The Matricellular Protein CCN1 Regulates Colitis, Colitis-Associated Cancer, And Neutrophil Mobilization

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LIST OF ABBREVIATIONS

AOM	Azoxymethane
BSA	Bovine serum albumin
C/EBP	CCAAT enhancer binding protein
CD	Crohn Disease
CRC	Colorectal cancer
CT	Carboxyl-terminal
CTGF	Connective tissue growth factor
DAI	Disease activity index
DAPI	4,6 diamidino-2-phenylindole
D125A-CCN1	Mutant D125A protein
DM-CCN1	Mutant DM protein
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
EGF	Epidermal growth factor
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte-colony stimulating factor

LIST OF ABBREVIATIONS

HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cell
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IGFBP	Insulin-like growth factor binding protein
i.p.	Intraperitoneally
IL6R	Interleukin-6 receptor
LRP-1	Lipoprotein receptor-related protein-1
mAb	Monoclonal antibody
MPO	Myeloperoxidase
MUC	Mucin
NOV	Nephroblastoma over-expressed
PAS	Periodic acid-schiff
PBS	Phosphate buffered-saline
PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species

LIST OF ABBREVIATIONS

SP	N-terminal secretory signaling peptide
TSP	Thrombospondin type 1 repeat
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling
UC	Ulcerative colitis
vWC	von-Willebrand factor type C repeat
Wisp	Wnt-1 induced secreted protein
WT	Wild-type
WT-CCN1	Wild-type CCN1 protein
YAMC	Young adult mouse colon

SUMMARY

The emergence of matricellular proteins has redefined the functions of extracellular matrix (ECM) proteins, which were traditionally viewed as an inert scaffolding that mainly provided structural support and anchorage for cells to be organized into tissues. As a subset of ECM proteins, matricellular proteins have been found to have integral regulatory activities in various cell functions and biological processes. CCN1, the first member in the CCN family, is a 40kDa matricellular protein that is dynamically expressed upon external stimuli, secreted into the ECM where it binds to cells through integrins and co-receptors, such as heparin-sulfate proteoglycan, to regulate cell behaviors including cell adhesion, motility, apoptosis, and growth in a cell type- and context-dependent manner. CCN1 is broadly expressed in many tissues and organs, and shown to be critical for proper development. This is highlighted by the finding that CCN1 null animals die in utero mainly due to cardiovascular defects. CCN1 expression is downregulated as animals approach adulthood, only to be upregulated once again during pathologic situations including inflammation, wound healing and cancer. Moreover, a recent study has shown that CCN1 can regulate cytokine expression in macrophages. Collectively, these findings are consistent with the notion that CCN1 may be playing a pivotal role in inflammation and inflammation-associated processes.

Growing evidence have demonstrated that CCN1 directly participates in inflammation. In addition, 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*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ knockin mice expressing a CCN1 mutant unable to bind integrin $\alpha_v\beta_3$ or integrin $\alpha_6\beta_1$ and $\alpha_M\beta_2$, respectively, as a model to probe CCN1 function

in dextran sodium sulfate (DSS)-induced colitis. We show that *Ccn1*$^{d125a/d125a}$ mice were highly susceptible to severe colitis resulting in high mortality and morbidity due a defective mucosal barrier and unregulated activation of pro-inflammatory cytokines, specifically involving the IL23/IL17/GCSF pathway, that lead to the development of chronic inflammation. In DSS-challenged *Ccn1*$^{dm/dm}$ mice, we observed increased mortality, albeit not as severe as *Ccn1*$^{d125a/d125a}$ mice, impaired mucosal healing, and diminished *IL-6* expression during the repair phase of DSS-induced colitis compared to wild type mice, despite having comparable severity of initial inflammation and tissue injury. 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. Furthermore, 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 maintaining mucosal barrier integrity to attenuate acute inflammation and prevent the development of chronic inflammation. In addition, CCN1 restores 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.

Chronic inflammation is strongly associated with cancer. Patients who suffer from inflammatory bowel disease, particularly ulcerative colitis, are at increased risk for developing colorectal cancer. Interestingly, CCN1 expression is correlated with various neoplasms including colorectal cancer. Preliminary results in azoxymethane-DSS induced carcinogenesis show

Ccn1$^{dm/dm}$ and *Ccn1*$^{d125a/d125a}$ mice had increased tumor incidence and multiplicity compared to wild type animals. This is consistent with notion that CCN1 may act as a tumor suppressor inflammation-associated cancer.

Neutrophils are an important effector of innate immunity. In the event of a bacterial or fungal infection, and/or tissue injury, neutrophils are released from the bone marrow storage into the circulation as a part of coordinated systemic response to mount a defense against the invading pathogens. Granulocyte-colony stimulating factor (G-CSF) is a critical hematopoietic growth factor and a prototypical cytokine that mobilizes neutrophils, providing the link between emergency granulopoiesis and neutrophil release. In this thesis, we show for the first time that administration of exogenous CCN1 in animals induces G-CSF to drive neutrophilia in a dose-dependent manner. *In vitro*, CCN1 upregulated expression of *G-CSF* in macrophages and fibroblasts. Notably, combining CCN1 and IL-6 had a synergistic effect in the induction of *G-CSF* expression in macrophages; however, this property was not observed *in vivo*. Given that CCN1 is an immediate-early gene that is rapidly induced in inflammation, these findings suggest that CCN1 may be contributing to the effort of neutrophil mobilization in acute inflammation through its regulation of G-CSF.

CCN1 is a matricellular protein that serves diverse key functions in complex biological processes. Specifically, through its ability to regulate cytokine expression in a cell type- and context-dependent manner, CCN1 promotes intestinal mucosal repair, suppresses

tumorigenicity, and coordinates neutrophil mobilization. Taken together, these findings support the notion that CCN1 is a critical modulator of inflammation and tissue homeostasis.

1. INTRODUCTION

1.1 Initiation and resolution of inflammation, and tissue repair

Humans and other organisms are constantly exposed to microbes and toxins in the environment that can be injurious to the host. Appreciably, the host is equipped with an evolving immune system that triggers a self-limiting inflammatory response to eliminate pathogens, neutralize toxins, and repair the injured tissue. However, when this process is deregulated, the immune system can also become the etiology of debilitating pathology such as auto-immune disease. Therefore, tight regulation of this complex system is essential for the health of the host.

Proper wound healing and tissue repair response requires precise coordination of three overlapping but distinct phases: inflammation, granulation tissue formation, and tissue remodeling (1). During inflammation, platelets aggregate and de-granulate to activate coagulation pathways to minimize bleeding and fluid loss, re-establish a physical barrier from the external environment, and form a matrix to accommodate immune cells at the site of injury (2). Within hours, neutrophils are recruited to combat pathogens and limit the spread of infection (3;4). Subsequently, monocytes arrive and differentiate into macrophages to begin clearance of necrotic and apoptotic cells (5). As leukocytes migrate into the damaged area, they express and secrete cytokines and growth factors to promote cell migration and proliferation of parenchymal and stromal cells. This marks the transition towards the second stage of tissue repair where deposition of extracellular matrix (ECM), re-epithelization or replacement of lost parenchymal cells, and angiogenesis are the critical events that are required for efficient new tissue formation (2). Lastly, approximately two weeks after the initial injury, mast cells and

macrophages undergo apoptosis to achieve resolution of inflammation and matrix remodeling continues to restore tissue integrity and homeostasis (6). However, in severe injury or chronic inflammation, multiple aspects of wound healing may be deregulated to induce excessive ECM deposition, which leads to tissue scarring and fibrosis (7). This ultimately compromises tissue and organ function contributing to significant morbidity and mortality (8). Therefore, a better understanding of the precise coordination and exquisite regulation of cells, cell-to-cell dynamics, and cell-to-matrix interactions during inflammation and wound healing response is needed to develop therapies that prevent chronic inflammation and stimulate restoration of tissue homeostasis.

1.2 Intestinal inflammation and mucosal homeostasis

The intestinal tract is home to trillions of microorganisms that reside in close proximity to host immune cells. Genetic studies and experimental models of colitis have revealed that the pathogenesis of intestinal inflammation often involves alterations in epithelial barrier function (9). This allows an increase in translocation of microbes and foreign antigens into the host tissue, skewing the balance between pro- and anti-inflammatory cytokine expressions (10). Consequently, deregulated activation of the mucosal immune system fuels chronic inflammation, preventing resolution of inflammation and proper tissue repair. Therefore, mucosal homeostasis and immune tolerance are essential for harmonious coexistence of microbes and the host immune cells in the gut.

The intestinal lining is composed of intestinal epithelial cells (IECs), goblet cells, and Paneth cells. These three cell types cooperatively function to provide a physical and biochemical barrier for the host from the external environment. Specifically, this essential task

is accomplished by secretion of mucin glycoproteins by goblet cells and antimicrobial peptides (e.g. defensins, resistins, regenerating islet-derived proteins) by Paneth cells (11). Notably, Paneth cells also produce resistin-like molecule β (RELMβ) to regulate intestinal inflammation (12). IECs are responsible for maintaining an intact mucosal barrier through regulation of occludin, claudins, tricelllulin, and E-cadherin to form functional apical junctional complexes in the intercellular gaps, which act as semi-permeable gates that permit flow of nutrients, fluid, and electrolytes while occluding bacteria (13). When any of these functions are defective, bacteria invade the host tissue, increasing the risk for inflammatory bowel disease (IBD)(14). Therefore, in addition to its digestive functions, the intestinal epithelial lining, which consists of specialized cells, fulfill an essential role in barrier function and immune homeostasis.

1.3 CCN1, a member of CCN family of matricellular proteins

CCN1/CYR61, a secreted matricellular protein, is the first member of CCN family, which includes CCN2/CTGF (connective tissue growth factor), CCN3/Nov (Nephroblastoma over-expressed), CCN4/Wisp-1 (Wnt-1 induced secreted protein 1), CCN5/Wisp-2, and CCN6/Wisp-3. This ~40 kDa protein containing 381 amino acids (of which 38 are conserved cysteine residues) was first discovered as a product of serum growth factor-inducible, immediate-early gene in mouse fibroblasts (15;16); since then, it has been shown to be an essential factor for cardiac and placental development (17;18). CCN1 shares approximately 30-50% amino acid sequence homology with its other CCN family members (19) and orthologs of CCN genes have been discovered in vertebrates (20). CCN proteins consist of an N-terminal secretory signal peptide, a non-conserved, protease-sensitive central hinge region, and four conserved modular domains that share sequence homology to insulin-like growth factor binding proteins (IGFBP), the von

Willebrand factor type C repeat (vWC), the thrombospondin type 1 repeat (TSP), and the carboxyl-terminal (CT) domain that contains a cysteine knot motif, with the exception for CCN5/Wisp-2, which lacks the CT domain. Sequence analysis of genes *ccn1*, *ccn2*, and *ccn3* revealed that each domain is encoded by a separate, but well-conserved, exon, raising the possibility that CCN genes are a product of gene shuffling (19-22). Each domain of CCN1 can interact with other ECM partners and cell surface integrins, independent of other domains, to regulate a diverse range of cellular processes.

1.4 CCN1 gene regulation

CCN1, as an immediate-inducible gene, is expressed at low levels in quiescent fibroblasts, but can be rapidly induced by serum or mitogenic growth factors without *de novo* synthesis (16). Analysis of the promoter region of CCN1 revealed that transcriptional activation is driven by a serum response element which binds to serum response factor upon incubation of fibroblasts with serum or platelet-derived growth factor (23). Since then, CCN1 has been found to be expressed in response to wide range of external stimuli including, but not limited to, hormones, vitamin D_3, cAMP, hypoxia, UV light exposure, oncogene activation, and mechanical stretch (22). Foreshadowing its involvement in inflammation, CCN1 has also been shown to be induced by bacterial and viral infections (24;25), lipid mediators of inflammation (26;27), and cytokines including IL-1β, IL-17 and TGF-β (22;28).

Consistent with its classification as a matricellular protein, CCN1 is dynamically expressed *in vivo*. During embryonic development, CCN1 is highly expressed in the placenta, skin, skeletal and cardiovascular systems (20). Accordingly, *Ccn1* is essential for proper formation of the placenta and cardiovascular development as *Ccn1*-null mice die *in utero* from

failure to form chorioallantoic fusion, placental vascular insufficiency, loss of embryonic vessel integrity, and cardiac atrioventricular septal defects (17;18). In adult tissues, CCN1 expression is associated with inflammation, tissue repair, and some cancers (29). In support of this finding, internal ribosome entry sites have been found in the 5' non-coding region and in the IGFBP domain of *Ccn1* mRNA, suggesting that translation of *Ccn1* mRNA occurs under conditions of stress (30;31). Induction of CCN1 expression is not organ-specific demonstrated by its induction in bone fracture repair, cutaneous skin wound healing, and liver injury and fibrosis (29). This suggests that the expression of CCN1 is tightly regulated *in vivo* and that CCN1 may serve critical functions in different organ systems undergoing inflammation, tissue repair, or tumorigenesis.

1.5 Receptors of CCN1

CCN1 is secreted upon synthesis and associates with broad range of matrix proteins, cell receptors, and growth factors (20). Through these associations, CCN1 serves to regulate cell behavior rather than to provide the traditional structural roles of ECM proteins. Mechanistically, much of its regulatory activity depends on its ability to function as a ligand to distinct integrins, which are transmembrane receptors that mediate cell-ECM and cell-cell interactions. Integrins are bidirectional, heterodimeric proteins that consist of at least one alpha and beta subunit combined from an existing pool of 18 alpha-subunits and 8 beta-subunits (10). First discovered as the link between the ECM and the cytoskeleton of the cell, integrins are now characterized as mechanosensors, signaling receptors, and fine tuners of cell signaling (32;33). CCN1 as a novel ligand to integrin was established in the direct binding of CCN1 to integrin $\alpha_v\beta_3$ in endothelial cells (34). To date, CCN1 has been shown to interact with

six other integrins ($\alpha_6\beta_1$, $\alpha_M\beta_2$, $\alpha_v\beta_5$, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_D\beta_2$) to regulate cell activities such as cell adhesion, migration, proliferation, survival, and apoptosis in a cell type- and context-dependent manner (20;22;29). In addition to integrins, heparan sulfate proteoglycans (HSPG) have been identified as an important co-receptor for CCN1 (35). Particularly, the participation of HSPG syndecan-4 in CCN1 signaling was demonstrated to be critical for many functions of CCN1 in fibroblasts and macrophages (36-38).

Binding sequences of CCN1 to respective integrins and HSPG were investigated through biochemical and functional approaches. These studies revealed that H1 (KGKKCSKTKKSPEPVR) and H2 (FTYAGCSSVKKYRPKY) motifs in the CT domain of CCN1 support cell adhesion of fibroblasts through integrin $\alpha_6\beta_1$ and cell surface HSPG (37;39). Consistently, in a mutational study in which all lysine (K) or arginine (R) residues of H1 and H2 motifs were substituted with alanine (A), CCN1 lost its ability to bind to integrin $\alpha_6\beta_1$ and HSPG, and CCN1 function as a cell adhesion molecule was abrogated (39). Moreover, the H2 sequence contains the binding site for α_MI domain and mediates $\alpha_M\beta_2$-dependent cell adhesion of monocytes and macrophages (40;41) indicating that the binding sites for $\alpha_6\beta_1$, $\alpha_M\beta_2$, and HSPG are juxtaposed or overlapping in the H1 and H2 region.

