

The matricellular protein CCN1 promotes mucosal healing in murine colitis through IL-6

JS Choi¹, K-H Kim¹ and LF Lau¹

The matricellular protein CCN1 (CYR61) is known to function in wound healing and is upregulated in colons of patients with Crohn's disease and ulcerative colitis, yet its specific role in colitis is unknown. Here we have used *Ccn1*^{dm/dm} knockin mice expressing a CCN1 mutant unable to bind integrins $\alpha_6\beta_1$ and $\alpha_M\beta_2$ as a model to probe CCN1 function in dextran sodium sulfate (DSS)-induced colitis. *Ccn1*^{dm/dm} mice exhibited high mortality, impaired mucosal healing, and diminished interleukin-6 (IL-6) expression during the repair phase of DSS-induced colitis compared with wild-type mice, despite having comparable severity of initial inflammation and tissue injury. CCN1-induced IL-6 expression in macrophages through integrin $\alpha_M\beta_2$ and in fibroblasts through $\alpha_6\beta_1$, and IL-6 promoted intestinal epithelial cell (IEC) proliferation. Administration of purified CCN1 protein fully rescued *Ccn1*^{dm/dm} mice from DSS-induced mortality, restored IEC proliferation and enhanced mucosal healing, whereas delivery of IL-6 partially rectified these defects. CCN1 therapy accelerated mucosal healing and recovery from DSS-induced colitis even in wild-type mice. These findings reveal a critical role for CCN1 in restoring mucosal homeostasis after intestinal injury in part through integrin-mediated induction of IL-6 expression, and suggest a therapeutic potential for activating the CCN1/IL-6 axis for treating inflammatory bowel disease.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis, two major subtypes of inflammatory bowel disease (IBD), are chronic, relapsing, and remitting inflammatory disorders of the gastrointestinal tract that affect 1.4 million people in the US.¹ These medically incurable diseases of poorly defined etiology generally begin in young adulthood and continue throughout life, often requiring lifelong management. Traditional treatment modalities have aimed at dampening inflammation in the gastrointestinal tract to alleviate symptoms in patients, and this rationale prompted the development of monoclonal antibodies (mAbs) targeting the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) for the treatment of IBD. Although blockade of TNF- α induces clinical remission and has become a critical component in the therapeutic arsenal for IBD, many patients (~40%) do not respond, lose their response during treatment, or develop complications due to side effects.^{2,3} Patients who are refractory to therapies may eventually require colectomy and are at increased risks of developing colorectal cancer, causing significant morbidity. Thus, there is an urgent need for alternative treatment options, and considerable effort has been focused on the identification of novel therapeutic targets.⁴

Among the targets under investigation for IBD therapy is interleukin-6 (IL-6), a multifunctional cytokine expressed by diverse cell types during inflammation.^{5,6} IL-6 is induced upon intestinal injury in the blood and colonic tissue of IBD patients, and is thought to be involved in the pathogenesis of IBD by inducing T-cell activation and suppressing T-cell apoptosis.^{6,7} Consistent with this notion, mAbs against IL-6 receptor (IL-6R) prevent T cell-mediated murine colitis,^{7,8} and a pilot clinical trial using anti-IL-6R mAbs showed symptomatic improvement in patients with CD.⁹ Paradoxically, IL-6-deficient mice suffer increased mortality, morbidity, and mucosal damage in infectious and non-infectious models of colitis, suggesting that IL-6 may have pleiotropic and potentially antithetical roles in IBD.^{10,11} Therefore, further elucidation of the regulation and function of IL-6 in colitis is likely needed to optimize its therapeutic potential.

CCN1 (CYR61), a secreted matricellular protein of ~40 kDa, is emerging as an important regulator of tissue homeostasis and injury repair.¹² Acting primarily through direct binding to integrin receptors, CCN1 regulates diverse cellular functions in a cell type- and context-dependent manner.¹² Whereas CCN1

¹Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois, USA. Correspondence: LF Lau (LFLau@uic.edu)

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is essential for angiogenesis and cardiovascular development during embryogenesis,¹³ its expression has been linked to inflammation and tissue repair in adulthood.^{14,15} However, the specific functions of CCN1 in various inflammation-associated pathologies are only beginning to be appreciated. For example, in cutaneous wound healing and in chronic liver injuries induced by hepatotoxin or cholestasis, CCN1 functions to diminish and restrict tissue fibrosis in the maturation phase of tissue repair by triggering cellular senescence in activated myofibroblasts.^{16,17} CCN1 can also contribute to inflammatory damage by inducing the expression of pro-inflammatory cytokines in macrophages and enhancing the cytotoxicity of TNF family cytokines.^{18–20} Importantly, CCN1 expression is elevated in biopsies of patients with ulcerative colitis or CD, and in mice with experimental colitis.²¹ However, the precise function of CCN1 in IBD remains unknown.

Here we provide the first evidence that CCN1 has a critical role in promoting recovery and mucosal healing in colitis, in part through integrin-mediated induction of *IL-6* expression during the repair phase. Moreover, administration of CCN1 protein accelerated recovery and mucosal healing in wild-type (WT) and *Ccn1* mutant mice. Our findings reveal CCN1 as a critical regulator of mucosal healing in colitis, uncover the importance of CCN1-induced *IL-6* in intestinal–epithelial restitution, and suggest a therapeutic potential in activating the CCN1/*IL-6* axis for the treatment of IBD.

RESULTS

Ccn1^{dm/dm} mice suffer increased mortality as well as impaired recovery and mucosal healing upon DSS challenge

Immunohistochemical analysis showed that CCN1 protein was mainly associated with the surface epithelial cells in the normal colon, but was detected in the entire mucosal epithelium when mice were challenged with 5% DSS to induce colitis²² (Supplementary Figure S1A online). This expression pattern was confirmed in CCN1-EGFP (enhanced green fluorescent protein) mice²³ in which *Ccn1* expression was visualized by anti-GFP staining, with GFP restricted to terminally differentiated intestinal epithelial cells (IECs) in the normal colon but expanded to the entire crypt upon DSS exposure (Supplementary Figure S1B). Consistently, *Ccn1* mRNA was increased in the colon of DSS-challenged mice compared with untreated mice ($P < 0.01$; Supplementary Figure S1C), suggesting that CCN1 is actively regulated during colitis. CCN1 exerts diverse effects in various cell types through distinct integrins, among them $\alpha_M\beta_2$ in macrophages and $\alpha_6\beta_1$ in fibroblasts.¹² To elucidate the role of CCN1 in IBD, we have used *Ccn1*^{dm/dm} knockin mice in which the *Ccn1* genomic locus is replaced by an allele encoding a CCN1 double mutant protein (DM-CCN1) disrupted in its overlapping binding sites for integrins $\alpha_M\beta_2$ and $\alpha_6\beta_1$.^{24,25} This genetic model circumvents the embryonic lethality of *Ccn1*-null mice,¹³ and avoids potential limitations of cell type-specific deletions as CCN1 may be secreted by multiple cell types in the colonic tissue microenvironment. *Ccn1*^{dm/dm} mice are viable, fertile,

morphologically, and behaviorally normal,^{16,18} and display a normal intestinal histology in the absence of pathogenic insults.

