantly ameliorated the development of TNBS colitis and 60 ng had the most profound effect; this dose resulted in a Wallace score of 2.3 ± 0.4 compared with 9.2 ± 0.84 in PBS-treated mice (P < 0.001), which was significantly better than that achieved with 180 ng, 4.4 ± 0.77 (P < 0.05).

Since 60 ng of CCL2 was the most effective dose in preventing development of TNBS colitis, we repeated the experiment with another 20 mice to evaluate additional disease parameters (10 treated with 60 ng CCL2 and 10 treated with PBS). Mice treated with 60 ng CCL2 showed better survival (Fig. 1B) and the shortening of their colons was less pronounced compared with controls (7.8 ± 0.09 versus 6.5 ± 0.08 cm, P < 0.001). Moreover, CCL2-treated mice lost less weight as well (Fig. 1C).

To verify that the reduction in Wallace score and the additional favorable effects were due to a decrease in inflammation, tissue levels of the inflammatory cytokines IL-12 and TNF-α were evaluated by ELISA. Figure 1D,E, respectively, demonstrates a significant reduction of IL-12 and TNF-α in the CCL2-treated groups.

Finally, treating mice with 60 ng of CCL2 resulted in almost normal histological structure of the colon and in decreased lymphocyte infiltration (Fig. 2A) compared with severely inflamed colons of mice that were treated with PBS (Fig. 2B).

Low Levels of CCL2 Inhibit the Inflammatory Response to DSS Colitis

To prove that the beneficial effects of low levels of CCL2 are not specific to one colitis model, we next evaluated its administration in the DSS colitis model. Our preliminary studies showed that the most effective dosage for preventing inflammation in this model in C57BL/6 mice is 120 ng of CCL2 (not shown). Figure 3 shows the comparison of histological scores between PBS and CCL2 (120 ng) administrated to DSS-induced colitis mice. CCL2 significantly inhibited the development of inflammation (Fig. 3A: 2.65 ± 0.98 versus 9 ± 0.71, P < 0.001), and had a
favorable effect on survival (Fig. 3B), as well as on weight gain (Fig. 3C). Assessment of tissue inflammatory cytokine levels provided additional evidence for the amelioration of inflammation by low-dose CCL2. Levels of IL-12 (Fig. 3D), TNF-α (Fig. 3E), and interferon-γ (Fig. 3F) were found to be lower in the CCL2-treated group. Nevertheless, differences reached statistical significance only for IL-12 and IFN-γ ($P < 0.05$).

Finally, pathological examination (Fig. 4) further demonstrated the beneficial effect of CCL2 on the prevention of inflammation. Severe inflammation was detected in the untreated group (Fig. 4B), compared with almost completely normal histology in the 120 ng CCL2-treated group (Fig. 4A).

### CCL2 Inhibits LPL Migration

We have previously shown that CCL2 (at pM levels) renders both murine and human T cells defective in their ability to develop CCR7-triggered activation of LFA-1, and LFA-1-mediated adhesion strengthening to endothelial ICAM-1 both in vitro and in vivo. CCL2 also attenuates peripheral blood lymphocyte chemotaxis toward lymph node chemokines. We therefore next examined whether LPL migration is similarly inhibited by pM levels of CCL2. In this assay (Fig. 5A), migration of LPL toward CXCL12, following treatment of cells with CCL2, was...
inhibited by 45% compared to control LPL preincubated in PBS ($P < 0.003$), suggesting local migration of LPL within the gut wall compartment may be inhibited, and indicating an additional possible mechanism by which CCL2 inhibits inflammation.

Monocytes/macrophages play an important role during inflammation. To determine whether pM levels of CCL2 inhibit the inflammatory response in these IBD models by attenuating monocyte migration, we evaluated whether exposing monocytes to low levels of CCL2 inhibits monocyte migration by analyzing their migration in a Transwell assay toward CXCL12. No difference was observed in migration of monocytes that were first incubated in low levels of CCL2 (0.1 ng/mL or 1 ng/mL) versus those that were not treated by CCL2 (Fig. 5B), suggesting that the effect of CCL2 is not monocyte-dependent.

**pM Levels of CCL2 Have Favorable Effects on a CRC Model**

We have shown that administration of low doses of CCL2 has a favorable effect on colitis. We further inquired whether these effects are sustained during long-term administration in prevention of inflammation-related CRC using the AOM-DSS CRC model. In this model, cancer is induced by i.p. injection of the genotoxic carcinogen AOM. The progression of cancer is accelerated by induction of inflammation by administration of DSS through the drinking water. Balb/c mice were shown to be sensitive to

**FIGURE 6.** Murine colonoscopy on week 7 of AOM-DSS CRC model on representative Balb/c mice. Negative control mouse not treated shows normal colon mucosa (A). CCL2-treated mouse with a methylene blue staining demonstrating normal gut mucosa (B). PBS-treated mouse with methylene blue staining demonstrating an ulcer and decrease in vascular pattern (C). Colitis with fibrin in the colon of PBS-treated mouse (D). Small adenomas in CCL2-treated mouse (E). A large tumor in PBS-treated mouse (F). Fifteen mice were examined by murine colonoscopy and methylene blue staining.
development of tumors in this model. At week 7 we assessed inflammatory changes and development of tumors by murine colonoscopy on some of the mice (Fig. 6). In the CCL2-treated mice we found a decrease in inflammatory changes (Fig. 6B) and in number and size of tumors (Fig. 6E) compared to the inflammation (Fig. 6C,D) and tumors (Fig. 6F) observed in PBS-treated mice.