In a separate set of studies, V2 (NCKHQCTCIDGAVGCIPLCP) sequence in the vWC domain of CCN1 was found to be a novel $\alpha_v\beta_3$ binding site despite CCN1 not containing the canonical arginine-glycine-aspartic acid sequence, which recognizes α_V integrins (42). V2 peptide was shown to support $\alpha_v\beta_3$-mediated cell adhesion of human umbilical vein endothelial cells; a single amino acid substitution for an aspartate (D125) with an alanine disrupted this activity suggesting that D125 is a key residue for binding $\alpha_v\beta_3$ (42). Notably, mutational analysis

revealed that each domain of CCN1 is capable of binding to its respective integrin independent of other domains, and that the combination of mutant proteins can restore the wild-type activity of CCN1 (36;42). This suggests that CCN1 can be cleaved in the protease-sensitive central hinge region to separately bind their respective integrins on different cells or even the same cell to differentially regulate cell behavior. Further investigation is required to assess if there are any functions of CCN1 that require CCN1 to remain intact as a whole and bind to multiple integrins simultaneously.

Other members of the CCN family are known to bind to receptors that do not typically interact with matricellular proteins (20). For example, CCN2 binds to low-density lipoprotein receptor-related protein-1 (LRP-1) to mediate cell adhesion in hepatic stellate cells (43). Similarly, a recent finding has demonstrated that CCN1 can also bind to LRP-1, and that this interaction is critical for synergism with TNFα (tumor necrosis factor-α) to stimulate production of reactive oxygen species (ROS), and subsequently, induce apoptosis in fibroblasts (44). In addition, CCN2 and CCN3 has been shown to bind to TrkA (45) and Notch (46), respectively; however, the interaction between these receptors and CCN1 remains unknown.

1.6 Functions and mechanisms of CCN1

Through its various binding sites to distinct integrins, CCN1 regulates diverse cell behavior in a cell type- and context-dependent manner (figure 1)(22). For example, CCN1 mediates cell adhesion of fibroblasts, endothelial cells, platelets, and macrophages in an integrin $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$-dependent manner, respectively (22). Further examination revealed that the adhesion of human skin fibroblasts to CCN1 may promote cell migration as CCN1 triggers the formation of focal adhesion complexes, actin cytoskeleton reorganization,

activation of focal adhesion kinase, paxillin and the small GTPase RAC, and formation of filopodia and lamellipodia (47). Like other ECM and matricellular proteins, CCN1 can enhance survival of endothelial cells but also promote apoptosis in fibroblasts as a cell adhesion substrate (36;48). The adaptability of CCN1 to either promote cell survival or apoptosis depending on the context and cell type suggests that CCN1 may play important roles in wound healing and tissue remodeling.

First evidence of CCN1 as a potent angiogenic factor was established in a corneal micropocket implant assay and subsequently confirmed in a rabbit ischemic hindlimb model (49;50). This function of CCN1 involves direct binding to integrin $\alpha_v\beta_3$ in microvascular endothelial cells to enhance proliferation, survival, chemotaxis, and formation of endothelial tubules (48;49). Furthermore, CCN1 promotes adhesion and recruitment of $CD34^+$ progenitor cells to potentiate endothelial proliferation and neovascularization (51). The angiogenic function of CCN1 is critical in the placenta and proper cardiovascular development as *Ccn1*-null mice are embryonic lethal, in part, due to placental vascular insufficiency and defects in embryonic vascular integrity (17). Additionally, CCN1 can indirectly affect angiogenesis by regulating expression of vascular endothelial growth factor A and vascular endothelial growth factor C, two prominent angiogenic inducers (17;52;53). Therefore, CCN1, as a potent angiogenic factor, may serve important roles in diverse physiological and pathological contexts including embryogenesis, inflammation and wound healing, and tumorigenesis.

In order to dissect the different functions of CCN1 *in vivo* with respect to its binding to $\alpha_6\beta_1/\alpha_M\beta_2$/HSPG or $\alpha_v\beta_3$, transgenic $Ccn1^{dm/dm}$ and $Ccn1^{d125a/d125a}$ knockin mice were constructed by replacing the *Ccn1* genomic locus with an allele encoding a CCN1 DM protein

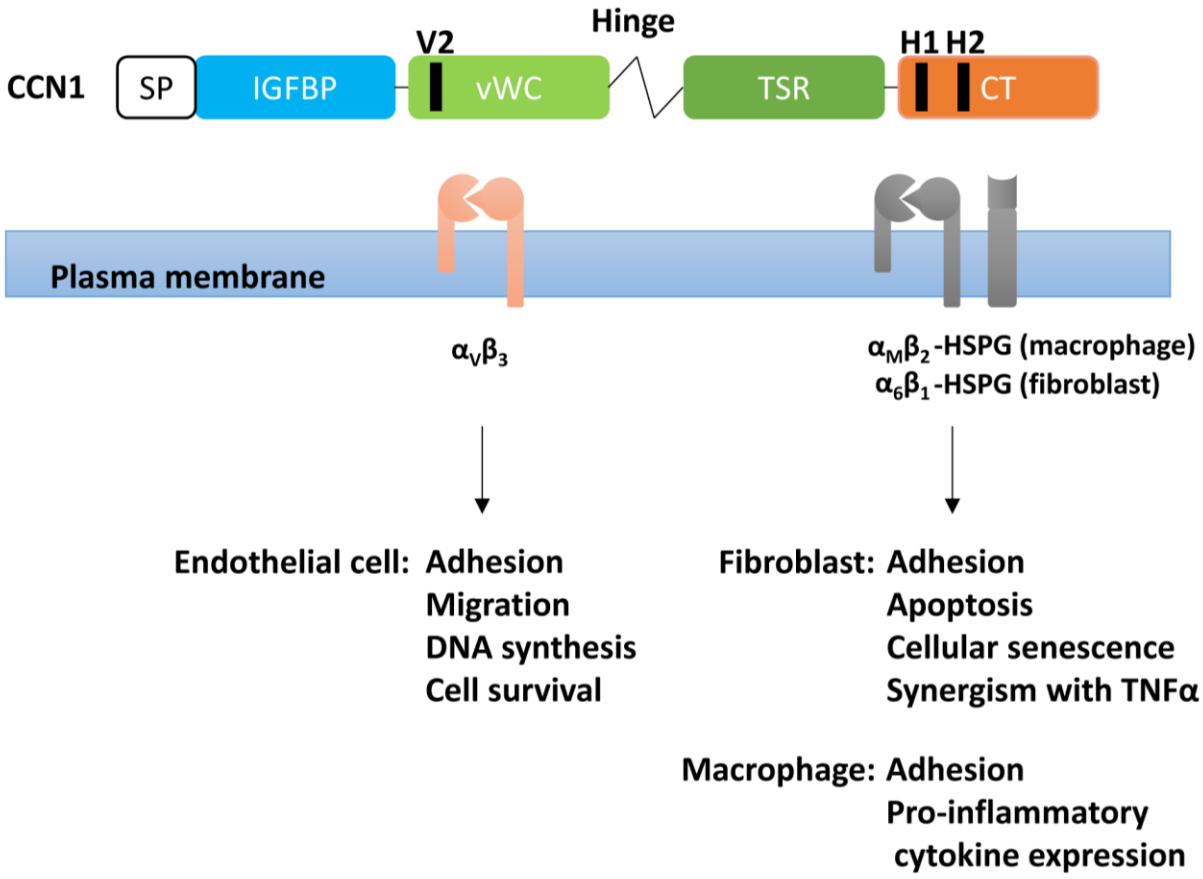


Figure 1. Modular domain structure of CCN1, receptor binding sites, and biological activities mediated through respective binding sites. Schematic diagram of CCN1 and its N-terminal secretory peptide followed by four conserved domains showing the identified integrin and HSPG binding sites. Listed below are examples of cell behaviors known to be regulated by CCN1 through different domains. "Hinge" refers to the central variable region susceptible to protease cleavage. SP = N-terminal secretory signal peptide; IGFBP = insulin-like growth factor binding protein; vWC = von Willebrand factor type C repeat; TSR = thrombospondin type 1 repeat; CT = carboxyl terminal domain.

(DM-CCN1) or D125A protein (D125A-CCN1), respectively (figure 2)(36;42;54). Both strains of mice are viable and fertile, and therefore, provides a genetic tool to probe the specific functions of CCN1 mediated through its binding to respective integrins.

1.7 Regulatory functions of CCN1 in inflammation and tissue repair

Expression of CCN1 is associated with various inflammatory-associated pathologies including atherosclerosis, rheumatoid arthritis, and nephritis (28). *In vitro*, CCN1 can regulate the inflammatory response of macrophages (40). Through binding to integrin $\alpha_M\beta_2$ and syndecan-4, CCN1 activates NF-κB to promote the expression of proinflammatory cytokines, *TNFα, iNOS, IFNγ*, and *IL-12b* while downregulating anti-inflammatory factors, *IL-10Rβ* and *TGF-β1* (40). This cytokine profile is consistent with M1, or classical, activation of macrophages, suggesting that CCN1 may be polarizing macrophages to participate in Th1 response. In addition to its pro-inflammatory activity in macrophages, CCN1 can unmask and enhance the cytotoxic function of TNFα and FasL through binding to $\alpha_6\beta_1$-HSPG and induce apoptosis in fibroblasts and hepatocytes, indicating that CCN1 can contribute to inflammatory damage (36;55). These findings raise the possibility that CCN1 may be directing the inflammatory response of other immune cells, including neutrophils, which express $\alpha_M\beta_2$ as its predominant integrin, and act in concert with other inflammatory mediators to regulate tissue injury and repair.

CCN1 expression is also highly upregulated during tissue repair in bone, cutaneous skin, and liver (54;56;57). Notably, CCN1 expression was increased in hepatocytes of human cirrhotic liver biopsies (57). Whereas CCN1 promotes healing of bone fracture through its proangiogenic activity, CCN1 induces cellular senescence in activated, ECM-producing myofibroblasts to restrict fibrosis in cutaneous wound healing and multiple models of chronic liver injury

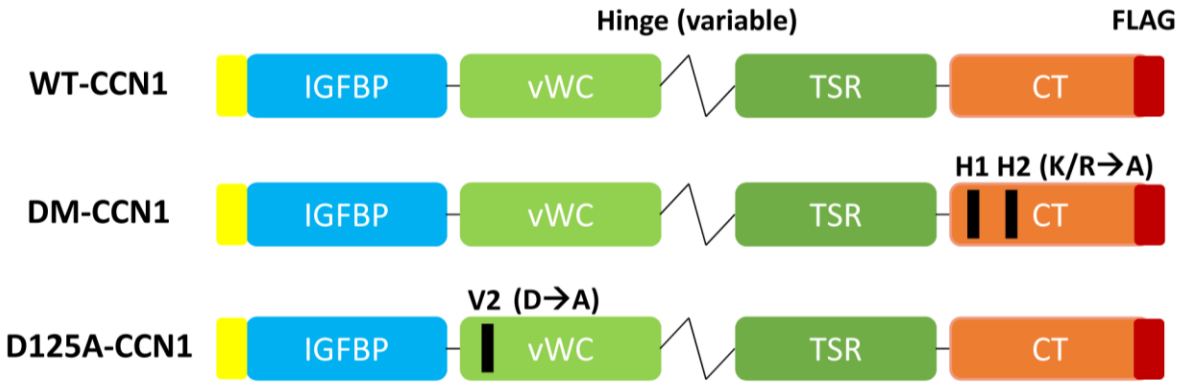


Figure 2. Constructs of WT-, DM-, and D125A-CCN1 proteins. Schematic diagram of WT-CCN1, and mutant DM- and D125A-CCN1 constructs. In DM-CCN1 protein, Lys and Arg residues of H1 and H2 motifs of the CT domain were substituted with Ala, whereas Asp125 in the V2 sequence of vWC domain was substituted with Ala in D125A-CCN1 protein, in the context of full-length of CCN1.

(54;56;57). Mechanistically, CCN1 binds to $\alpha_6\beta_1$-HSPG in myofibroblasts and hepatic stellate cells to activate the RAC1-dependent NAPDH oxidase 1, which stimulates a sustained production of ROS to activate cellular tumor antigen p53 and the retinoblastoma-associated protein pRb via the DNA damage response or ERK and p38 MAPK pathways, respectively(54). Consistently, senescent activities of CCN1 are abrogated in DM-CCN1, and *Ccn1*$^{dm/dm}$ mice develop excessive fibrosis due to decreased senescent myofibroblasts after skin injury or chronic liver damage (54;57). Furthermore, delivery of purified CCN1 protein to injured tissues dampen fibrotic response and accelerated regression of established fibrosis, exhibiting its therapeutic potential as an anti-fibrotic factor (54;57). Therefore, CCN1 has powerful and significant functions in tissue repair and remodeling.

2. MATERIALS AND METHODS

2.1 Cell culture, proteins, antibodies, and reagents

Immortalized splenic macrophage cell line I-13.35 (CRL-2471, ATCC) derived from TLR4-defective C3H/HeJ strain and human 18Co colonic fibroblasts (CCL-228, ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) containing 20% LADMAC conditioned media and Eagle's minimum essential medium (EMEM), respectively. 1077Sk (CRL-2076, ATCC) human skin fibroblasts derived from foreskin of a healthy newborn was cultured in DMEM. All cell media were supplemented with 10% fetal bovine serum (Hyclone) at 37^{o}C with 5% CO_2.

The conditionally immortalized young adult mouse colon (YAMC) cell line (a gift of R. Whitehead, Vanderbilt University Medical Center, Nashville, TN) was derived from the colonic epithelium of H-2K^{b}-tsA58 Immortomouse, a transgenic animal that expresses a heat-labile SV40 large T antigen under the control of an interferon (IFN)-γ-dependent promoter (58). YAMC cells were cultured on rat tail collagen (BD Biosciences)-coated plates in RPMI 1640 medium with 5% FBS, 2 mM L-gluatamine, 5 U/ml IFN-γ (Peprotech), and 5 μg/ml of insulin (Gibco) at 32^{o}C (permissive condition). Cells were transferred to RPMI 1640 containing 1% FBS without IFN-γ or insulin at 37^{o}C (nonpermissive condition) for at least 24 hrs prior to experiments. Recombinant mouse IL-6 was obtained from Peprotech (Rocky Hill, NJ) and recombinant TNFα was obtained from *Apotech* (Epalinges, Switzerland). Neutralizing monoclonal antibodies against intergrin 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, integrin α_6 (clone GoH3) from Beckman Coulter, IL-6Rα, IL-6 (clone MP5-20F3), and IL-17 (clone 50104) from R&D.

2.2 Mice, DSS-induced colitis, and tumor induction

Ccn1$^{dm/dm}$ and *Ccn1*$^{d125a/d125a}$ mice were generated in an svJ129-C57BL/6 mixed background and backcrossed >10 times or 4 times, respectively, into the C57BL/6 background (36;54;57). Wild type, *Ccn1*$^{dm/dm}$, and *Ccn1*$^{d125a/d125a}$ female mice (12 to 16-week old) were given 5% or 3.5% (w/v) dextran sodium sulfate (DSS; 36-50 kDA, MP Biomedicals, Solon, OH) in the drinking water for five days, followed by 14 days of regular water. This cycle was repeated for a total of three times in survival experiments. Where indicated, mice were treated intraperitoneally (i.p.) with 5 μg of purified recombinant CCN1 protein (59) or 100 ng of recombinant IL-6 in 200 μL of PBS for 5 consecutive days after 5 days of DSS feeding. PBS alone was injected as control. Mice were anesthetized with i.p. injection of ketamine/xylazine (100 mg/kg) and blood was collected via cardiac puncture. Colon length (rectum to cecum) was measured at endpoint of experiments. For colitis-associated carcinogenesis, wild type, *Ccn1*$^{dm/dm}$, and *Ccn1*$^{d125a/d125a}$ mice were injected i.p. with azoxymethane (AOM; 10mg/kg of body weight)(Sigma-Aldrich, St. Louis, MO) five days prior to three cycles of DSS treatment. Each cycle consisted of 2.5% DSS for five days followed by 14 days of recovery with normal drinking water. Animals were sacrificed on day 62 post-AOM injection and numbers of tumors were counted macroscopically. All animal procedures were approved by the University of Illinois Animal Care Committee.