WT, *Ccn1*^{wt/wt} and *Ccn1*^{dm/dm} mice were subjected to a single or repeated cycles of DSS challenge, which induced acute or chronic colitis, respectively, in an established protocol for the induction of intestinal inflammation.²² When challenged with 5% DSS (w/v), only 60 and 20% of *Ccn1*^{dm/dm} mice survived through the first and third cycles of DSS feeding, respectively, compared with 95 and 80% of WT mice ($P < 0.05$ and $P < 0.001$, respectively; Figure 1a), suggesting that CCN1 serves an important protective role in both acute and chronic DSS-induced colitis. However, increased lethality of *Ccn1*^{dm/dm} mice was not due to exacerbated disease as judged by body weight loss, disease activity index (DAI), and colonic shortening (Figure 1b–d). The intestinal barrier function after DSS feeding, as evaluated by detection of gavaged fluorescein isothiocyanate-conjugated dextran in the serum, was also similar between the two genotypes (Figure 1e). Histological analyses of the distal colon showed comparable crypt loss and epithelial damage (Figure 1f) with similar numbers of apoptotic cells (Figure 1g). Lastly, myeloperoxidase activity in the colonic tissue was comparable in WT and *Ccn1*^{dm/dm} mice, indicating similar extent of neutrophil infiltration into the mucosal epithelium and lamina propria (Figure 1h). These results show that CCN1 functions mediated through its $\alpha_6\beta_1/\alpha_M\beta_2$ binding sites are critical for animal survival in DSS-induced colitis, although loss of these functions did not result in exacerbated morbidity or inflammatory damage.

To test the hypothesis that CCN1 may function to promote recovery and mucosal healing in colitis, we lowered the dose of DSS to 3.5% (w/v) to avoid death of *Ccn1*^{dm/dm} mice, and examined recovery (as judged by DAI) and mucosal healing (as judged by epithelial restitution). When administered 3.5% DSS, *Ccn1* mRNA expression was elevated throughout the recovery phase compared with the untreated mice (day 8 and 14, $P < 0.01$; day 11, $P < 0.05$; Supplementary Figure S1D). As expected, WT and *Ccn1*^{dm/dm} mice showed comparable body weight loss, DAI, colonic shortening, and histological score during the onset of disease from days 0 to 8 (Figure 2a–d). However, as recovery commenced on day 9 and the DAI began to decline in WT, *Ccn1*^{dm/dm} mice experienced a prolonged disease course with elevated DAI (Figure 2b), slow recovery of body weight and colon length (Figure 2a and c), and unrelenting epithelial erosion and ulceration through day 14 ($P < 0.001$; Figure 2d), indicating that *Ccn1*^{dm/dm} mice suffer impaired recovery and mucosal healing from colitis.

CCN1 protein rescues *Ccn1*^{dm/dm} mice from lethality and accelerates mucosal healing

To assess whether increased mortality of *Ccn1*^{dm/dm} mice is indeed due to compromised CCN1 activity during recovery, we tested the effect of supplying WT-CCN1 protein during the recovery phase. After 5 days of 5% DSS feeding, *Ccn1*^{dm/dm} mice were injected (intraperitoneally) daily with purified WT-CCN1 for 5 consecutive days. Strikingly, 90% of CCN1-treated *Ccn1*^{dm/dm} mice survived through three cycles of DSS challenge,

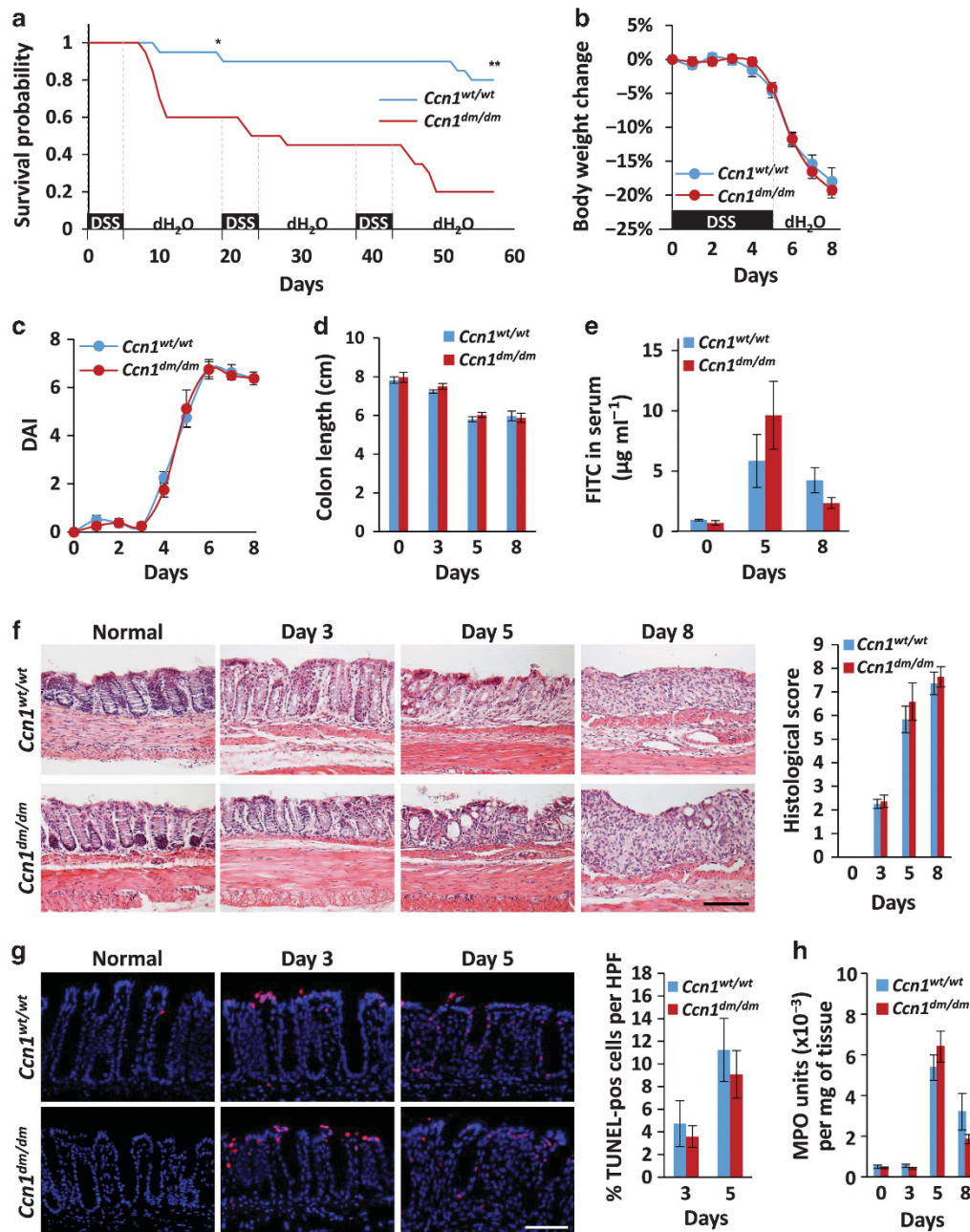


Figure 1 Higher mortality but not morbidity in DSS-challenged *Ccn1*^{dm/dm} mice. (a) Wild-type and *Ccn1*^{dm/dm} mice ($n = 20$ per genotype) were given 5% (w/v) of DSS water for 5 days followed by 14 days of recovery with normal drinking water as indicated. Survival was monitored through three cycles of DSS challenge. $*P < 0.05$, $**P < 0.001$ (log-rank test). The disease course of acute colitis was monitored by measuring (b) body weight change, (c) DAI, and (d) colon lengths on indicated days after initiation of DSS exposure. (e) Orally gavaged FITC particles were detected in the serum to evaluate intestinal barrier integrity ($n = 3$). (f) Mucosal damage was assessed by H&E staining of paraffin-embedded sections of the distal colon; histological scores are shown on right ($n = 4-6$). Scale bar = 100 μm . (g) Cell death in the distal colon was measured by TUNEL, and cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Quantitation is shown on the right ($n = 3$). Scale bar = 50 μm . (h) MPO activity from colonic tissue ($n = 3-6$). DAI, disease activity index; DSS, dextran sodium sulfate; FITC, fluorescein isothiocyanate; MPO, myeloperoxidase.