Mice were sacrificed on week 11 and the number of tumors was assessed macroscopically (Fig. 7A) and confirmed by histology (Fig. 7B,C). There was a significant difference in number of adenocarcinomas between the groups, 0 in the CCL2-treated group versus 7 in the PBS-treated group ($P < 0.028$). Analysis of the total number of neoplasias (adenomas and carcinomas, by histology) demonstrated an even larger difference between the two groups, four in the CCL2-treated group versus 19 tumors in the PBS-treated group ($P < 0.005$) (Fig. 7D).

In order to evaluate whether the mechanism accounting for the decreased number of tumors in the CCL2-treated group is due to decreased inflammation, we assessed inflammation on the histological slides of the two groups by a pathologist blinded to the treatment group of each sample (E.B.). The histologic inflammatory score in the PBS group ($5.92 \pm 0.69$) was significantly higher than that in the CCL2-treated group ($1.8 \pm 0.9$; $P < 0.002$; Fig. 7E).

Monocytes/macrophages play an important role during inflammation and in the pathogenesis of CRC, either by supporting tumor growth (tumor-associated macrophages) or by destruction of the tumor through the immune system by anti-tumor effects. However, since CCL2 did not attenuate monocyte migration, we suggest that the effect of CCL2 on tumor formation is not monocyte-dependent.
DISCUSSION

In this study we demonstrated that low doses of CCL2 inhibit development of colitis in two models of IBD, induced by DSS and by TNBS. We found that administration of pM levels of CCL2 almost completely inhibits the development of colitis as assessed by clinical score, histologically, and as reflected by tissue cytokine levels. Moreover, CCL2 treatment improved survival in the treated groups. Disease scores of the PBS-treated mice indicate that the induced colitis was severe. The effect of CCL2 was dose-dependent; while the lowest concentration (30 ng) had only minimal effect on colitis score, dosages of 60–120 ng optimally obliterated colitis.

Intriguingly, long-term administration of low-dose CCL2 in an inflammation-enhanced carcinoma model (AOM-DSS model) almost completely prevented development of tumors compared with PBS-treated mice.

Treatment of colitis is a clinical challenge. Research efforts have been aimed at all levels of known mechanisms, targeting antigens, lymphocytes and dendritic cells, cell trafficking, and cytokines. In addition, targeting proinflammatory cytokines has proved to be an effective strategy in the treatment of IBD. One of the most important advances in the last decade in the treatment of IBD is the use of anti-TNF-α agents, which were found to be effective against both UC and CD. Other emerging treatments that have already shown their effectiveness in animal models, and in preliminary human trials target IL-12 as well as IFN-γ.

In this study we show that treatment with a low dose of CCL2 ameliorates levels of these cytokines as well as preventing colitis progression. Our studies suggest that CCL2 has a dual role in the inflammatory process. We have previously shown that CCL2 exerts inhibitory effects on B and T cells, both in vivo and in vitro. Here we show that CCL2 has a similar inhibitory effect on LPL isolated from the colon. Inhibition of local migration of LPL may represent an additional mechanism by which exposure of lymphocytes to pM CCL2 levels interferes with the inflammatory process in the gut wall. CCL2 downregulates homing of naïve T cells to the lymph nodes, and thereby reduces the exposure of these T cells to antigen, and as a consequence their activation is prevented. In addition, CCL2 dramatically inhibits migration of effector cells, probably to sites of inflammation where they exert their function. Thus, exposure of T cells to pM levels of CCL2 probably inhibits both the sensitization of naïve T cells and migration of effector cells, which together dramatically attenuate the inflammatory response.

A major threat to IBD patients is the risk of CRC. It has been suggested that there is a correlation between the severity of colitis and CRC risk. Use of 5-aminosalicylate (5-ASA) preparations has an apparently protective effect against CRC in UC patients, despite only a minor effect on inflammatory score in this disease. Thus, the mechanisms responsible for cancer in the inflammatory state are incompletely understood. However, it was recently shown that in a chronic DSS colitis model inflammation has a central role in cancer development by inducing oxidative stress, resulting in genetic mutations and DNA damage.

We suggested that administration of low-dose CCL2 may prevent the development of CRC by at least two mechanisms, primarily by decreasing the severity of inflammation through downregulation of migration of effector lymphocytes to the colon, CCL2 could decrease tumorogenic mechanisms, such as oxidative stress. Second, by affecting migration of monocytes, as well, either through inhibition of monocyte migration to the inflamed gut, thereby decreasing the number of potential tumor-associated macrophages that are crucial for the support of tumor development, or by regulating chemotaxis of antitumor monocytes. Indeed, CCL2 treatment dramatically reduced the number of tumors in the CCL2-treated mice and, similar to the acute colitis models, inflammation was significantly attenuated in those mice as well. However, we found that low levels of CCL2 had no effect on migration of monocytes and, thus, we believe that the principal mechanism for inhibition of tumor development is due to the inhibition of inflammatory colitis.

Our results demonstrate that CCL2, although a key inflammatory chemokine, does not always augment inflammatory processes. Rather, pM levels of circulating CCL2 can exert global suppressive effects on murine inflammatory colitis and may be clinically effective as an antiinflammatory and tumor-suppressing agent in acute and chronic colitis models in mice. Further research is needed to understand these effects in murine colitis genetic models and perhaps, in the future, in humans.

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