2.3 Disease activity index (DAI) and histological studies

Mice were examined for clinical signs of colitis daily to determine disease activity index (DAI), which includes body weight loss, stool consistency, and rectal bleeding, all graded on a scale from zero to three based on severity as previously described (60). The scoring for body

weight loss is as follows: 0, <1%; 1, 1-4.99%; 2, 5-10%; 3, >10%. The scoring for stool consistency is as follows: 0, normally formed pellets; 1, soft pellets not adhering to the anus, pellets on walls of cages; 2, very soft pellets adhering to the anus; 3, liquid stool, wet anus. The scoring for rectal bleeding is as follows: 0, none; 1, small spots of blood in stool; 2, large spots of blood in stool or gross bleeding on anal orifice; 3, deep red stool; blood spreads largely around the anus. Values were added to give a maximal disease score of 9.

Distal portion of colons were excised on days 0, 5, 8, 11, or 14 after initiation of DSS challenge, formalin-fixed, and embedded in paraffin. Tissue sections (7 μm thick) were stained with hematoxylin and eosin (H&E). Photomicrographs were taken using a Leica DM4000B microscope mounted with QI Click CCD digital camera. Histological score was based on 3 parameters. Severity of inflammation: 0, none; 1, slight; 2, moderate; 3, severe. Extent of inflammation: 0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural. Crypt damage: 0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost. Values were added to give a maximal histological score of 10.

For detecting goblet cells or neutrophils in paraffin-embedded sections of the distal colon, periodic acid-schiff (PAS) kit (Sigma-Aldrich) or naphthol AS-D chloroacetate esterase kit (Sigma-Aldrich) was used, respectively, according to manufacturer's instructions.

2.4 ***In vivo*** **permeability assay**

Mice were gavaged with fluorescein isothiocyanate (FITC)-dextran (MW 4000; Sigma-Aldrich, St. Louis, MO) at 600 mg/kg of body weight 4 hrs prior to sacrifice. Blood was collected via cardiac puncture, and fluorescence intensity in serum was measured (excitation, 485 nm; emission, 520 nm) using Fluostar Omega multimode microplate reader. FITC-dextran

concentrations were determine from a standard curve generated by a serial dilution of FITC-dextran (Enzo Lifesciences).

2.5 Intraperitoneal injection of CCN1

WT male and female mice were injected i.p. with purified recombinant WT-CCN1, DM-CCN1, or D125A-CCN1 diluted into 200 µl of PBS. PBS alone was injected as control. Animals were sacrificed at indicated times and blood was obtained via cardiac puncture. Complete blood counts with white blood cell type differential were performed by University of Illinois at Chicago Biological Resources Laboratory using the flow cytometry method.

2.6 Immunofluorescence microscopy and TUNEL

Formalin-fixed, paraffin-embedded colon sections were double stained with Rabbit polyclonal anti-E-cadherin (Clone H-108; Santa Cruz Biotechnology) and a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Abcam). Secondary antibodies, anti-rabbit IgG Texas Red (Invitrogen) and anti-mouse IgG Alexa Fluor 488 (Invitrogen), were used for visualization. Samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using ApopTag Red detection kit (Millipore) according to manufacturer's protocol. Four randomly selected high-power microscopic fields of each sample were examined using Leica DM 4000B microscope mounted with QI Click digital CCD camera (Q Imaging, Surrey, Canada). Number of PCNA- or TUNEL-positive cells were scored and normalized by total number of cells.

2.7 RNA isolation and qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen), and reverse transcribed to complementary DNA using SuperScript Reverse Transcriptase III following the manufacturer's protocol (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) was performed with iCycler thermal cycler (Bio-Rad) using iQ SYBR Green supermix. *Cyclophilin E* was used as an internal standard and compared with specific primers by the $2^{-\Delta\Delta Ct}$ method, where Ct is the threshold cycle. Primer sequences used for qRT-PCR are shown in table 1.

Table 1. Primer sequences used for qRT-PCR.

Gene	qPCR Sense Primer (5' to 3')	qPCR Antisense Primer (5' to 3')
Tnfα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Ifnγ	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
Il17	ACCGCAATGAAGACCCTGAT	TCCCTCCGCATTGACACA
Il4	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA
Il1ß	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il6	GAGGATACCACTCCCAACGAGCC	AAGTGCATCATCGTTGTTCATACA
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Il22	TTCGAGGAGTCAGTGCTAAA	AGAACGTCTTCCAGGGTGAA
Il23	AGAGCCAGCCAGATTTGAGA	CAGCAGCATTACAGCTCTGC
Muc2	TTTCAAGCACCCCTGTAACC	AAGTCCTGGTGTTGAACCTG
Gcsf	CCCACCTTGGACTTGCTTCA	TAGGTTTTCCATCTGCTGCCA
Tgfß	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
Cxcl1	AACCGAAGTCATAGCCACACT	TTGTCAGAAGCCAGCGTTCA
Arg1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC
Ccn1	GGAGGTGGAGTTAACGAGAAAC	GTGGTCTGAACGATGCATTTC
CypE	TTCACAAACCACAATGGCACAGGG	TGCCGTCCAGCCAATCTGTCTTAT
IL6	AAATTCGGTACATCCTCGACGGCA	AGTGCCTCTTTGCTGCTTTCACAC
CYPE	GCAGACAAGGTCCCAAAGACA	CACCCTGACACATAAACCCTG
GCSF	AGCTTCCTGCTCAAGTGCTTA	GTAGGTGGCACACTCACTCAC

2.8 ELISA

ELISA in cell culture conditioned media was conducted using mouse and human IL-6 Quantikine ELISA kit (R&D). Serum IL-6 levels were quantified IL-6 and G-CSF Quantikine ELISA kit (R&D) or IL-6 and G-CSF single analyte ELISA kit (Qiagen) following manufacturer's protocol.

2.9 Cell growth curve and proliferation assays

For growth curve, YAMC cells were plated in 12-well dishes ($1x10^4$ cells/well) and maintained under nonpermissive conditions for 24 hrs before the beginning of treatments. Cell cultures were exposed to purified WT-CCN1, DM-CCN1 or BSA as indicated. Cells were stained with 1% Trypan blue and total cell numbers were counted using hemacytometer. For Ki67 immunostaining of cells, cells were seeded in 24-well plates ($2.5x10^4$ cell/well) and treated with WT-CCN1, DM-CCN1, IL-6, EGF (epidermal growth factor), or BSA for 24 hrs. Cells were then fixed in 4% paraformaldehyde, washed, incubated with Rabbit polyclonal antibody against Ki67 (Abcam), and visualized with Anti-Rabbit IgG Alexa Fluor 488 (Invitrogen). Fluorescence micrographs were taken in random fields in each using Leica DM4000B microscope mounted with QI Click CCD digital camera. Ki67-positive cells were scored and normalized to DAPI. All assays were done in triplicates, and more than 300 cells were counted in each sample from randomly selected fields.

2.10 MPO assay

Distal colon tissue were excise from mice on indicated days, weighted, and homogenized in 50 mM potassium phosphate buffer pH 6.0. After centrifugation, pellet was resuspended in 50 mM potassium phosphate buffer pH 6.0 containing 1% hexadecyltrimethylammonium bromide (HTA-Br, Sigma, H9151). Cell lysate was sonicated,

freeze-thawed for a total of 3 times, and centrifuged at 10,000 RPM for 10 minutes. Supernatant (10µL) was added to 1% HTA-Br in 50 mM Potassium Phosphate pH6.0 containing 0.167 mg/ml *o*-dianisidine hydrochloride (O-DA, Sigma) and 0.0005% H_2O_2. 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 MPO enzyme (Enzo Life Sciences, Farmingdale, NY).

2.11 Statistical analysis

Date are expressed as mean ± SEM. 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, and for tumor incidence was evaluated by Fisher's exact test.

3. CCN1 AMELIORATES INTESTINAL INFLAMMATION IN MURINE COLITIS

3.1 Background

Crohn's disease (CD) and ulcerative colitis (UC), 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 United States (11). These medically incurable diseases of poorly defined etiology generally begin in young adulthood and continue throughout life, often requiring lifelong management to temper episodic manifestations of abdominal pain, diarrhea, malabsorption and weight loss, and rectal bleeding. Incidences of IBD are increasing worldwide, especially in developing countries (61), and prolonged UC is an important risk factor for developing colorectal cancer (62).

Genetic studies and mouse models of IBD reveal that an imbalance between pro- and anti-inflammation cytokines play a critical role in the pathogenesis of intestinal inflammation (10). For example, deletion of genes encoding regulatory cytokines, such as *IL-2* or *IL-10*, in mice resulted in the development of spontaneous colitis (63;64). Furthermore, treating animals with recombinant anti-inflammatory cytokines or blockade of pro-inflammatory cytokines demonstrated beneficial effects in multiple murine model of colitis (65-68). Lastly, genome-wide association studies have identified IBD risk loci that contain genes that encode cytokines and its signaling receptors (69). Taken together, these findings support the notion that cytokines are key players in directing intestinal inflammation. Therefore, there is a need to better understand the functions of cytokines that are differentially expressed in IBD patients and identify the factors that regulate those cytokines to develop novel therapeutic agents.

CCN1, a secreted matricellular protein of ~40 kDa, is emerging as an important modulator of inflammation (28). Acting primarily through direct binding to integrin receptors, CCN1 regulates diverse cellular functions, including cytokine expression, in a cell-type and context-dependent manner (22). Whereas CCN1 is essential for angiogenesis and cardiovascular development during embryogenesis (17;18), its expression has been linked to inflammation and tissue repair in adulthood (29;70). However, the specific functions of CCN1 in various inflammation-associated pathologies are only beginning to be appreciated. Importantly, *CCN1* expression is elevated in biopsies of patients with UC or CD, and in mice with experimental colitis (71;72). However, the precise function of CCN1 in IBD remains unknown.

Integrin α_v-deficient mice develop spontaneous colitis and are predisposed to colon adenocarcinomas (73). Since many known functions of CCN1 are mediated through its vWC domain-dependent binding to integrin $\alpha_v\beta_3$, we postulated that CCN1 may act as a critical ligand for α_v integrins and contribute to the protective signaling mediated through α_v integrins in murine colitis. To assess this function, we utilized *Ccn1*$^{d125a/d125a}$ knockin mice, which expresses a mutant CCN1 protein (D125A-CCN1) that is defective in its ability to interact with integrin $\alpha_v\beta_3$. *Ccn1*$^{d125a/d125a}$ mice are viable and fertile, which allows us to circumvent the embryonic lethality of *Ccn1*-null mice (17;18) and avoids potential limitations of cell type-specific deletions as CCN1 may be secreted by multiple cell types in the colonic tissue microenvironment. To elicit intestinal inflammation, the mice were challenged with dextran sodium sulfate (DSS) in an established protocol for the induction of IBD (74).

3.2 DSS-challenged *Ccn1*$^{d125a/d125a}$ mice exhibit exacerbated disease and inflammatory damage

When WT and *Ccn1*$^{d125a/d125a}$ mice were subjected to 5% (w/v) DSS feeding for 5 days, mice in both groups began to demonstrate clinical manifestations of disease, defined by body weight loss, rectal bleeding, and loose stool/diarrhea on day 4. WT animals showed the highest disease activity score on day 8 but began their recovery in subsequent days, and all WT mice survived (figure 3A). In striking contrast, *Ccn1*$^{d125a/d125a}$ mice exhibited progressive weight loss and an inability to recover, which resulted in death beginning on day 7 with no survival recorded after day 11 of the experiment (figure 3A-C). This indicates that *Ccn1*$^{d125a/d125a}$ mice are highly sensitive to DSS-induced colitis. To further investigate this finding, we lowered the concentration of DSS to 3.5% (w/v) to avoid death of *Ccn1*$^{d125a/d125a}$ mice. Consistently, *Ccn1*$^{d125a/d125a}$ mice exhibited more severe and prolonged body weight loss, disease activity index (DAI), and colonic shortening than WT (figure 3D-F). Moreover, histological evaluation of the distal colon showed increased tissue damage in *Ccn1*$^{d125a/d125a}$ mice beginning on day 5 (p<0.05) and continuing to day 14 (p<0.001)(figure 4). Notably, many *Ccn1*$^{d125a/d125a}$ mice developed neutrophilic abscesses on day 11 and 14 after initiation of DSS feeding suggesting that *Ccn1*$^{d125a/d125a}$ mice suffered from chronic inflammation. TUNEL staining in the distal colon section of wild type and *Ccn1*$^{d125a/d125a}$ mice revealed that there was an increase in the number of cell deaths during the initial stages of intestinal inflammation (figure 5), consistent with notion that *Ccn1*$^{d125a/d125a}$ mice are more sensitive to DSS toxicity. Collectively, these results show that CCN1 functions through its $\alpha_V\beta_3$-binding site to ameliorate tissue damage, attenuate disease severity, and minimize mortality of mice in a chemically-induced model of colitis.

Figure 3. Higher morbidity and mortality in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice. Wild type and *Ccn1*$^{d125a/d125a}$ mice (*n*=6 per genotype) were given 5% (w/v) DSS water for 5 days followed by regular drinking water. (A) Body weight change, (B) DAI, and (C) survival was monitored daily. The disease course of acute colitis and recovery from intestinal injury of wild type and *Ccn1*$^{d125a/d125a}$ mice challenged with 3.5% (w/v) DSS water for 5 days followed by regular drinking water was monitored by measuring (D) body weight change, (E) DAI, and colon lengths on indicated days after initiation of DSS exposure (*n*>5). *p<0.05, **p<0.01, ***p<0.001.