compared with only 20% survival in vehicle-treated controls ($P < 0.01$; **Figure 3a**) and 80% in WT mice (**Figure 1a**). Thus, CCN1 therapy fully restored survival in *Ccn1*^{dm/dm} mice to WT level. To evaluate the role of CCN1 during recovery, mice fed 3.5% DSS were similarly injected with WT-CCN1. These mice recovered their body weight ($P < 0.05$) and colon length

significantly faster than vehicle controls (day 8, $P < 0.05$; days 11 and 14, $P < 0.01$; **Figure 3b and c**), comparable to WT animals (**Figure 2a and c**). Histology showed that CCN1-treated *Ccn1*^{dm/dm} mice completely restored the crypt architecture in the distal colon as early as day 11 after initiation of DSS exposure ($P < 0.001$; **Figure 3d**), further

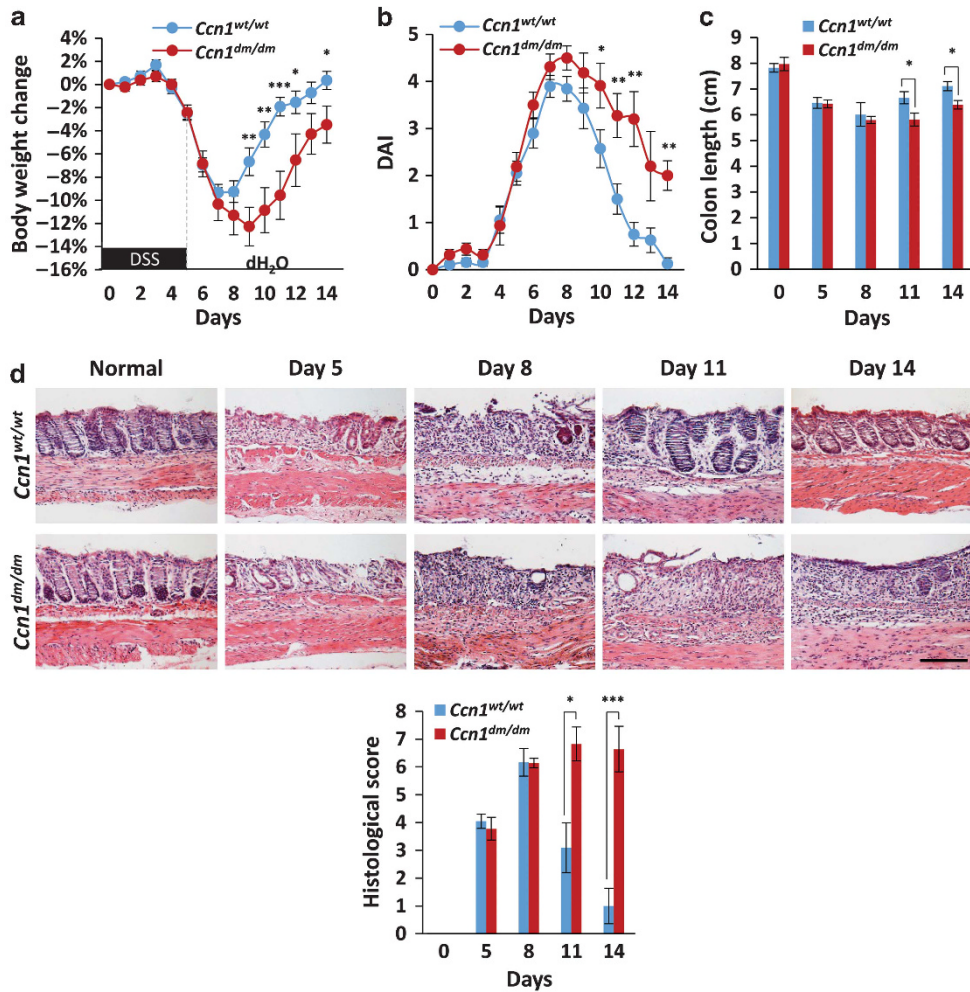


Figure 2 Impaired recovery and mucosal healing in DSS-challenged *Ccn1^{dm/dm}* mice. Wild-type and *Ccn1^{dm/dm}* mice were given DSS water (3.5% w/v) for 5 days followed by normal drinking water. (a) Changes in body weight and (b) DAI were recorded daily, and (c) colonic length was measured ($n = 7-8$). (d) H&E staining of paraffin-embedded sections of the distal colon from mice 5, 8, 11, and 14 days after initiation of DSS feeding. Histological scores are shown on bottom ($n = 4-6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bar = 100 μ m. DAI, disease activity index; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin.

indicating that CCN1 promotes tissue repair after DSS-induced colitis.

Proliferation of IECs is a critical component in intestinal tissue regeneration. Therefore, we postulated that CCN1 may have a direct role in intestinal tissue repair by promoting proliferation of colonic epithelial cells. To test this possibility, we treated young adult mouse colon (YAMC) IECs with bovine serum albumin, purified WT-CCN1 or DM-CCN1. Surprisingly, neither WT-CCN1 nor DM-CCN1 had any effect on the proliferation of YAMCs in culture as judged by cell number counts and Ki67 staining (Supplementary Figure S2), suggesting that CCN1 may indirectly mediate tissue repair after DSS-induced injury.

CCN1 induces IL-6 *in vivo* and in macrophages and fibroblasts

As cytokines are important in inflammation and repair, we measured cytokine expression in the WT and *Ccn1^{dm/dm}* colon

after 3.5% DSS challenge. Most differences were small: WT mice transiently expressed somewhat higher levels of the pro-inflammatory cytokines interferon (*IFN*)- γ and *IL-17*, but *TNF- α* expression was the same in both genotypes (Supplementary Figure S3A). Likewise, *Ccn1^{dm/dm}* mice showed similar or higher levels of the anti-inflammatory cytokines *IL-4*, *TGF- β* , and *IL-22*, whereas *IL-10* expression was the same (Supplementary Figure S3B). Despite the expression of reparative cytokines such as *IL-22*, *IL-10*, and *TGF- β* ,²⁶ *Ccn1^{dm/dm}* mice showed impaired mucosal repair (Figure 2), suggesting that expression of these cytokines was insufficient to initiate efficient recovery and repair in *Ccn1^{dm/dm}* mice. By contrast, whereas *IL-6* expression was similar in *Ccn1^{dm/dm}* and WT mice at day 5 when inflammatory damage began to be observed, it was dramatically lower in *Ccn1^{dm/dm}* mice (>7-fold) by day 8 when damage had peaked and repair began (Figure 4a). Thus, *Ccn1^{dm/dm}* mice suffered a large deficit in *IL-6* expression in the repair phase but not the initiation phase