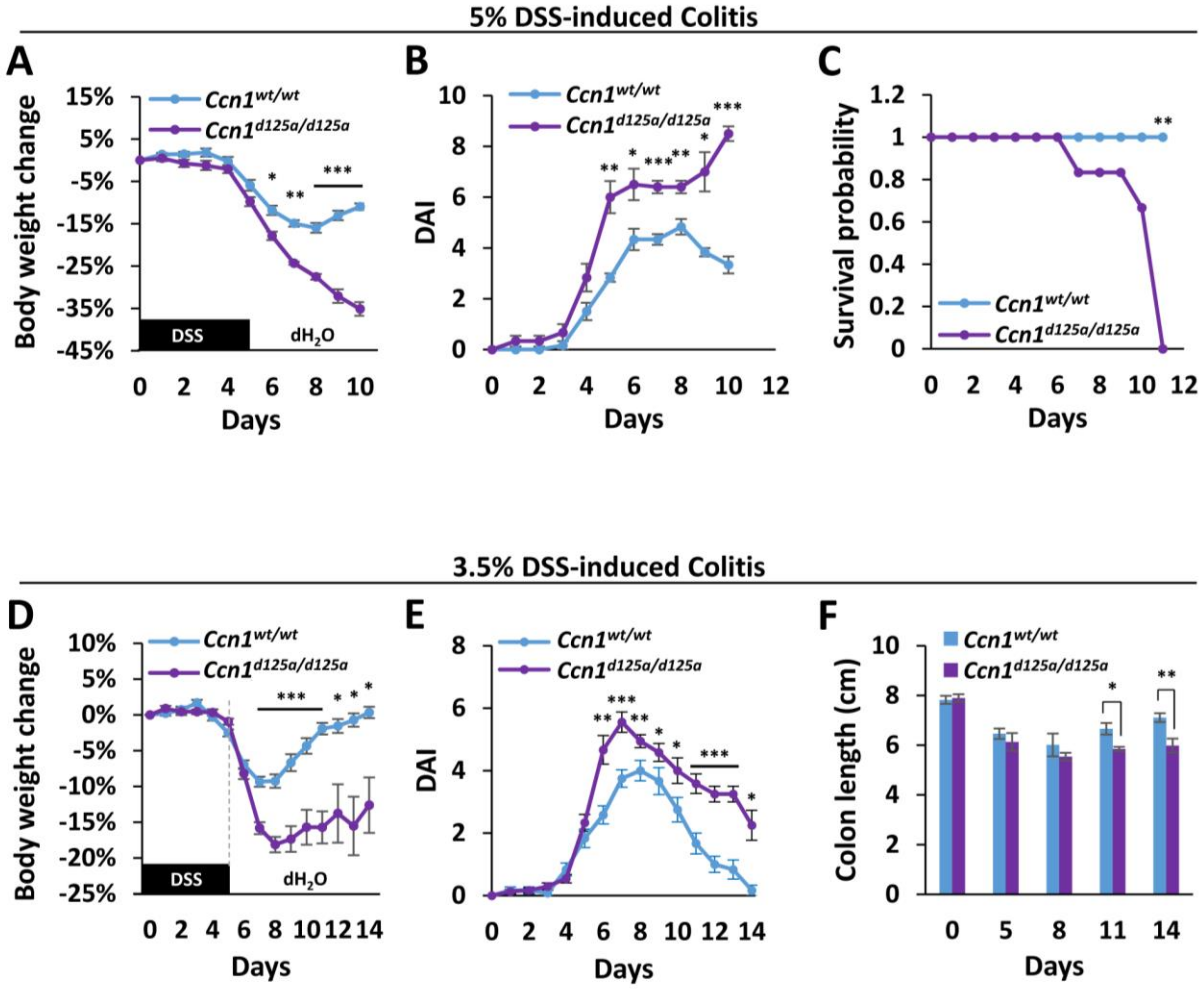
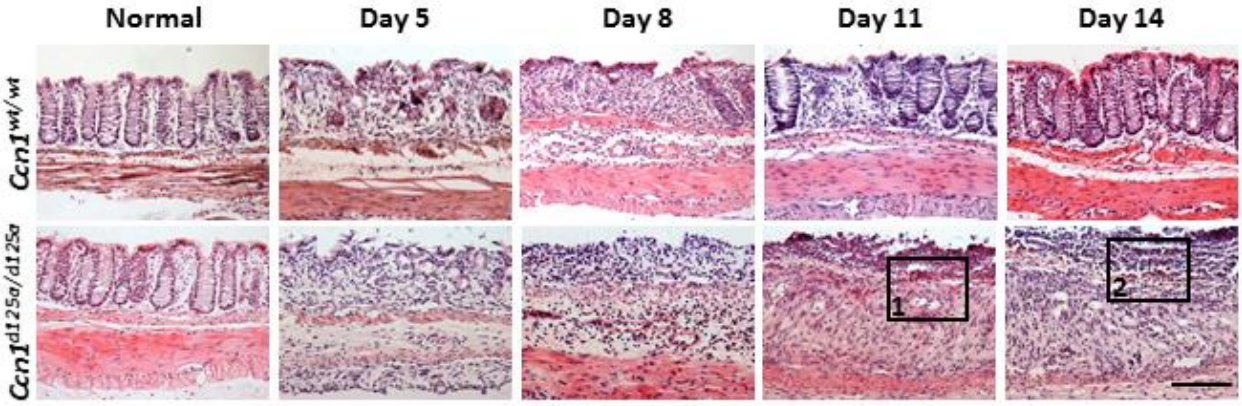
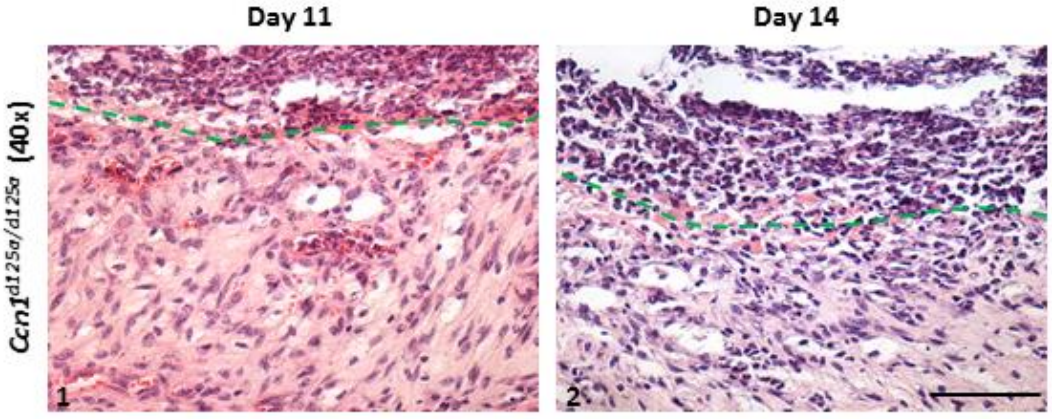

5% DSS-induced Colitis
A
Body weight change
15%
5%
-5%
-15%
-25%
-35%
-45%
Ccn1wt/wt
Ccn1d125a/d125a
DSS
dH2O
0 2 4 6 8 10
Days
B
DAI
10
8
6
4
2
0
Ccn1wt/wt
Ccn1d125a/d125a
0 2 4 6 8 10 12
Days
C
Survival probability
1.2
1
0.8
0.6
0.4
0.2
0
Ccn1wt/wt
Ccn1d125a/d125a
0 2 4 6 8 10 12
Days
3.5% DSS-induced Colitis
D
Body weight change
10%
5%
0%
-5%
-10%
-15%
-20%
-25%
Ccn1wt/wt
Ccn1d125a/d125a
DSS
dH2O
0 2 4 6 8 10 12 14
Days
E
DAI
8
6
4
2
0
Ccn1wt/wt
Ccn1d125a/d125a
0 2 4 6 8 10 12 14
Days
F
Colon length (cm)
10
8
6
4
2
0
Ccn1wt/wt
Ccn1d125a/d125a
0 5 8 11 14
Days

Figure 4. Increased tissue damage in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice. Mucosal damage in the distal colon of Wild type and *Ccn1*$^{d125a/d125a}$ mice challenged with 3.5% (w/v) DSS water for 5 days was analyzed by H&E staining on indicated days. Scale bar=100 µm. Higher magnification of intestinal mucosa with neutrophilic abscesses from *Ccn1*$^{d125a/d125a}$ mice on day 11 and 14 after DSS feeding are shown in the middle. Dashed lines demarcate the border of neutrophilic abscess (above) and intestinal mucosa (below). Scale bar=50 µm. Histological scores are shown on the bottom (n=3-6). *p<0.05, **p<0.01, ***p<0.001.


Normal
Day 5
Day 8
Day 11
Day 14
Ccn1wt/wt
Ccn1d125a/d125a
1
2


Day 11
Day 14
Ccn1d125a/d125a (40x)
1
2

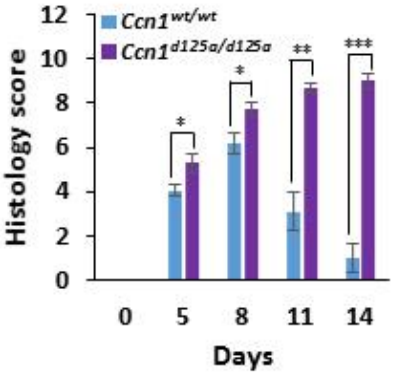
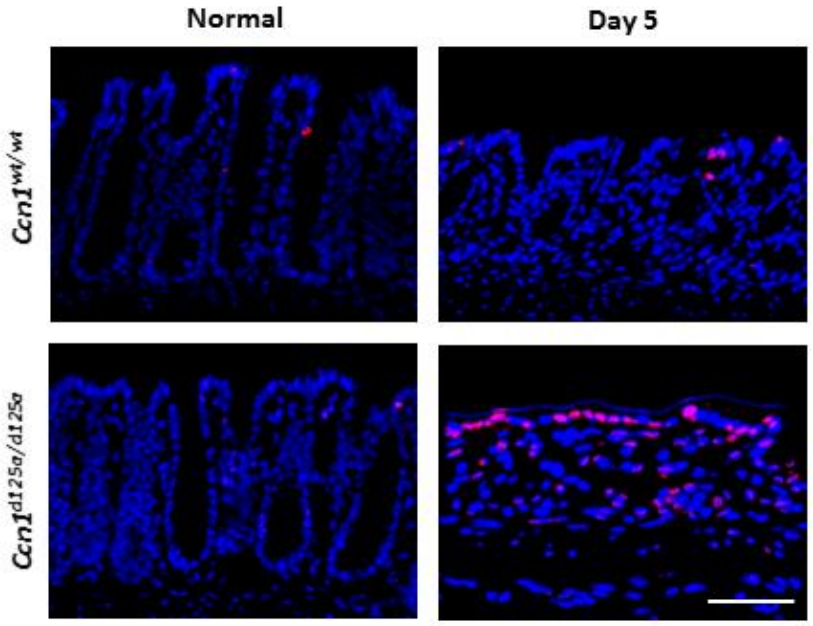
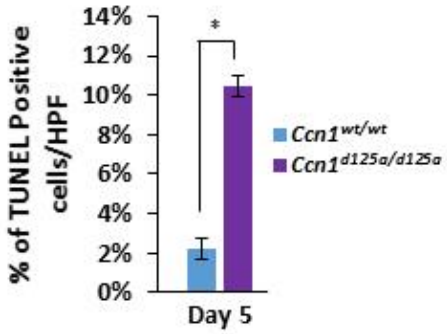

Ccn1wt/wt
Ccn1d125a/d125a
Histology score
Days
0
5
8
11
14
*
*
**
***

Figure 5. Increased cell death in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice. Cell death in the distal colon on day 5 after initiation of DSS feeding was measured by TUNEL, and cells were counterstained with 4,6-diamidino-2-phyenylindole (DAPI). Quantitation is shown below (*n*=3). Scale bar=50 μm. *p<0.05

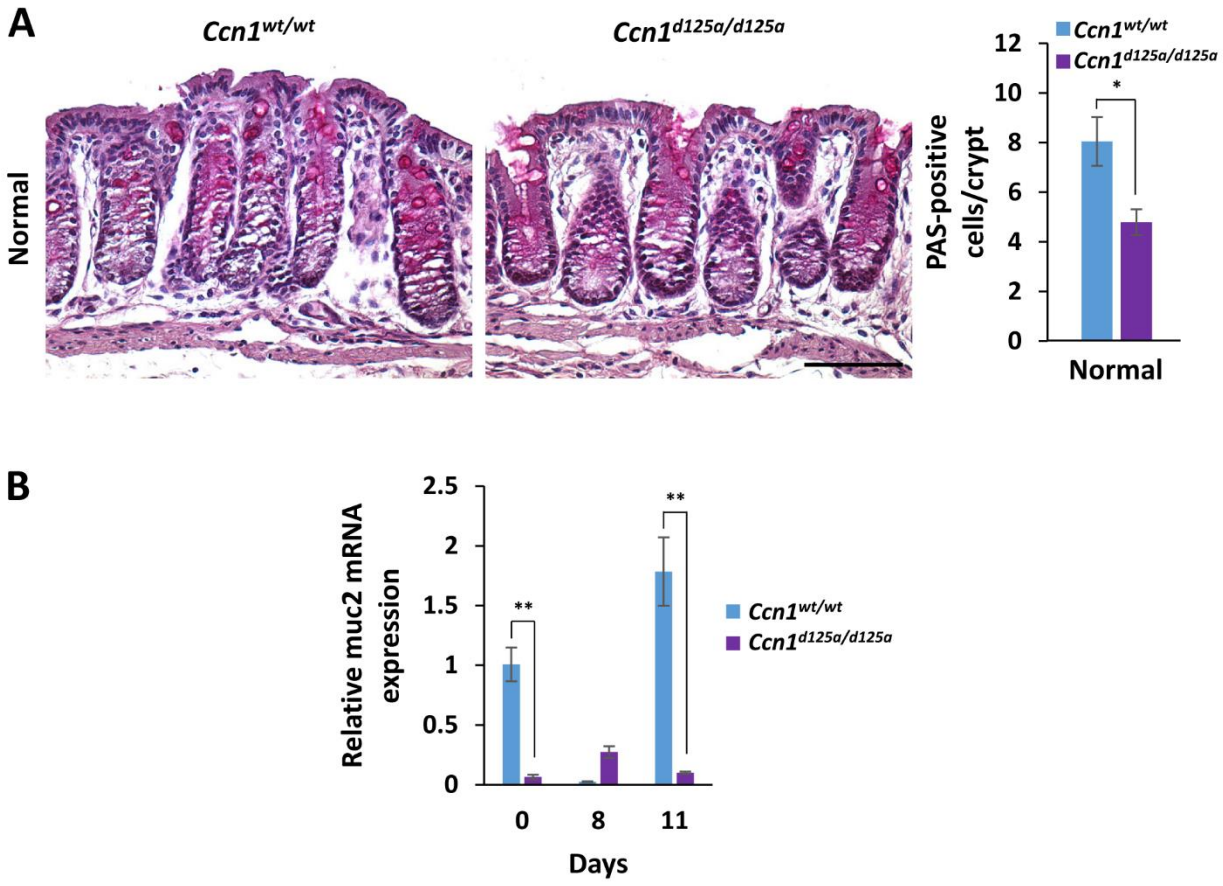

Normal
Day 5
Ccn1wt/wt
Ccn1d125a/d125a


% of TUNEL Positive cells/HPF
14%
12%
10%
8%
6%
4%
2%
0%
*
Day 5
Ccn1wt/wt
Ccn1d125a/d125a

3.3 *Ccn1*$^{d125a/d125a}$ mice have intestinal barrier dysfunction

A critical component of the innate defense mechanism to protect IECs from the toxic and noxious agents in the gut lumen is the mucus layer that forms a physical barrier between the luminal content and the underlying tissue (75). An outer (loose) layer and inner (adherent) mucus layers are organized by mucin glycoproteins (MUC*),* trefoil factor peptides, and Fc-γ binding protein (76). Among the 20 different mucin genes identified, *MUC2* is the major secretory mucin synthesized and secreted by the goblet cells in healthy and inflamed colon (77). Accordingly, *Muc2*-deficient mice develop spontaneous colitis, and *Muc2*-haploinsufficient mice show exacerbated disease and greater tissue injury compared to wild type littermates in DSS-induced colitis, indicating that Muc2 is critical for protection against intestinal inflammation (78). Therefore, we postulated that *Ccn1*$^{d125a/d125a}$ mice may also have a deficit in *Muc2*. To test this hypothesis, we stained the normal distal colon tissue sections of wild type and *Ccn1*$^{d125a/d125a}$ mice with periodic acid-schiff (PAS) staining and counted the number of goblet cells in the crypts. We found that *Ccn1*$^{d125a/d125a}$ mice had fewer goblet cells compared to wild type (4.8 vs 8.0, respectively, $p<0.05$)(figure 6A). Furthermore, *MUC2* gene expression was dramatically reduced (>10-fold) in the normal and inflamed colon (day 11) of *Ccn1*$^{d125a/d125a}$ mice (figure 6B). These findings reveal that *Ccn1*$^{d125a/d125a}$ mice have a defect in barrier function which may contribute to its increase in sensitivity to DSS toxicity, and suggests that CCN1 regulates goblet cells and *MUC2* expression through its $\alpha_V\beta_3$ binding site.