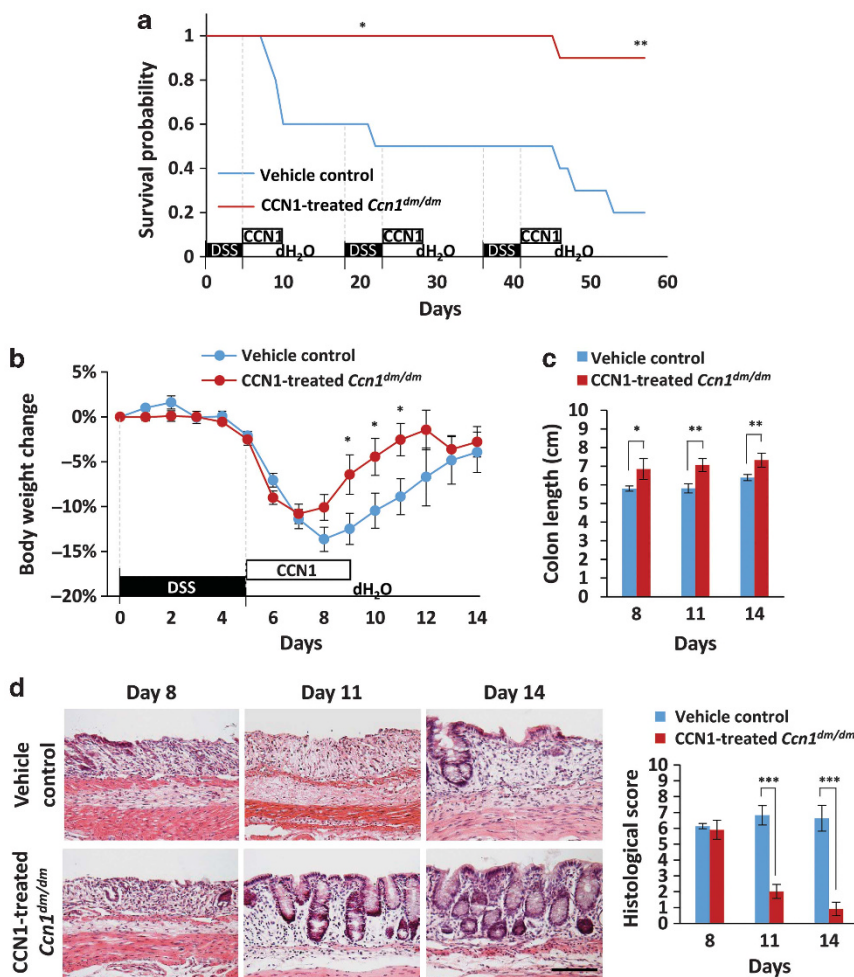


Figure 3 Treatment with WT-CCN1 promotes survival and mucosal repair. (a) *Ccn1^{dm/dm}* mice ($n = 10$) were injected i.p. with 5 μg of WT-CCN1 or vehicle control daily for 5 days after each cycle of 5% DSS feeding and monitored for survival. $*P < 0.05$, $**P < 0.01$ (log-rank test). (b) Mice similarly treated except with 3.5% DSS were monitored for body weight change and (c) colon lengths ($n = 6-8$). (d) Mucosal recovery was evaluated by H&E staining of paraffin-embedded colonic tissue. Histological scores are shown on right. Data are presented as mean \pm s.e.m.; $n = 3-4$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Scale bar = 100 μm . DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; i.p., intraperitoneally; WT, wild type.

of colitis. *IL-6* expression remained induced but at a lower level at later times, and the difference between the two genotypes diminished, suggesting an exquisite control of *IL-6* by CCN1 in colitis. To assess the ability of CCN1 to induce the expression of *IL-6* and other cytokines *in vivo*, we injected WT-CCN1 intraperitoneally into WT mice in the absence of induced colitis. We found that WT-CCN1 upregulated serum *IL-6* level in a time- and dose-dependent manner as judged by enzyme-linked immunosorbent assay (ELISA), whereas DM-CCN1 had no effect ($P < 0.05$; **Figure 4b** and **c**), indicating that CCN1 acts through its $\alpha_6\beta_1/\alpha_M\beta_2$ binding sites to induce *IL-6*. Moreover, we did not detect an increase in systemic levels of *IL-1\alpha*, *IL-1\beta*, *IL-2*, *IL-4*, *IL-12*, *IL-17\alpha*, *IFN-\gamma*, *TNF-\alpha*, and granulocyte-macrophage colony-stimulating factor after injection of WT-CCN1 protein by ELISA, indicating that *IL-6* is a specific target of CCN1 regulation (**Figure 4b**). In the context of DSS-induced colitis, WT-CCN1 elevated *IL-6* in blood from 18 to 787 pg/ml in *Ccn1^{dm/dm}* mice on day 8 after DSS exposure ($P < 0.05$;

Figure 4d), further confirming that CCN1 can induce *IL-6* during the repair phase.

IL-6 is thought to be produced by several cell types in the lamina propria, including macrophages and fibroblasts.²⁷ To identify the cellular source of CCN1-induced *IL-6*, we first tested CCN1 functions in I-13.35 macrophages. WT-CCN1-induced *IL-6* mRNA and protein levels in these cells, whereas DM-CCN1 did not (**Figure 5a** and **b**). Pre-incubation of macrophages with mAbs against integrin α_M or β_2 abrogated CCN1-induced *IL-6* expression, showing the requirement of $\alpha_M\beta_2$ (**Figure 5c**). In addition, WT-CCN1, but not DM-CCN1, greatly upregulated *IL-6* mRNA and protein levels in 18Co human colonic fibroblasts (**Figure 5d** and **e**). As CCN1 and *TNF-\alpha* are known to be co-expressed in inflammation, we tested whether they act synergistically to regulate *IL-6*. Remarkably, CCN1 in combination with *TNF-\alpha* induced *IL-6* mRNA in 18Co cells by nearly 250-fold, or 15- and 23-fold higher than stimulation by CCN1 or *TNF-\alpha* alone, respectively

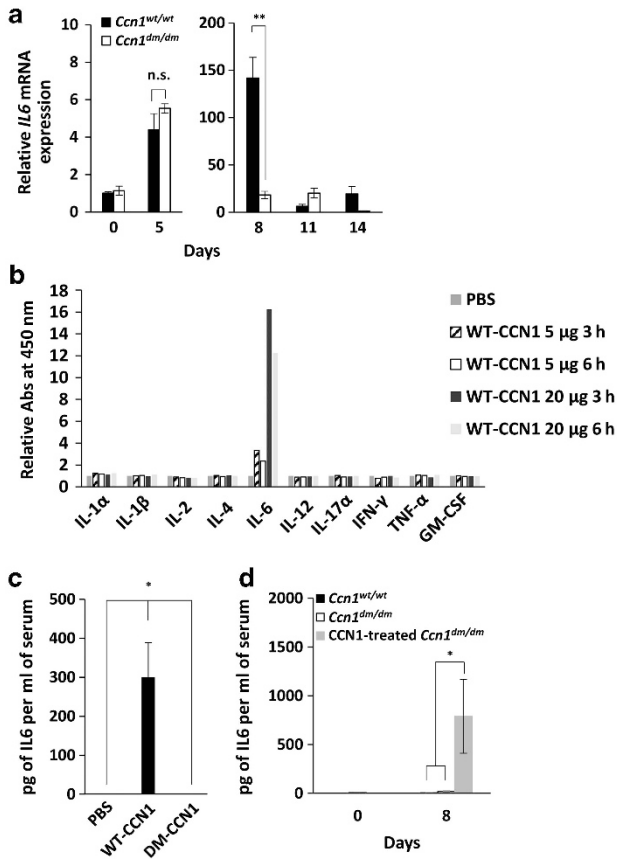


Figure 4 WT-CCN1, but not DM-CCN1, induces IL-6 *in vivo*. **(a)** IL-6 mRNA in the colon of wild-type and *Ccn1^{dm/dm}* mice treated with 3.5% DSS for 5 days was measured by qRT-PCR, and normalized to healthy wild-type colon with cyclophilin E as internal reference. Data are shown as mean \pm s.d.; $n = 3-4$. ** $P < 0.003$. **(b)** IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-12, IL-17 α , IFN- γ , TNF- α , and GM-CSF protein was measured by ELISA in the serum of wild-type mice at indicated times after a single injection i.p. of purified WT-CCN1 protein (5 or 20 μ g), or **(c)** 24 h after i.p. injection of 10 μ g of WT-CCN1, DM-CCN1 protein, or PBS ($n = 4$). **(d)** IL-6 was measured from the serum of 3.5% DSS-challenged wild type and *Ccn1^{dm/dm}* mice with and without CCN1 treatment by ELISA. Data are presented as mean \pm s.e.m.; $n = 7$; * $P < 0.05$. DM, double mutant; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; H&E, hematoxylin and eosin; IFN- γ , interferon; IL, interleukin; i.p., intraperitoneally; NS, not significant; qRT-PCR, quantitative reverse transcription-PCR; TNF- α , tumor necrosis factor- α ; WT, wild type.

(Figure 5d). This synergistic effect was also observed by measuring IL-6 protein levels in the conditioned media of 18Co cells (Figure 5e). Consistent with integrin $\alpha_6\beta_1$ being the principal CCN1 receptor in fibroblasts,²⁸ pretreatment of 18Co cells with anti- α_6 mAb blocked CCN1-induced IL-6 expression (Figure 5f). Although α_6 can heterodimerize with β_1 and β_4 subunits, β_4 is predominantly localized to hemidesmosomes and is not expressed in fibroblasts. Together, these results show that CCN1 upregulates IL-6 mRNA and protein in macrophages through $\alpha_M\beta_2$ and in fibroblasts through $\alpha_6\beta_1$, consistent with the deficiency in IL-6 expression in *Ccn1^{dm/dm}* mice (Figure 4b) and inability of DM-CCN1 to induce IL-6 *in vivo* (Figure 4d).

CCN1-induced IL-6 promotes intestinal epithelial healing

To determine whether deficits in IL-6 expression contributed to impaired healing in *Ccn1^{dm/dm}* mice, we delivered five daily injections of recombinant IL-6 (rIL-6) to *Ccn1^{dm/dm}* mice following 5% DSS feeding. Remarkably, rIL-6 treatment increased survival from 60 to 95% in the first cycle of DSS challenge ($P < 0.01$) and from 20 to 65% by the third cycle ($P < 0.01$; Figure 6a). In *Ccn1^{dm/dm}* mice fed 3.5% DSS, rIL-6 treatment also enhanced body weight recovery, although the numbers did not reach statistical significance (Figure 6b). Histological evaluation showed that rIL-6 treatment accelerated mucosal restitution in *Ccn1^{dm/dm}* mice compared with vehicle controls, although improvements in histological scores still lagged behind WT mice (Figures 2d and 6c). These results indicate that IL-6 treatment partially rescued lethality and accelerated intestinal repair in *Ccn1^{dm/dm}* mice following DSS-induced colitis.

To examine whether CCN1-induced IL-6 stimulated cell proliferation during tissue repair, we counted the number of proliferating cells in tissue sections of WT, *Ccn1^{dm/dm}*, rIL-6-treated and CCN1-treated *Ccn1^{dm/dm}* mice stained with antibodies against proliferating cell nuclear antigen, a proliferation marker. There were significantly fewer proliferating cells in *Ccn1^{dm/dm}* mice compared with WT mice ($P < 0.01$), and treatment with rIL-6 or WT-CCN1 partially or completely restored cell proliferation, respectively, to WT levels (Figure 6d). Furthermore, proliferating cell nuclear antigen-positive cells co-localized with E-cadherin, an epithelial cell marker, in WT and rIL-6 or CCN1-treated *Ccn1^{dm/dm}* mice, but not in *Ccn1^{dm/dm}* mice, indicating that there was a deficit in IECs undergoing proliferation in *Ccn1^{dm/dm}* mice that was rectified by treatment with rIL-6 or CCN1.

To assess the activity of IL-6 in IEC proliferation directly, YAMC cells were treated with bovine serum albumin, rIL-6, or epidermal growth factor as a positive control and stained for Ki67. Treatment of YAMCs with rIL-6 increased Ki67-positive cells in a dose-dependent manner and to the same extent as epidermal growth factor, a known mitogen for IECs, when both were applied at 100 ng ml⁻¹ (Figure 6e). Pretreatment of cells with anti-IL-6R α antibody 1 h before rIL-6 addition blocked the increase in Ki67-positive cells, indicating that rIL-6 acts directly through its specific receptor to enhance IEC proliferation.

CCN1 promotes recovery and mucosal healing in DSS-challenged WT animals

To determine whether the healing effect of CCN1 in colitis is limited to mice with deficiency in CCN1 activity or IL-6 expression, and to test whether CCN1 may have therapeutic value in treating colitis, we delivered five daily injections of purified WT-CCN1 (10 μ g intraperitoneally) into WT animals with colitis induced by 5% DSS. WT-CCN1 protein accelerated body weight recovery and attenuated DAI from days 8 to 11 in WT mice with colitis (Figure 7a and b). To investigate whether the improved clinical outcome was a result of enhanced intestinal mucosal repair, we harvested the distal colon on day 11 and evaluated for tissue injury. Histology of the distal colon

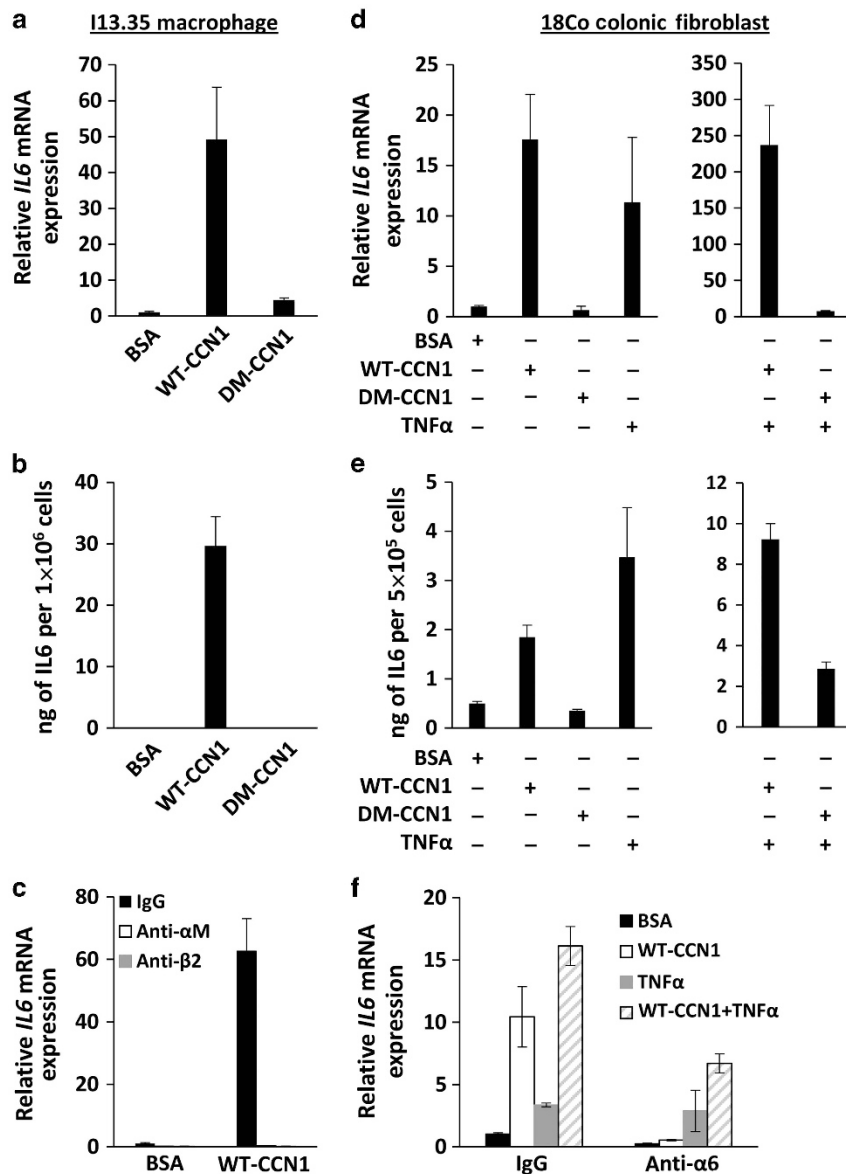


Figure 5 WT-CCN1, but not DM-CCN1, induces IL-6 in macrophages and fibroblasts. (a) *IL-6* mRNA was measured by qRT-PCR and (b) IL-6 protein from conditioned media was measured by ELISA in serum-starved (overnight) I13.35 macrophages treated with $5 \mu\text{g ml}^{-1}$ of purified WT-CCN1 or DM-CCN1, or BSA for 24 h. (c) I13.35 macrophages were incubated with blocking mAbs against integrin α_M ($50 \mu\text{g ml}^{-1}$) or β_2 ($50 \mu\text{g ml}^{-1}$) 1 h prior to treatment with WT-CCN1 for 24 h. *IL-6* mRNA was measured by qRT-PCR. (d) IL-6 mRNA was measured by qRT-PCR and (e) IL-6 protein from conditioned media was measured by ELISA in serum-starved 18Co fibroblasts treated with $5 \mu\text{g ml}^{-1}$ of purified WT-CCN1, DM-CCN1, BSA, or 25 ng ml^{-1} of TNF- α for 24 h. (f) 18Co fibroblasts were incubated with blocking mAb against integrin α_6 for 1 h, then treated with WT-CCN1, TNF- α , or BSA for 24 h as above. *IL-6* mRNA was measured by qRT-PCR. Data shown as mean \pm s.d. of triplicate experiments. BSA, bovine serum albumin; DM, double mutant; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IFN- γ , interferon- γ ; IL, interleukin; mAbs, monoclonal antibodies; qRT-PCR, quantitative reverse transcription-PCR; TNF- α , tumor necrosis factor- α ; WT, wild type.

showed significantly fewer areas of ulceration and a higher number of organized crypts compared with vehicle controls ($P < 0.01$; **Figure 7c**). Thus, CCN1 therapy enhances recovery and promotes mucosal healing in colitis even in WT mice with a fully functional CCN1.