Figure 6. *Ccn1*$^{d125a/d125a}$ mice have fewer goblet cells and a decreased *MUC2* production. (A) Paraffin-embedded sections of normal distal colon of wild type and *Ccn1*$^{d125a/d125a}$ mice were stained with periodic acid-schiff (PAS) staining kit. Quantitation of PAS-positive cells in the intestinal crypt are shown on the right (*n*=3). Scale bar=50 μm. (B) *MUC2* mRNA in the normal and inflamed colon (day 8 and 11 after initiation of 3.5% DSS feeding) of wild type and *Ccn1*$^{d125a/d125a}$ mice was measured by qRT-PCR, and normalized to normal wild type colon with *cyclophilin E* as an internal reference. Data shown as mean $\pm$ SEM; *p<0.05, **p<0.01.


A
Ccn1wt/wt
Ccn1d125a/d125a
Normal
PAS-positive cells/crypt
Ccn1wt/wt
Ccn1d125a/d125a
*
12
10
8
6
4
2
0
Normal
B
Relative muc2 mRNA expression
2.5
2
1.5
1
0.5
0
**
**
0
8
11
Days
Ccn1wt/wt
Ccn1d125a/d125a

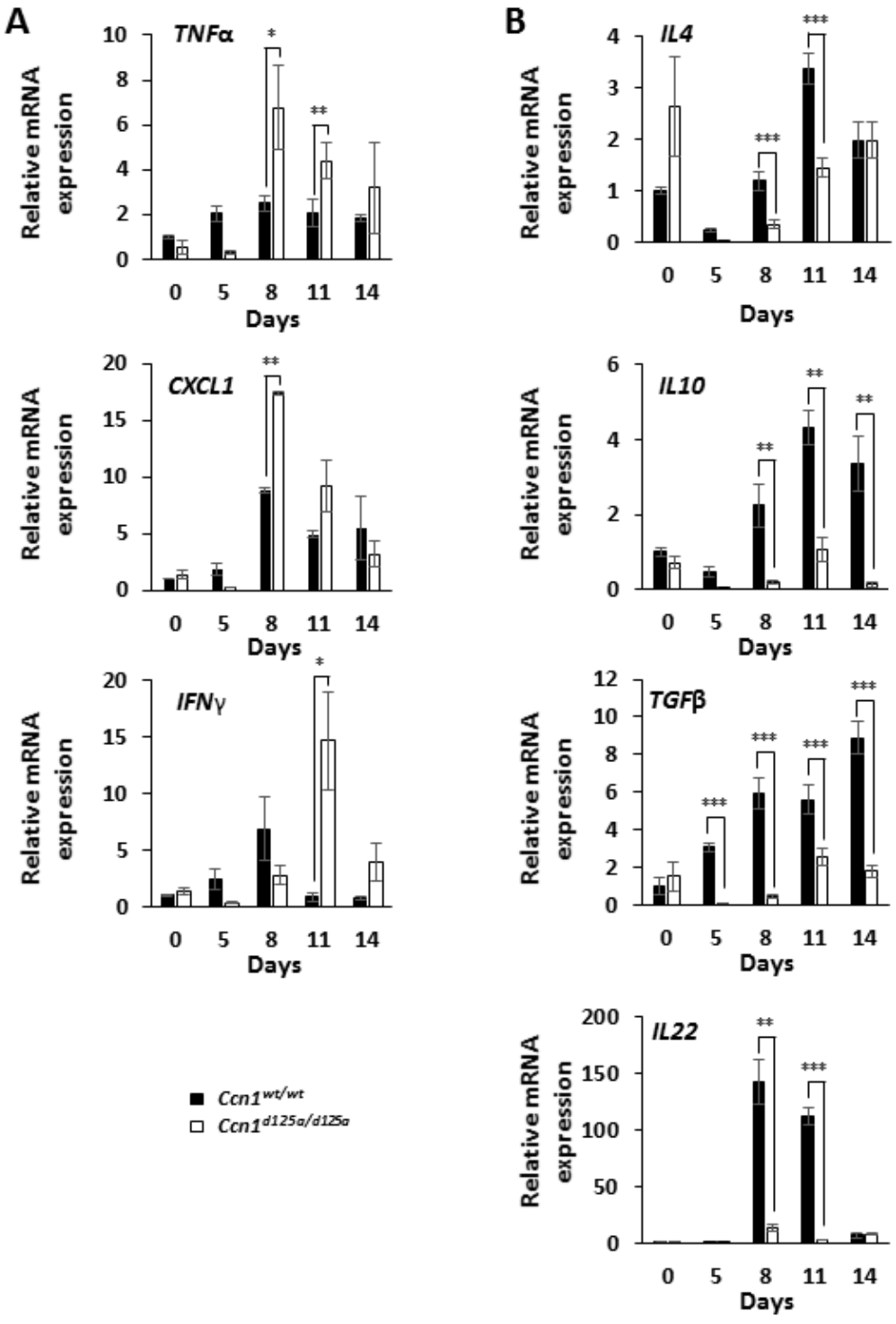
3.4 DSS-challenged *Ccn1*$^{d125a/d125a}$ mice show aberrant expression of pro- and anti-inflammatory cytokines

To examine if DSS-challenged *Ccn1*$^{d125a/d125a}$ mice suffer an increase in severity of inflammation as a result of heightened sensitivity to DSS toxicity, we isolated total RNA from the distal colons of wild type and *Ccn1*$^{d125a/d125a}$ mice at the indicated times post-DSS feeding and measured expression of pro- and anti-inflammatory cytokines by qRT-PCR. We observed that *Ccn1*$^{d125a/d125a}$ mice expressed higher levels of *TNFα*, *CXCL1*, and *IFN-γ* on days 8 and/or 11 post-DSS feeding while showing a deficit in expression of anti-inflammatory cytokines, namely *IL-4, IL-10*, and *TGF-β* (figure 7). Furthermore, *Ccn1*$^{d125a/d125a}$ mice showed a striking deficit for *IL-22*, a member of the IL-10 family of cytokines that has been previously demonstrated to ameliorate murine colitis (79). Interestingly, the protective effect of IL-22 in experimental colitis involved enhanced mucus production (79), further supporting the finding that *Ccn1*$^{d125a/d125a}$ mice lack an adequate mucus barrier. These results indicate that DSS-challenged *Ccn1*$^{d125a/d125a}$ mice have lost the ability to maintain a balance between pro- and anti-inflammatory cytokines, which contributes to the exacerbated inflammatory damage.

3.5 DSS-challenged *Ccn1*$^{d125a/d125a}$ mice have excessive and prolonged neutrophil recruitment as well as a deregulated IL23/IL17/G-CSF axis

In addition to the increase in severity of initial inflammation in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice, we observed that many *Ccn1*$^{d125a/d125a}$ mice developed neutrophilic abscesses that formed in the mucosal lining of the colon starting from day 11 and persisting to day 14 after initiation of DSS administration (figure 4). Measure of MPO activity from the distal colon of *Ccn1*$^{d125a/d125a}$ mice confirmed the increase in neutrophil accumulation, particularly on

Figure 7. DSS-challenged *Ccn1*$^{d125a/d125a}$ mice exhibit an imbalance in pro- and anti-inflammatory cytokine expression. (A) Pro-inflammatory (*TNFα*, *CXCL1, IFNγ*), (B) anti-inflammatory (*IL-4*, *IL-10*, *TGF-β*, *IL-22*) cytokine expression was measured on day 5, 8, 11, and 14 from isolated colon RNA of 3.5% DSS-challenged wild type and *Ccn1*$^{d125a/d125a}$ mice by qRT-PCR. Data are represented as mean ± SEM; *n*=3-4; **p*<0.05, ***p*<0.01, ****p*<0.001.

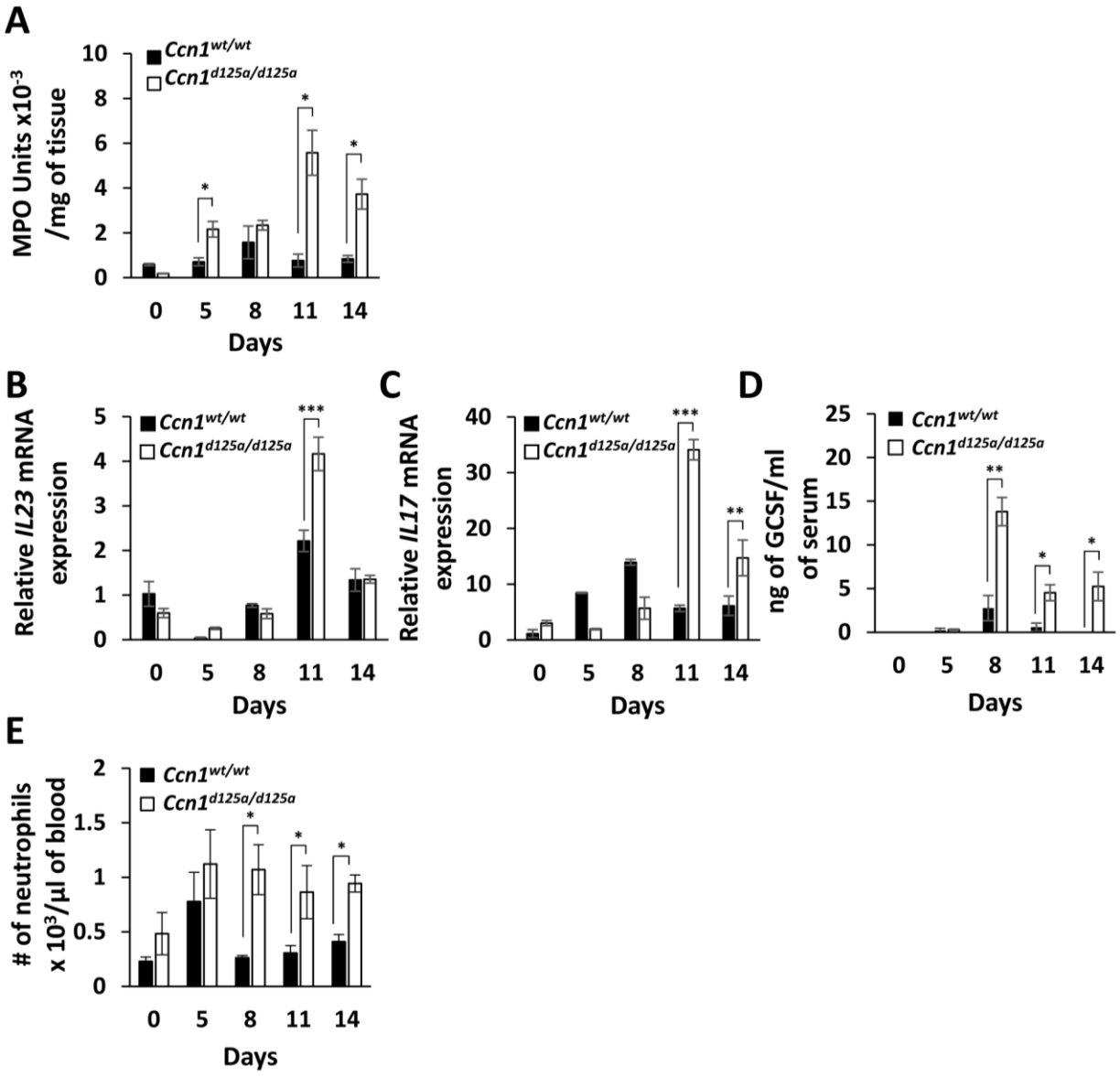

A
B
TNFα
IL4
CXCL1
IL10
IFNγ
TGFβ
IL22
Relative mRNA expression
Days
0
5
8
11
14
Ccn1wt/wt
Ccn1d125a/d125a

day 11 and 14, compared to wild type (figure 8A). One pathway that has been previously described to regulate neutrophil homeostasis is the IL23/IL17/GCSF axis (80). To ascertain if this pathway is deregulated in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice, we measured gene expression of IL-23 and IL-17 from the inflamed colon by qRT-PCR and sampled protein levels of G-CSF in the serum on corresponding days. Indeed, *IL-23* and *IL-17* gene expression were elevated on day 11 (figure 8B-C), and the serum levels of G-CSF peaked on day 8 and remained elevated through day 14 post-DSS feeding (figure 8D). In addition, we counted the number of neutrophils in the blood to examine if the aberrant activation of the IL23/IL17/GCSF axis coincided with excessive neutrophil recruitment. Consistent with elevated G-CSF levels in the serum, there was an increase in the number of circulating neutrophils in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice from day 8 to 14 (figure 8E), indicating an activation of systemic inflammatory response. These findings show that a mutation in the $\alpha_V\beta_3$ binding site of CCN1 is associated with a deregulated IL23/IL17/GCSF axis and neutrophil-mediated chronic inflammation, suggesting that CCN1 is a critical regulator of the IL23/IL17/GCSF pathway and neutrophil mobilization during inflammation.

3.6 IL-17 drives chronic inflammation in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice

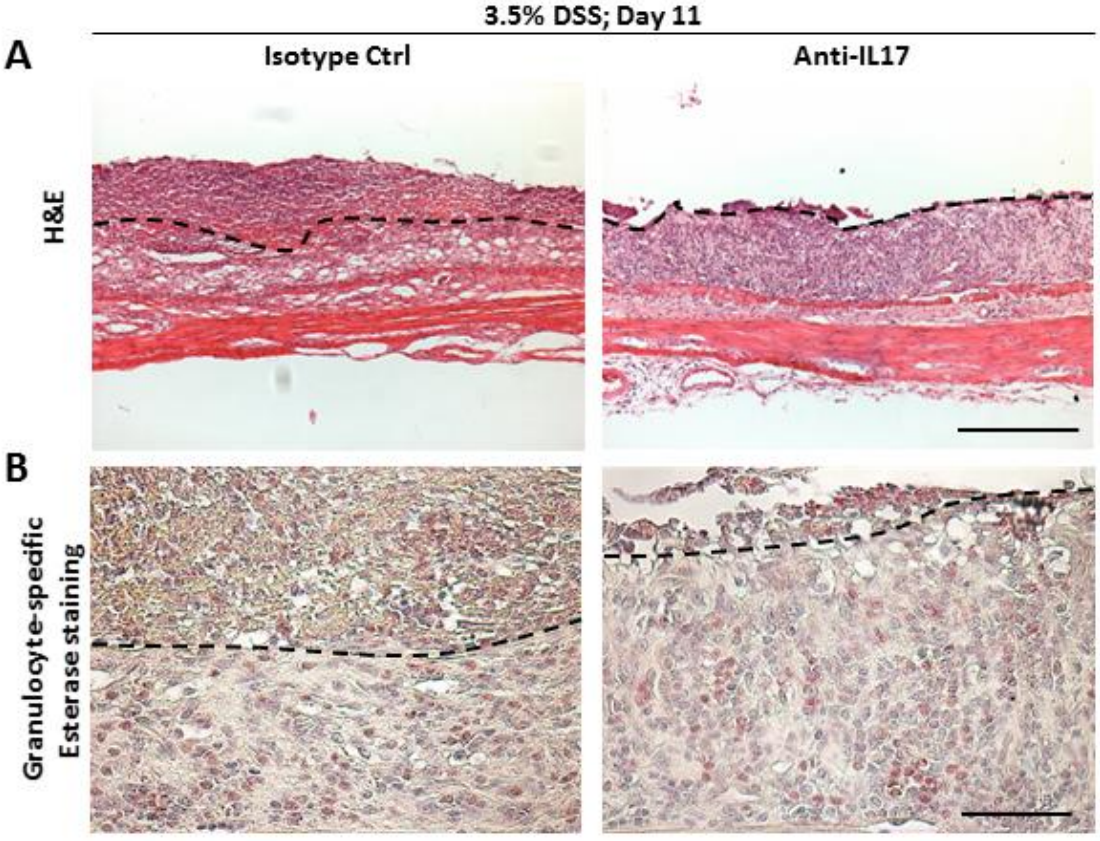
To confirm the involvement of IL-17 in excessive neutrophil recruitment and accumulation in the distal colon of *Ccn1*$^{d125a/d125a}$ mice, we injected *Ccn1*$^{d125a/d125a}$ mice i.p. with 200 µg of neutralizing monoclonal antibodies against IL17 (anti-IL17) or Isotype IgG control on day 10 post-DSS feeding and sacrificed the mice 24 hours later. Treatment with anti-IL17, compared to isotype control, reduced the formation of neutrophilic abscesses as observed in the paraffin-embedded section of the distal colon by H&E and naphthol AS-D chloroacetate

Figure 8. DSS-challenged *Ccn1*$^{d125a/d125a}$ mice have a deregulated IL23/IL17/GCSF axis and excessive neutrophil recruitment. In wild type and *Ccn1*$^{d125a/d125a}$ mice challenged with 3.5% DSS for 5 days, (A) *IL23* and (B) *IL17* gene expression was measured from the distal colon on indicated days by qRT-PCR. Data shown as mean ± SD; *n*=3. (C) G-CSF protein was measured from the serum by ELISA (*n*=4-6). (D) Neutrophil mobilization was assessed by counting the number of neutrophils in the blood (*n*=3-8) and (E) neutrophil infiltration into the distal colon was measured by MPO activity (*n*=3). Data shown as mean ± SEM; **p*<0.05, ***p*<0.01, ****p*<0.001.