DISCUSSION

Traditional treatment modalities for IBD have aimed at dampening inflammation in the gastrointestinal tract to

alleviate symptoms in patients. Recent studies have shown that IBD patients who achieve and maintain mucosal healing have more favorable long-term outcomes than patients who do not, and thus mucosal healing is emerging as a critical endpoint in clinical trials and practice.^{6,29} Although anti-TNF- α therapy for IBD was initially developed to reduce inflammation, it was found to improve symptoms as well as induce endoscopic remission and sustained mucosal healing.^{30,31} In this report, we provide the first evidence that CCN1 promotes mucosal healing

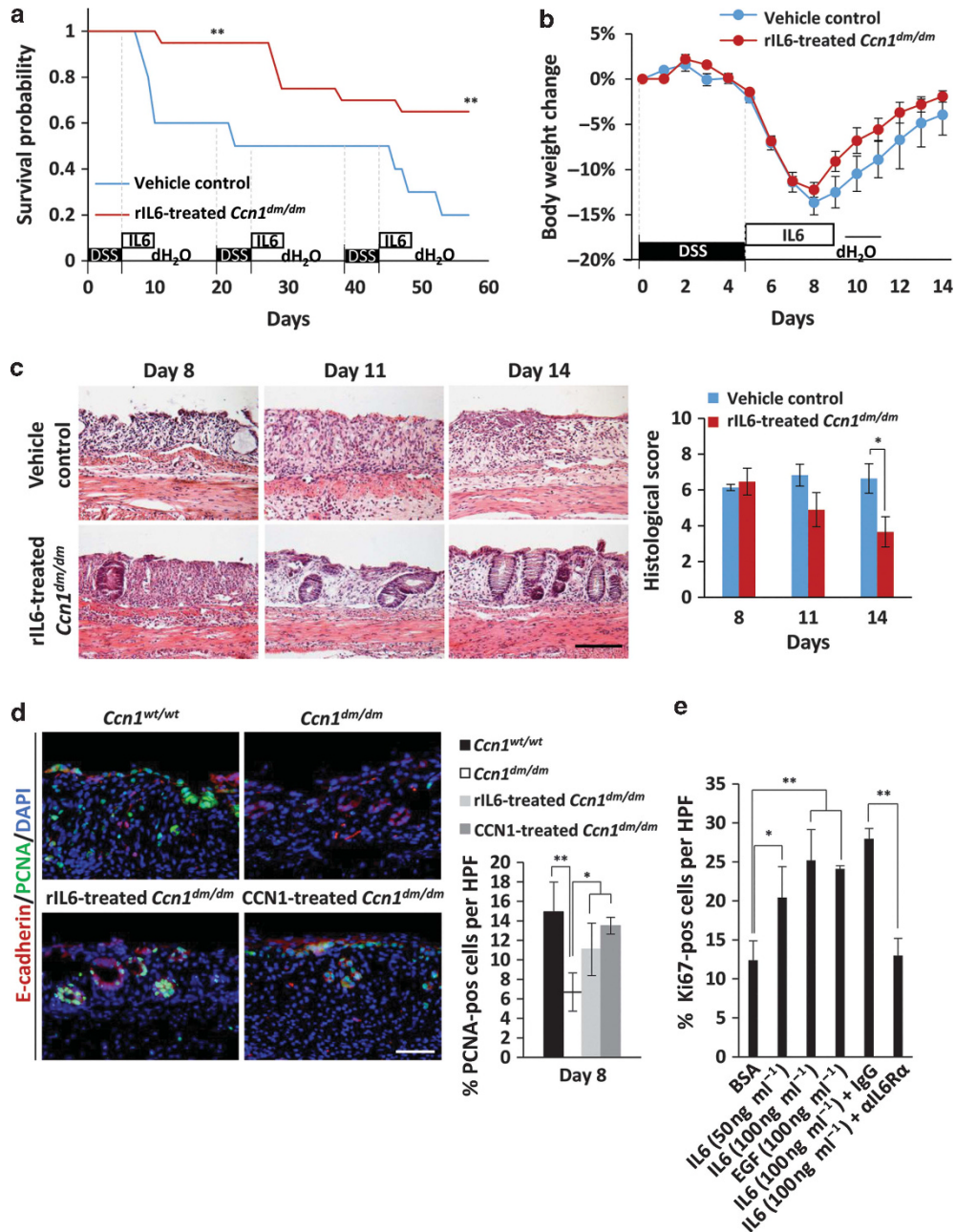


Figure 6 Delivery of IL-6 enhances survival and mucosal repair of *Ccn1^{dm/dm}* mice by restoring IEC proliferation. **(a)** *Ccn1^{dm/dm}* mice ($n=20$) were injected i.p. with rIL-6 (100 ng) or vehicle control daily for 5 consecutive days after each cycle of 5% DSS feeding and were monitored for survival. $**P<0.01$ (log-rank test). **(b)** Body weight change was monitored daily in 3.5% DSS-challenged *Ccn1^{dm/dm}* mice treated with 100 ng of rIL-6 or vehicle control for 5 consecutive days after DSS feeding. **(c)** Mucosal damage in mice treated in **b** was evaluated by H&E staining of paraffin-embedded sections of the distal colon. Histological scores are shown on the right ($n=3-4$; $*P<0.05$). Scale bar = 100 μm . **(d)** Wild-type and *Ccn1^{dm/dm}* mice were challenged with 3.5% DSS, and *Ccn1^{dm/dm}* mice were further treated with WT-CCN1 (5 μg) or rIL-6 (100 ng) as indicated. PCNA and E-cadherin was detected by immunofluorescence microscopy to assess IEC proliferation. DAPI was used for counterstaining. Quantitation of PCNA staining is shown on right ($n=3-4$; $*P<0.05$, $**P<0.01$). Scale bar = 50 μm . **(e)** YAMC cells were treated with BSA, IL-6 (50 or 100 ng ml⁻¹), or EGF (100 ng ml⁻¹) for 24 h and stained for Ki67 by immunofluorescence and quantitated. Where indicated, YAMC cells were pre-incubated with blocking polyclonal antibodies for IL-6 receptor- α (IL-6R α) or IgG for 1 h before IL-6 addition. Data are shown as mean \pm s.d.; $*P<0.05$, $**P<0.01$. Scale bar = 300 μm . BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; DSS, dextran sodium sulfate; EGF, epidermal growth factor; H&E, hematoxylin and eosin; IEC, intestinal epithelial cell; IFN- γ , interferon; IgG, immunoglobulin G; IL, interleukin; i.p., intraperitoneally; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative reverse transcription-PCR; rIL-6, recombinant IL-6; TNF- α , tumor necrosis factor- α ; WT, wild type; YAMC, young adult mouse colon.

in murine colitis. Administration of exogenous CCN1 can accelerate mucosal restitution from colitis in both WT and *Ccn1* mutant mice, underscoring a therapeutic potential for CCN1 in IBD.

As a multifunctional matricellular protein, secreted CCN1 is associated with the extracellular matrix and regulates diverse cellular functions through interaction with distinct integrins in various cell types.¹² Although gene array studies have linked

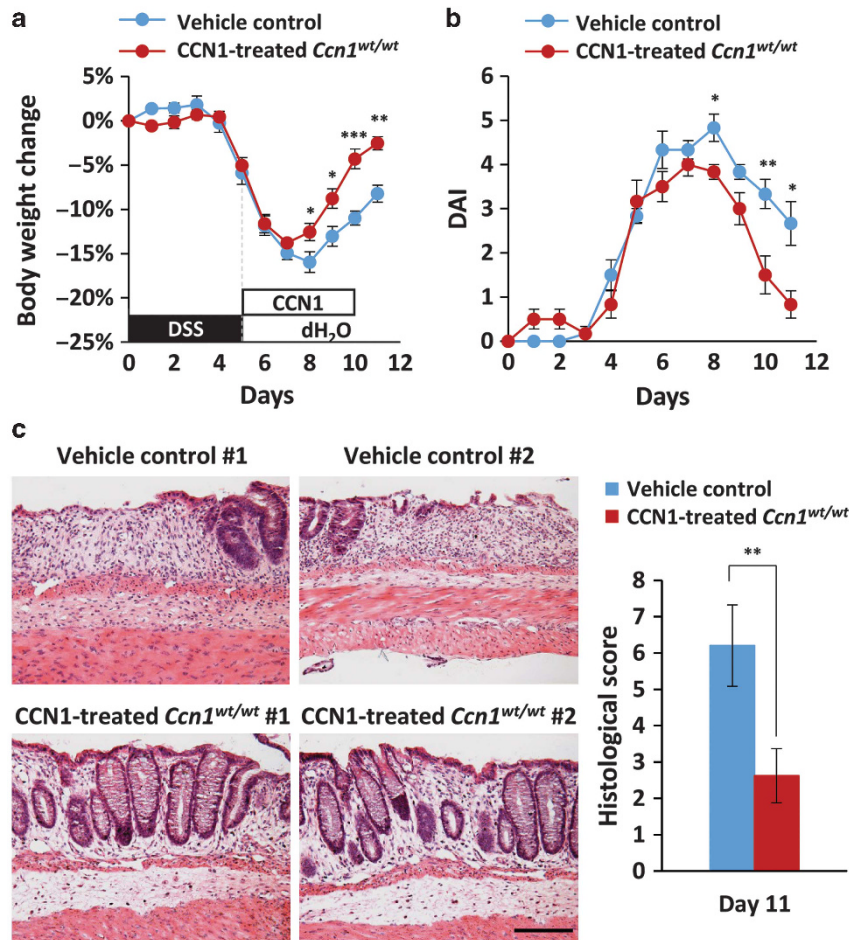


Figure 7 CCN1 treatment accelerates recovery and tissue repair in wild-type mice. Wild-type mice were challenged with 5% DSS for 5 days, then injected i.p. with vehicle control or 10 μ g of purified WT-CCN1 daily for 5 consecutive days post-DSS feeding. (a) Body weight change and (b) DAI were monitored. (c) Histological evaluation by H&E staining of paraffin-embedded sections of the distal colon. Histological scores are on the right. Data are represented as mean \pm s.e.m.; $n = 6$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bar = 100 μ m. DAI, disease activity index; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; i.p., intraperitoneally; WT, wild type.

aberrant *CCN1* expression with a multitude of inflammation-related diseases, the specific functions of CCN1 in various pathological contexts are only now beginning to emerge.¹⁴ Interestingly, different aspects of CCN1 activities may be revealed depending on the specific tissue and nature of injury. For example, in skin wound healing and in chronic inflammatory hepatic injuries that induce liver fibrosis, CCN1 functions to dampen fibrosis by acting through integrin $\alpha_6\beta_1$ to induce cellular senescence and expression of the antifibrotic senescence-associated secretory phenotype in myofibroblasts.^{16,17} In experimental hepatitis induced by concanavalin A, anti-CD95 antibody, or alcohol gavage, CCN1 exacerbates hepatocyte apoptosis by acting in synergy with TNF- α and FasL.^{18,19} Additionally, a role for CCN1 in bone fracture repair has been attributed to its angiogenic activity.³²

Ccn1^{dm/dm} mice suffer deficient *IL-6* expression in the repair phase of colitis, culminating in increased mortality and impaired mucosal healing. These defects can be substantially rescued by delivery of exogenous *IL-6*, indicating that CCN1 promotes mucosal healing in part through *IL-6* (Figure 6).

DSS-induced colitis is linked to damage in the intestinal epithelium, leading to an exuberant inflammatory response due to invasion of the intestinal mucosa by the intraluminal microbiota. Pattern recognition receptors that respond to invading bacteria such as toll-like receptors and the nucleotide-binding oligomerization domain-containing protein-like receptors can induce *IL-6* through activation of NF κ B, and both receptor systems have been implicated in the pathogenesis of IBD.^{1,33,34} Furthermore, transcriptional factor IFN regulatory factor 4 has been demonstrated to drive the expression of *IL-6* in T lymphocytes and contribute to the pathogenesis of the colitis.³⁵ Thus, it is not surprising that upregulation of *IL-6* expression in the initiation phase (\sim 4-fold; day 5) of colitis is independent of CCN1 (Figure 4a). Remarkably, CCN1 exerts exquisite control on *IL-6*, and a large upregulation of *IL-6* expression in the repair phase (\sim 140-fold; day 8) is largely CCN1-dependent. Therefore, *Ccn1*^{dm/dm} mice provide a unique model in which *IL-6* expression in the initiation and repair phases of colitis can be dissociated. CCN1 and TNF- α can act synergistically to induce a high level of *IL-6* expression

(Figure 5d). It is of interest to note that *Ccn1^{dm/dm}* mice suffered impaired healing even though they expressed a higher level of *IL-22* (Supplementary Figure S3B), a cytokine known to participate in mucosal repair,³⁶ suggesting that CCN1 and IL-6 activities are indispensable for efficient mucosal healing.

A rare though significant adverse effect in patients undergoing anti-IL-6 therapy for rheumatoid arthritis³⁷ and CD (ClinicalTrials.gov identifier NCT01545050)⁵ is intestinal perforation. This observation is consistent with our finding that downregulation of *IL-6* compromises gastrointestinal mucosal healing. However, we have not observed direct evidence of intestinal perforation in *Ccn1^{dm/dm}* mice, as they suffered similar initial inflammatory damage as WT mice as measured by body weight loss, DAI, colonic length, and intestinal barrier function (days 0–8, Figure 1b–e). Furthermore, bacterial culture of serum and peritoneal lavage fluid from *Ccn1^{dm/dm}* mice also did not show evidence of intestinal perforation (data not shown). The overwhelming number of deaths in *Ccn1^{dm/dm}* mice occurred (days 9–10) when loss of body weight exceeded that in WT mice in the recovery phase (Figure 2a), suggesting that acute volume depletion might be a significant factor contributing to mortality.

CCN1 regulates diverse cellular functions in a cell type- and integrin-dependent manner.¹² Here we show that CCN1 induces *IL-6* in macrophages through integrin $\alpha_M\beta_2$ and in fibroblasts through $\alpha_6\beta_1$ (Figure 5). These activities are abrogated in DM-CCN1, consistent with impaired *IL-6* expression in *Ccn1^{dm/dm}* mice. CCN1 may also induce the expression of genes other than *IL-6* or provide additional functions that promote mucosal repair, as delivery of CCN1 fully rescued defects in *Ccn1^{dm/dm}* mice whereas *IL-6* was only partially effective (Figures 6 and 7). However, it is also possible that the *IL-6* treatment regimen has not been fully optimized. CCN1 is also known to induce angiogenesis through integrin $\alpha_V\beta_3$ in endothelial cells,³⁸ although the binding site for $\alpha_V\beta_3$ is unaffected in DM-CCN1 and we did not observe angiogenic defects in the colon of *Ccn1^{dm/dm}* mice (data not shown).