A
MPO Units x10-3 /mg of tissue
Ccn1wt/wt
Ccn1d125a/d125a
Days
B
Relative IL23 mRNA expression
C
Relative IL17 mRNA expression
D
ng of GCSF/ml of serum
E
# of neutrophils x 10³/µl of blood

esterase staining, which specifically stains granules in neutrophils (figure 9A-B). MPO activity confirmed the decrease in neutrophil accumulation in the distal colon of anti-IL17-treated *Ccn1*$^{d125a/d125a}$ mice (figure 10A). Furthermore, anti-IL17 treatment reduced the number of neutrophils in the blood (figure 10B) along with G-CSF protein level in the serum (figure 10C) showing that IL-17 was involved in systemic neutrophil recruitment. This indicates that the persistent and excessive inflammation in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice is IL17-dependent, and suggests that CCN1 is a negative regulator of IL-17 in chronic intestinal inflammation.

Figure 9. IL-17 blockade decreases sizes of neutrophil abscesses in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice. *Ccn1*$^{d125a/d125a}$ mice challenged with 3.5% DSS for 5 days followed by regular drinking water for 6 days were injected i.p. with 200 μg of neutralizing monoclonal antibodies against IL-17 (anti-IL17) or isotype control on day 10 and sacrificed 24 hours later (*n*=3-6). Distal colon of mice were harvested, embedded in paraffin, and stained with (A) H&E for histological evaluation (Scale bar=300 μm) or (B) naphthol AS-D chloroacetate esterase kit for detection of neutrophils (Scale bar=50 μm). Dashed lines demarcate the border between neutrophilic abscess (above) and intestinal mucosa (below)


3.5% DSS; Day 11
A
Isotype Ctrl
Anti-IL17
H&E
B
Granulocyte-specific Esterase staining

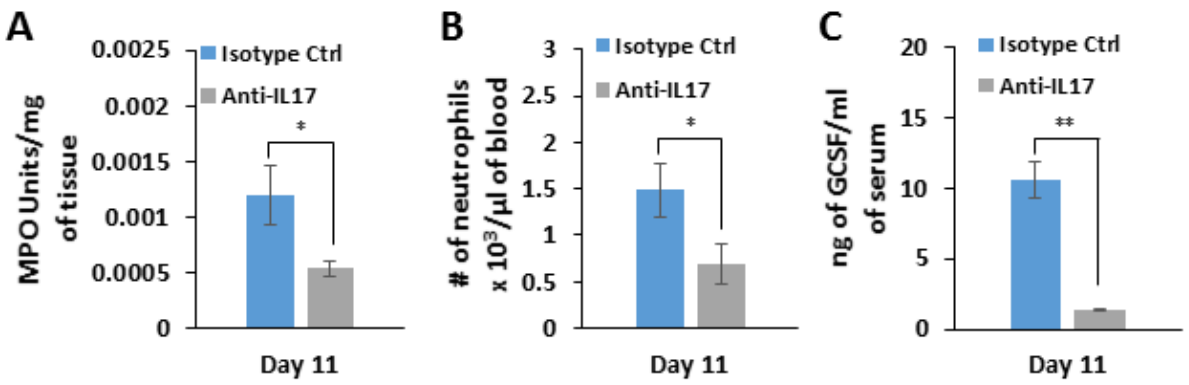


Figure 10. Aberrant neutrophil recruitment in DSS-challenged *Ccn1*$^{d125a/d125a}$ mice is IL17-dependent. (A) MPO activity from colonic tissue was measured to quantitate neutrophil accumulation. (B) Number of neutrophils in the blood were counted and (C) G-CSF protein was measured from the serum by ELISA. Data shown as mean ± SEM; *p<0.05, **p<0.001.

4. CCN1 PROMOTES MUCOSAL HEALING AFTER INTESTINAL INJURY

4.1 Background

Most of the existing therapies for IBD target the adaptive immune system to suppress the activity of pro-inflammatory cytokines and attenuate disease symptoms in patients (81). Based on this model, a breakthrough was made with the clinical development of monoclonal antibody against of TNF-α, and blockade of TNF-α has become a critical component in the therapeutic arsenal for IBD. However, despite its ability to induce clinical remissions, many patients (~40%) do not respond, lose their response during treatment, or develop complications due to side effects (82;83). Thus, there is an urgent need for alternative treatment options, and considerable effort has focused on the identification of novel therapeutic targets (84).

Among the targets under investigation for IBD therapy is interleukin-6 (IL-6), a multifunctional cytokine expressed by diverse cell types during inflammation (81;85). IL-6 is thought to be involved in the pathogenesis of IBD by inducing T-cell activation and suppressing T-cell apoptosis (65;81), and monoclonal antibodies (mAbs) against IL-6 receptor (IL-6R) prevent T cell-mediated murine colitis (65;68). Paradoxically, *IL-6*-deficient mice suffer increased mortality, morbidity, and mucosal damage in colitis, suggesting that IL-6 may play pleiotropic and potentially antithetical roles in IBD (86;87). Furthermore, a pilot clinical trial using anti-IL6R mAbs showed symptomatic improvement in patients with CD, but not enhancement in mucosal restitution (88). Therefore, further elucidation of the regulation and function of IL-6 in colitis is likely needed to optimize its therapeutic potential.

Mounting evidence suggests that CCN1 is emerging as an important regulator of tissue homeostasis and injury repair (22). For example, in cutaneous wound healing and in chronic

liver injuries induced by hepatotoxin or cholestasis, CCN1 functions to diminish and restrict tissue fibrosis by triggering cellular senescence in activated myofibroblasts (54;57). However, 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 (36;40;55). To further 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 $\alpha_6\beta_1$ (39;41). Similar to *Ccn1*$^{d125a/d125a}$ mice, *Ccn1*$^{dm/dm}$ mice are viable, fertile, morphologically and behaviorally normal (36;54;57), and display normal intestinal histology in the absence of pathogenic insults.

Here we provide the first evidence that CCN1 plays 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 and *Ccn1* mutant mice. Our findings reveal CCN1 as a critical regulator of mucosal healing in colitis, underscore 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.

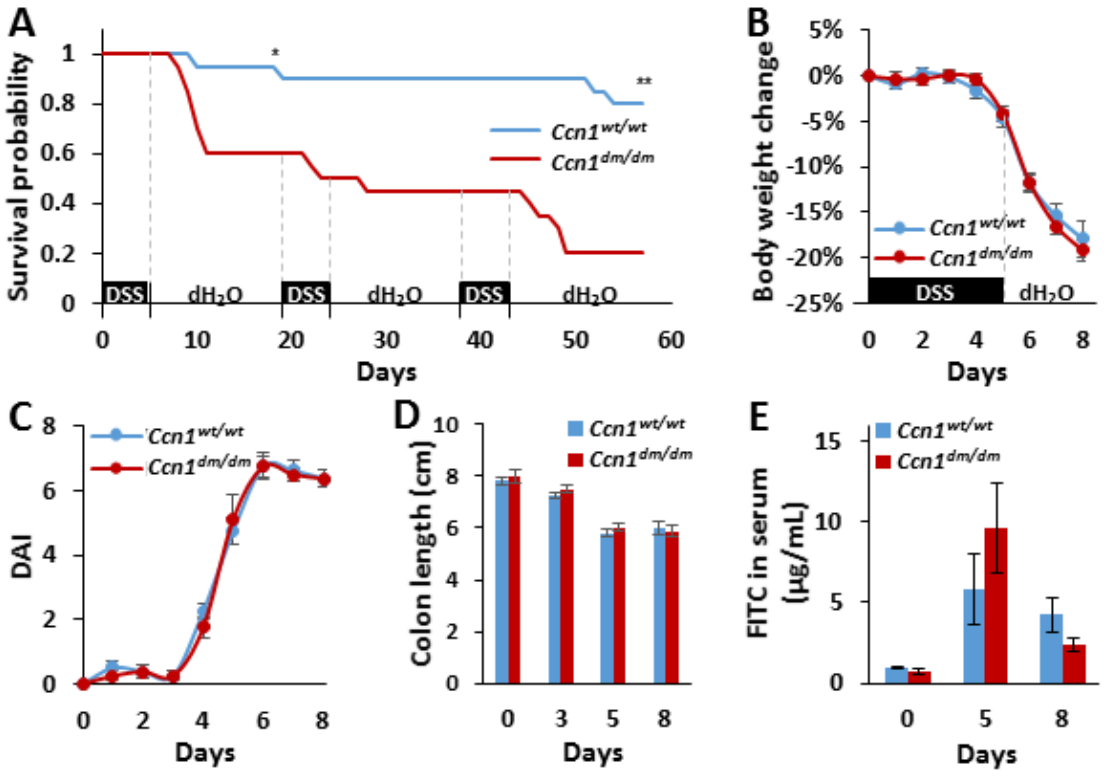
4.2 *Ccn1*$^{dm/dm}$ mice suffer increased mortality as well as impaired recovery and mucosal healing upon DSS challenge

Wild type (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. 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 to 95% and 80% of wild type mice (p<0.05 and p<0.001, respectively)(figure 11A), 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, DAI, and colonic shortening (figure 11B-D). The intestinal barrier function after DSS feeding, as evaluated by detection of gavaged FITC-conjugated dextran in the serum, was also similar between the two genotypes (figure 11E). Histological analyses of the distal colon showed comparable crypt loss and epithelial damage (figure 12), with similar numbers of apoptotic cells (figure 13). Lastly, MPO activity in the colonic tissue was comparable in wild type and *Ccn1*$^{dm/dm}$ mice, indicating similar extent of neutrophil infiltration into the mucosal epithelium and lamina propria (figure 14). 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). As expected, wild type and *Ccn1*$^{dm/dm}$ mice showed comparable body weight loss, DAI, colonic shortening, and histological score during the onset of disease from days 0-8 (figure 15). However, as recovery commenced on day 9 and the DAI began to decline, *Ccn1*$^{dm/dm}$ mice experienced a prolonged disease course with elevated DAI, slow recovery of body weight and colon length, and unrelenting epithelial erosion and ulceration through day 14 (p<0.001)(figure 16), indicating that *Ccn1*$^{dm/dm}$ mice suffer impaired recovery and mucosal healing from colitis.

Figure 11. 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 are detected in serum to evaluate intestinal barrier integrity (*n*=3).


A
Survival probability
1
0.8
0.6
0.4
0.2
0
*
**
Ccn1wt/wt
Ccn1dm/dm
DSS
dH2O
DSS
dH2O
DSS
dH2O
0
10
20
30
40
50
60
Days
B
Body weight change
5%
0%
-5%
-10%
-15%
-20%
-25%
Ccn1wt/wt
Ccn1dm/dm
DSS
dH2O
0
2
4
6
8
Days
C
DAI
8
6
4
2
0
Ccn1wt/wt
Ccn1dm/dm
0
2
4
6
8
Days
D
Colon length (cm)
10
8
6
4
2
0
Ccn1wt/wt
Ccn1dm/dm
0
3
5
8
Days
E
FITC in serum (µg/mL)
15
10
5
0
Ccn1wt/wt
Ccn1dm/dm
0
5
8
Days

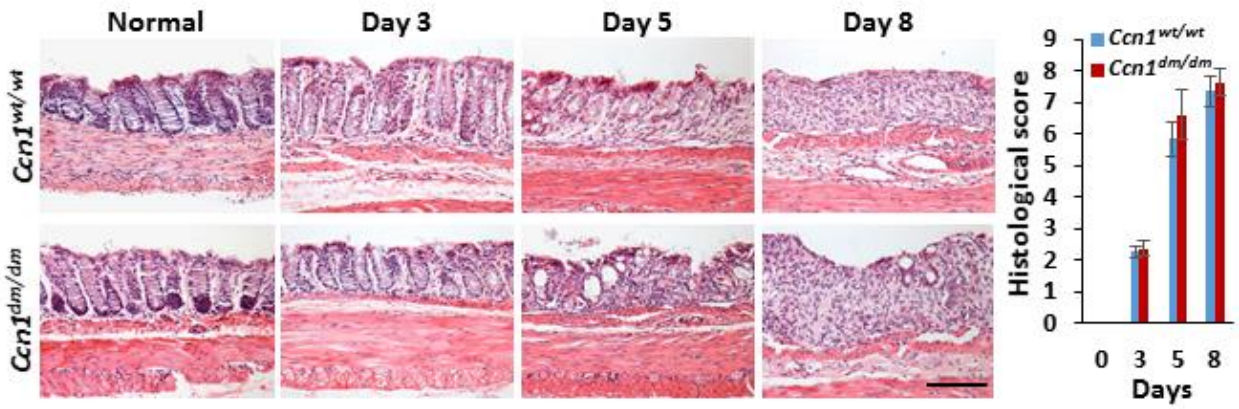


Figure 12. *Ccn1*$^{wt/wt}$ and *Ccn1*$^{dm/dm}$ mice have comparable levels of tissue injury in acute colitis. Mucosal damage was assessed by H&E staining of paraffin-embedded sections of the distal colon; histological scores are shown on right (*n*=4-6). Scale bar=100 µm.