Several lines of evidence indicate that *IL-6* is a pro-inflammatory cytokine required for the establishment of IBD,^{7,8} and a pilot clinical trial showed symptomatic improvements in CD patients treated with anti-*IL-6R* antibodies.⁹ Thus, *IL-6* has been identified as a therapeutic target for IBD and clinical trials to evaluate the efficacy of anti-*IL-6* therapies are in progress.^{4–6} However, accumulating evidence also indicates that *IL-6* can serve important protective and homeostatic functions in the intestinal mucosa by promoting IEC proliferation and survival, and regulating intestinal barrier function in mice.^{39–42} Existing results and findings presented herein are consistent with the interpretation that *IL-6* has a dual role in IBD: it is important for the development of colitis as a pro-inflammatory cytokine, but also critical for mucosal repair. Thus, *IL-6*-deficient mice showed reduced inflammation during colitis induction,^{43,44} but suffered impaired mucosal healing during recovery.^{10,45} These findings suggest that although anti-*IL-6* therapy may afford benefit by dampening the pro-inflammatory functions of *IL-6*, it may also impede

mucosal healing. Therefore, further optimization of anti-*IL-6* treatment regimens and/or selective induction of *IL-6* during mucosal healing, potentially through the CCN1/*IL-6* axis, may enhance the therapeutic value of targeting *IL-6* in IBD.

METHODS

Cell culture, proteins, antibodies, and reagents. Immortalized splenic macrophage cell line I-13.35 (CRL-2471, ATCC, Manassas, VA) derived from TLR4-defective C3H/HeJ strain and human 18Co colonic fibroblasts (CCL-228, ATCC) were maintained in Dulbecco's modified Eagle's medium containing 20% LADMAC conditioned media and Eagle's minimum essential medium, respectively, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37 °C with 10% CO₂. The conditionally immortalized YAMC cell line (a gift of R. Whitehead, Vanderbilt University Medical Center), which carries a heat-labile SV40 large T antigen under the control of an IFN- γ -dependent promoter,⁴⁶ was cultured on rat tail collagen (BD Biosciences, San Jose, CA)-coated plates in RPMI 1640 medium with 5% fetal bovine serum, 2 mM L-glutamine, 5 U ml⁻¹ IFN- γ (Peprotech, Rocky Hill, NJ), and 5 μ g ml⁻¹ of insulin (Gibco, Grand Island, NY) at 32 °C (permissive condition). Cells were transferred to RPMI 1640 containing 1% fetal bovine serum without IFN- γ and insulin at 37 °C (nonpermissive condition) 24 h prior to experiments. WT-CCN1 and DM-CCN1 proteins were purified as described.^{25,47} Recombinant mouse *IL-6* was from Peprotech (Rocky Hill, NJ) and recombinant TNF- α was from Apotech (Epalings, Switzerland), and epidermal growth factor from Sigma-Aldrich (St Louis, MO). Neutralizing mAbs against integrin α_M (clone M1/70.15) was from AbD Serotec (Raleigh, NC), integrin β_2 (clone M18/2.a.12.7) from Santa Cruz Biotechnology (Dallas, TX), integrin α_6 (clone GoH3) from Beckman Coulter (Indianapolis, IN), and *IL-6R α* from R&D (Minneapolis, MN).

Mice and DSS-induced colitis. *Ccn1^{dm/dm}* mice were backcrossed 20 \times into the C57BL/6 background.^{16–18} Transgenic CCN1(CYR61)-EGFP mice in FVB/N-Swiss Webster background harboring a modified bacterial artificial chromosome with an EGFP inserted upstream of the initiating ATG codon of the first coding exon of the *Cyr61* gene was obtained from the Mutant Mouse Regional Resource Centers at University of California, Davis.²³ Female WT C57BL/6, *Ccn1^{dm/dm}*, CCN1-EGFP mice (12–16 weeks) were given 5% or 3.5% (w/v) DSS (36–50 kDa; MP Biomedicals, Solon, OH) in the drinking water for 5 days followed by 14 days of regular water. This cycle was repeated 3 \times in survival experiments. Where indicated, mice were injected intraperitoneally with CCN1 or *IL-6* in 200 μ l of phosphate-buffered saline. All animal procedures were approved by the University of Illinois Animal Care Committee.

DAI and histological analysis. Mice were examined daily and DAI was assessed based on symptom severities as described.⁴⁸ Distal colons were formalin fixed and paraffin embedded; sections were stained with hematoxylin and eosin and photomicrographed. Histological score was based on published criteria⁴⁹ with each parameter multiplied by percentage of tissue involvement for a maximum score of 10.

RNA isolation, quantitative reverse transcription-PCR, and ELISA. RNA was extracted using TRIzol (Invitrogen, Grand Island, NY) and RNeasy Mini Kit (Qiagen, St. Louis, MO), and reverse transcribed to complementary DNA using SuperScript Reverse Transcriptase III following the manufacturer's protocol (Invitrogen). Quantitative reverse transcription-PCR was performed with iCycler thermal cycler (Bio-Rad, Hercules, CA) using iQ SYBR Green supermix. Cyclophilin E was used as an internal standard. List of primer sequences used for quantitative reverse transcription-PCR is provided in Supplementary Table S1. ELISA was conducted using *IL-6* Quantikine ELISA kit (R&D); serum protein levels were quantified using Inflammatory Cytokine and Chemokine Multi-Analyte ELISA Array and *IL-6* single analyte ELISA kits (Qiagen) with manufacturer's protocol.

In vivo permeability assay. Mice were gavaged with fluorescein isothiocyanate-dextran (MW 4000; Sigma-Aldrich) at 60 mg kg⁻¹ of body weight 4 h prior to sacrifice. Blood was collected via cardiac puncture, and fluorescence intensity in the serum was measured (excitation, 485 nm; emission, 520 nm) using Fluostar Omega multimode microplate reader. Fluorescein isothiocyanate-dextran concentrations were determined from a standard curve generated by a serial dilution of fluorescein isothiocyanate-dextran.

TUNEL and myeloperoxidase assays. TUNEL assay was performed using ApopTag Red detection kit (Millipore, Billerica, MA) according to manufacturer's protocol. Four randomly selected high-power microscopic fields of each sample were examined using Leica DM 4,000B microscope mounted with QI Click digital CCD camera. Number of TUNEL-positive cells were scored and normalized by total number of cells.

Myeloperoxidase activity was measured as previously described.⁵⁰ Enzymatic activity was measured at 450 nm using 96-well Multiskan microplate reader and determined from a standard curve generated by a serial dilution of purified myeloperoxidase enzyme (Enzo Life Sciences, Farmingdale, NY).

Immunofluorescence microscopy and cell proliferation assay. Paraffin-embedded colon sections were stained with anti-E-cadherin antibody (Santa Cruz Biotechnology), anti-GFP (Abcam, Cambridge, MA), or mAb against proliferating cell nuclear antigen (Abcam), and visualized with anti-rabbit immunoglobulin G Texas Red and anti-mouse immunoglobulin G Alexa Fluor 488 (Invitrogen). For cell proliferation, Ki67 was detected using rabbit polyclonal antibodies (Abcam) and anti-rabbit immunoglobulin G Alexa Fluor 488 (Invitrogen). All assays were done in triplicates, and > 300 cells were counted in each sample from random fields.

Statistical analysis. Data are expressed as mean ± s.e.m. All experiments were performed in triplicates unless stated otherwise. Statistical significance was determined by Student's *t* test. Survival curves were calculated using Kaplan-Meier method. Statistical significance for survival between populations was analyzed by log-rank test.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declare no conflict of interest.

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