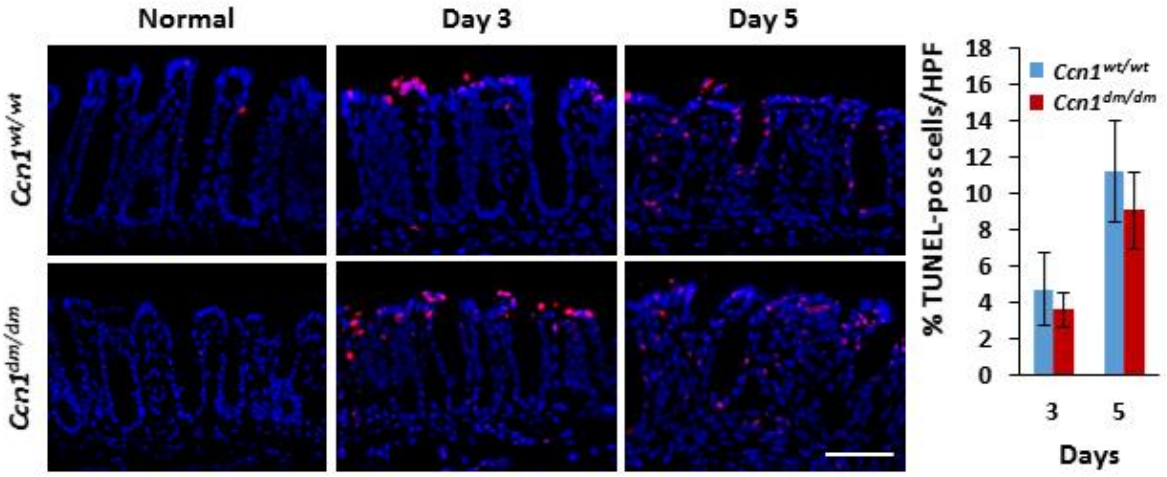


Figure 13. *Ccn1*$^{dm/dm}$ mice do not have increased number of cell deaths during DSS-induced colitis. Cell death in the distal colon was measured by TUNEL, and cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Quantitation is shown on the right (*n*=3). Scale bar=50 µm.

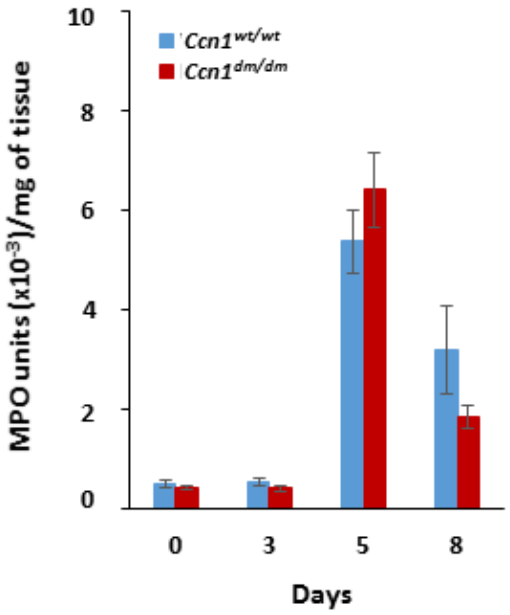


Figure 14. Innate inflammation is not more severe in DSS-challenged *Ccn1*$^{dm/dm}$ mice. MPO activity from the colonic tissue (*n*=3-6).

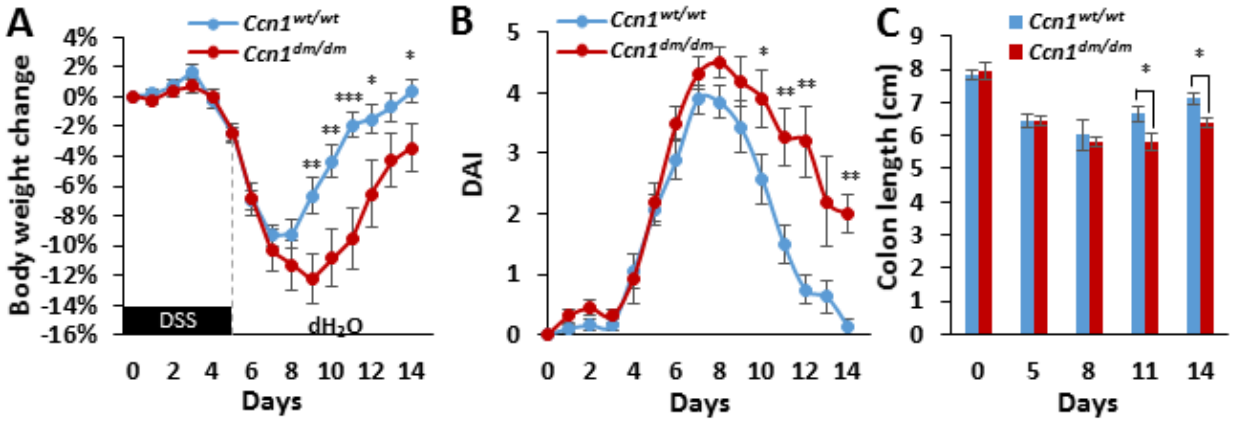


Figure 15. Impaired recovery in DSS-challenged *Ccn1*$^{dm/dm}$ mice. Wild type and *Ccn1*$^{dm/dm}$ mice were given DSS water (3.5% w/v) for five days followed by regular water. (A) Changes in body weight and (B) DAI were recorded daily, and (C) colonic length was measured (n=7-8). *p<0.05, **p<0.01, ***p<0.001.

4.3 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 (i.p.) 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, compared to only 20% survival in PBS-treated controls (p<0.01)(figure 17A) and 80% in wild type mice (figure 11A). Thus, CCN1 therapy fully restored survival in *Ccn1*$^{dm/dm}$ mice to wild type 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 PBS-treated controls (day 8, p<0.05; days 11 and 14, p<0.01)(figure 17B-C), comparable to wild type animals (figure 15A,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 18), further indicating that CCN1 promotes tissue repair after DSS-induced colitis.
Proliferation of intestinal epithelial cells (IECs) is a critical component in intestinal tissue regeneration. Surprisingly, neither WT-CCN1 nor DM-CCN1 had any effect on the proliferation of YAMC cells in culture as judged by cell number counts and Ki67 staining (figure 19-20), suggesting that CCN1 may indirectly mediate tissue repair after DSS-induced injury.

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

Since cytokines are important in inflammation and repair, we measured cytokine expression in the wild type and *Ccn1*$^{dm/dm}$ colon after 3.5% DSS challenge. Most differences were small: wild type mice transiently expressed somewhat higher levels of the pro-

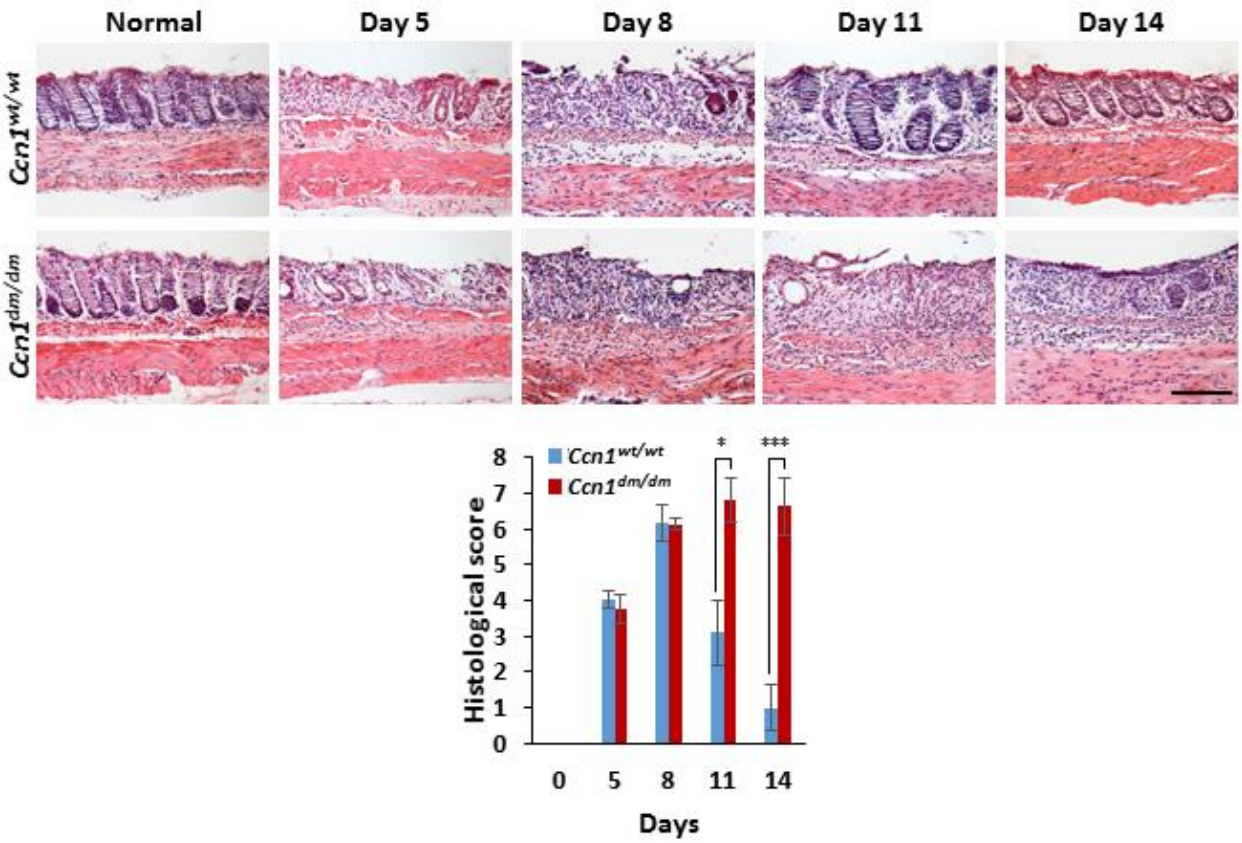
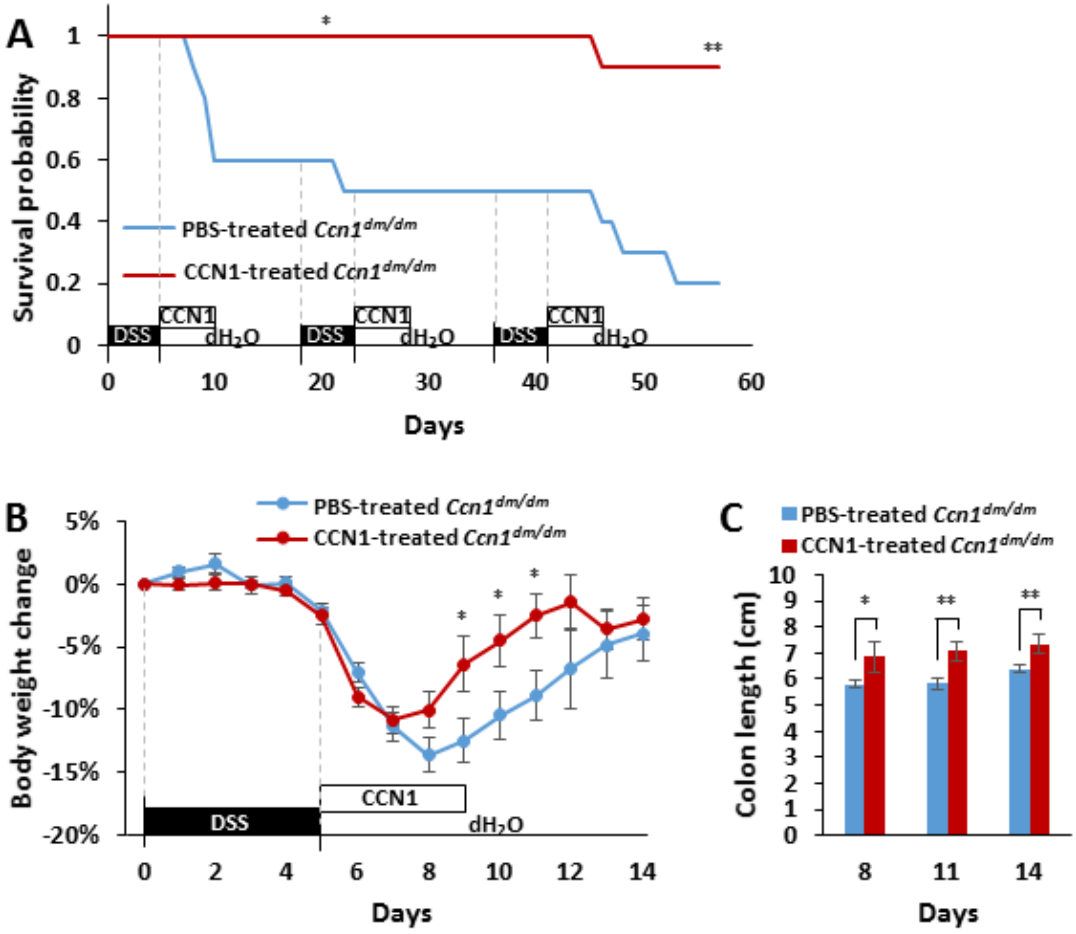


Figure 16. Impaired mucosal healing in DSS-challenged *Ccn1*$^{dm/dm}$ mice. H&E staining of paraffin-embedded sections of the distal colon from mice 5, 8, 11, and 14 days after initiation of DSS feeding. Histological scores are shown below (n=4-6). *p<0.05, ***p<0.001. Scale bar=100 μm.

Figure 17. Treatment with WT-CCN1 promotes recovery and survival in DSS-challenged *Ccn1*$^{dm/dm}$ mice. (A) *Ccn1*$^{dm/dm}$ mice (*n*=10) were injected i.p. with 5 µg of WT-CCN1 or PBS 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). Data are presented as mean ± SEM; *n*=3-4; **p*<0.05, ***p*<0.01, ****p*<0.001. Scale bar=100 µm.


A
Survival probability
1
0.8
0.6
0.4
0.2
0
*
**
PBS-treated Ccn1dm/dm
CCN1-treated Ccn1dm/dm
DSS
CCN1
dH2O
0
10
20
30
40
50
60
Days
B
Body weight change
5%
0%
-5%
-10%
-15%
-20%
PBS-treated Ccn1dm/dm
CCN1-treated Ccn1dm/dm
DSS
CCN1
dH2O
0
2
4
6
8
10
12
14
Days
C
PBS-treated Ccn1dm/dm
CCN1-treated Ccn1dm/dm
Colon length (cm)
10
9
8
7
6
5
4
3
2
1
0
*
**
**
8
11
14
Days

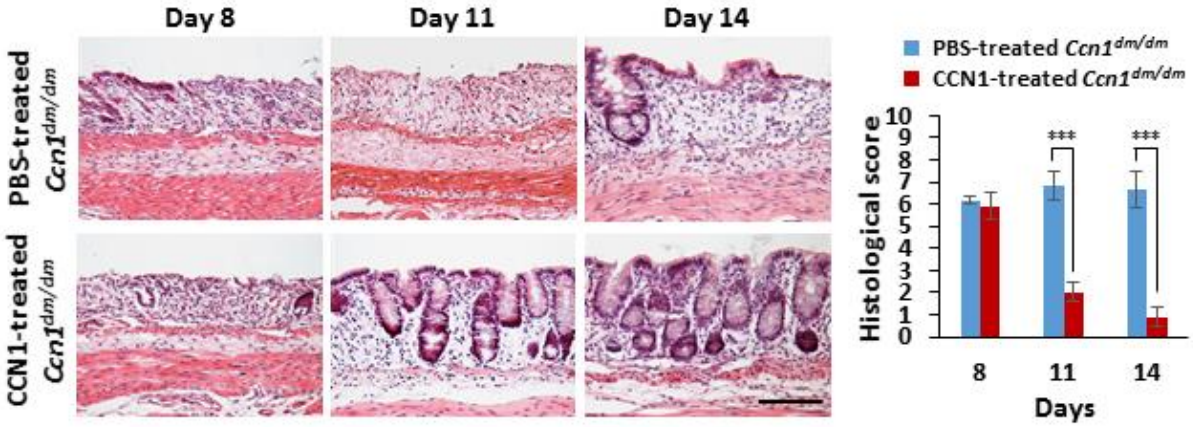


Figure 18. Treatment with WT-CCN1 accelerates mucosal healing DSS-challenged *Ccn1*$^{dm/dm}$ mice. Mucosal recovery was evaluated by H&E staining of paraffin-embedded colonic tissue. Histological scores are shown on right. Data are presented as mean ± SEM; *n*=3-4; ***p<0.001. Scale bar=100 µm.

inflammatory cytokines *IFN-γ* and *IL-17*, but *TNFα* expression was the same in both genotypes (figure 21A). Likewise, *Ccn1*$^{dm/dm}$ mice showed slightly higher levels of the anti-inflammatory cytokines *IL-4* and *TGF-β*, whereas *IL-10* expression was the same (figure 21B). However, *Ccn1*$^{dm/dm}$ mice did not exhibit less inflammation and more repair as this expression profile might suggest, but instead showed similar inflammatory damage and impaired mucosal repair (figures 15-16), indicating that the small differences in expression of these cytokines were insufficient to drive the *Ccn1*$^{dm/dm}$ phenotypes. By contrast, whereas *IL-6* expression was similar in WT and *Ccn1*$^{dm/dm}$ mice at day 5 when inflammatory damage began to be observed, it was dramatically higher (>7-fold) in wild type mice by day 8 when damage had peaked and repair began (figure 22A). Thus, *Ccn1*$^{dm/dm}$ mice suffered a large deficit in *IL-6* expression in the repair phase but not the initiation phase of colitis. *IL-6* expression remained induced but at a lower level at later times, and the difference between the two genotypes diminished, suggesting exquisite control of *IL-6* by CCN1 in colitis. Further, injection of WT-CCN1 into wild type mice upregulated serum IL-6 level in a time and dose-dependent manner, whereas DM-CCN1 had no effect (p<0.05)(figure 22B-C), indicating that CCN1 acts through its $\alpha_6\beta_1/\alpha_M\beta_2$ binding sites to induce IL-6. Moreover, 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 22D), 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 (10). To identify the cellular source of CCN1-induced IL-6, we first tested CCN1 functions in I13.35 macrophages. WT-CCN1 induced *IL-6* mRNA and protein levels in these cells, whereas DM-CCN1 did not (figure 23A-B). Pre-incubation of macrophages with

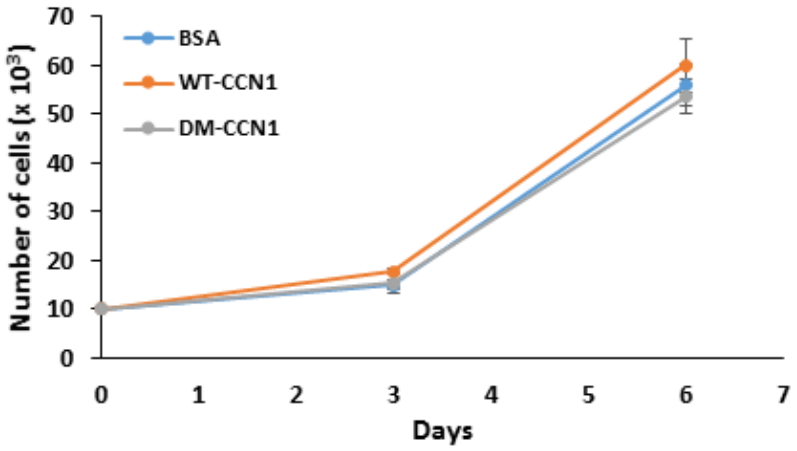


Figure 19. CCN1 does not directly induce IEC growth.
YAMC cells were grown in presence of 10 µg/ml of BSA, WT-CCN1, or DM-CCN1 for indicates days in culture. Cells were trypsinized, stained with 1% trypan blue and counted using a hemocytometer.

Figure 20. CCN1 does not directly induce IEC proliferation.
YAMC cells were treated with BSA, WT-CCN1, or DM-CCN1 for 24 hrs and immunostained for Ki67. Number of Ki67-positive cells were counted and normalized to the total number of cells (right). Scale bar=300 μm.

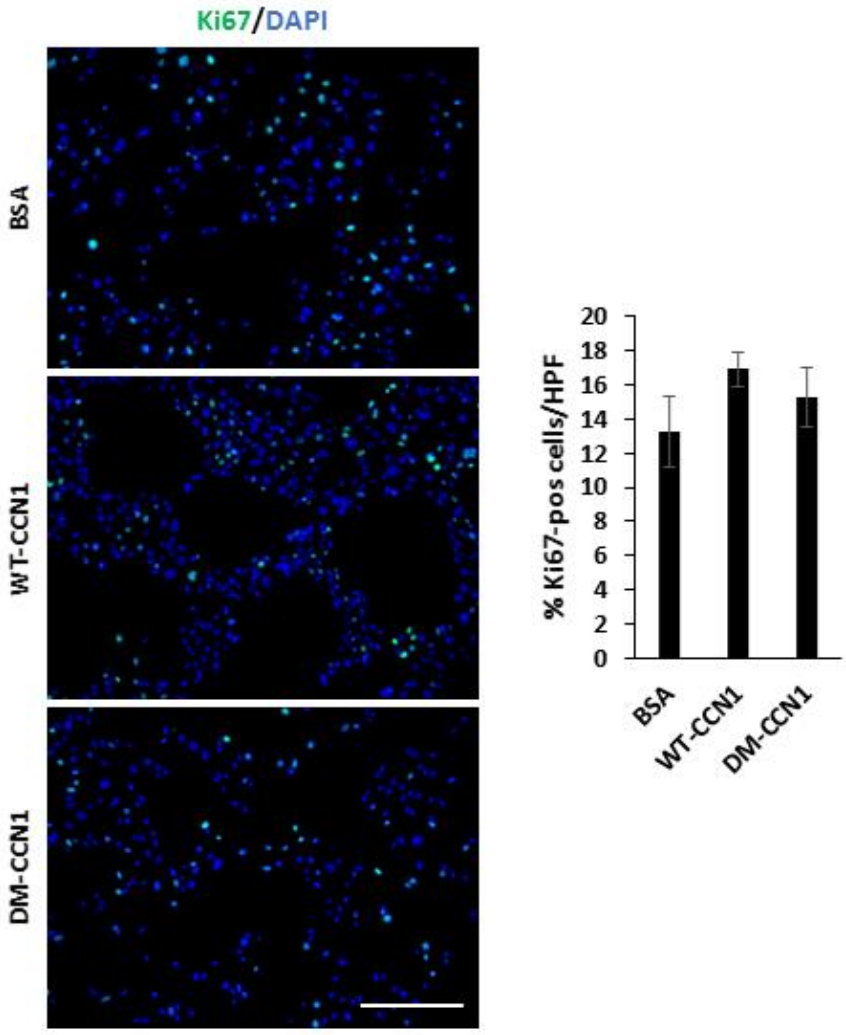

Ki67/DAPI
BSA
WT-CCN1
DM-CCN1
% Ki67-pos cells/HPF
20
18
16
14
12
10
8
6
4
2
0
BSA
WT-CCN1
DM-CCN1

Figure 21. Cytokine expression in DSS-challenged wild type and *Ccn1*$^{dm/dm}$ mice. (A) Pro-inflammatory (*TNFα*, *IFNγ*, *IL-17*, *IL-1β*), (B) anti-inflammatory (*IL-4*, *IL-10*, *TGF-β*) cytokine, and (C) *Ccn1* gene expression was measured on day 5 and 8 from isolated colon RNA of 3.5% DSS-challenged wild type and *Ccn1*$^{dm/dm}$ mice by qRT-PCR. Data are represented as mean ± SD; n = 3-4; *p<0.05, **p<0.01, ***p<0.001.

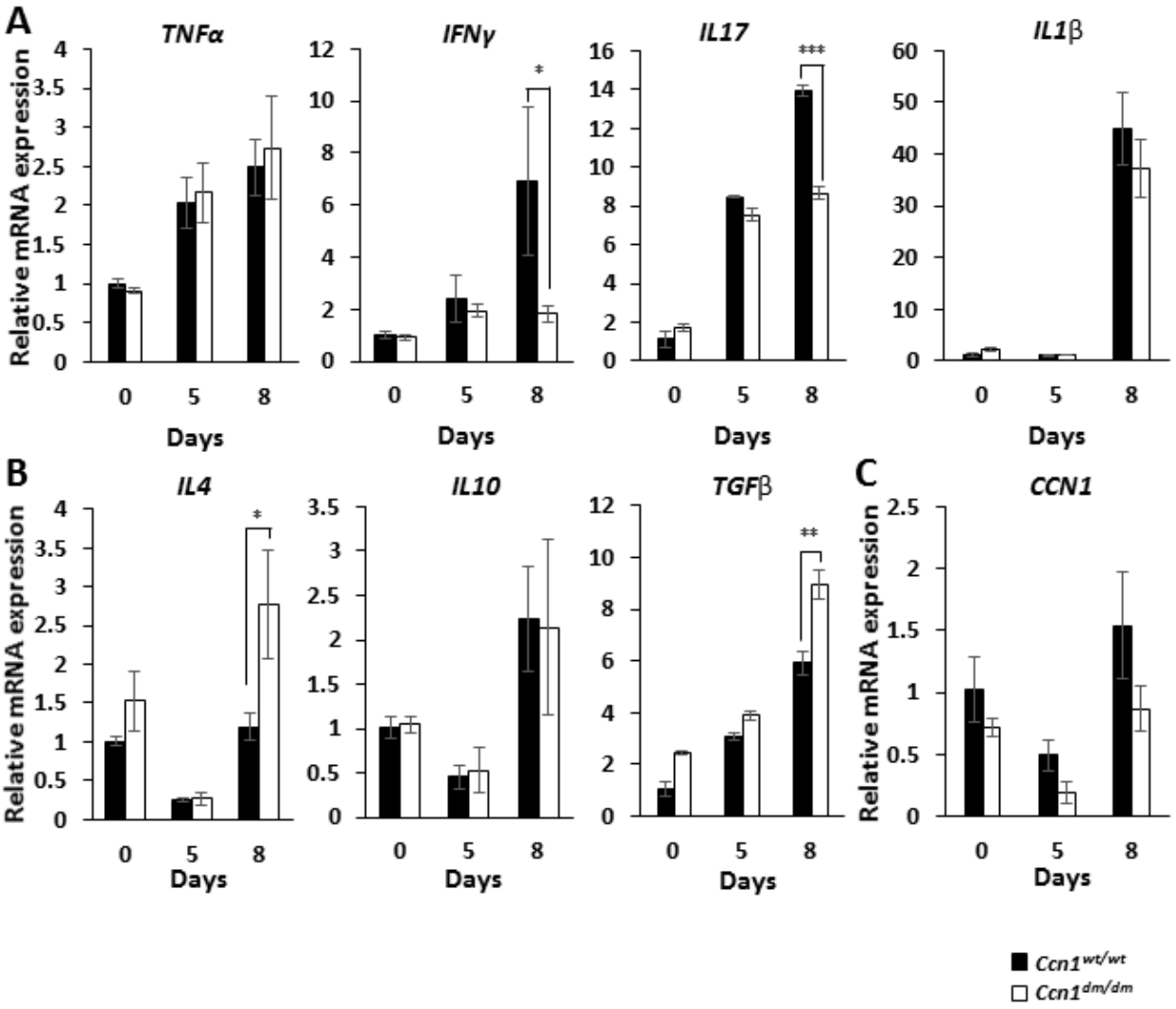
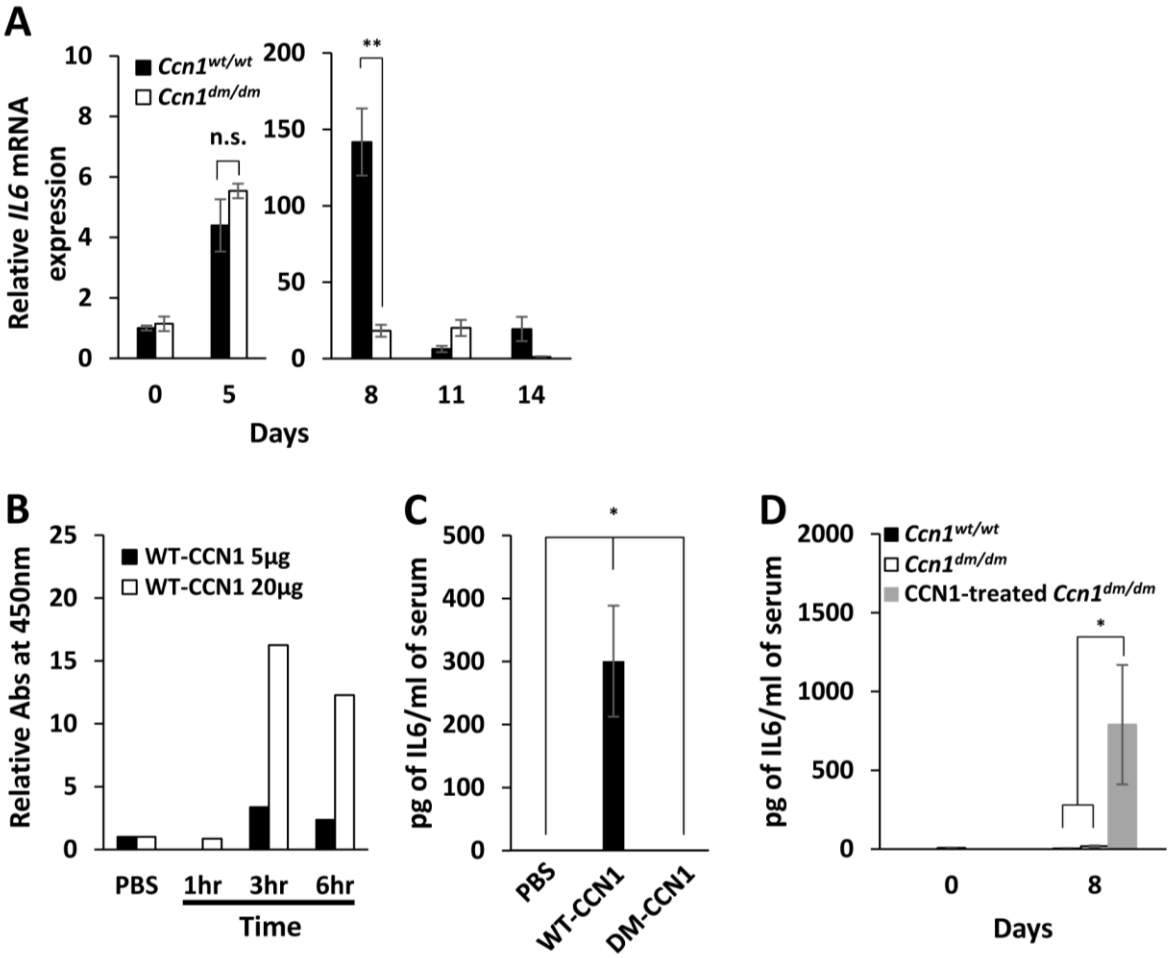

A
TNFα
IFNγ
IL17
IL1β
Relative mRNA expression
Days
B
IL4
IL10
TGFβ
C
CCN1
Ccn1wt/wt
Ccn1dm/dm

Figure 22. WT-CCN1, but not DM-CCN1, induces IL-6 *in vivo*. (A) *IL-6* mRNA in colon of wild type and *Ccn1*$^{dm/dm}$ mice treated with 3.5% for 5 days was measured by qRT-PCR, and normalized to healthy wild type colon with *cyclophilin E* as internal reference. Data shown as means ± SD; *n*=3-4; n.s., not significant. ***p*<0.003. (B) IL-6 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 μg), or (C) 24 hours after i.p. injection of 10 μg 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 ± SEM; *n*=7; **p*<0.05.


A
Relative IL6 mRNA expression
Ccn1wt/wt
Ccn1dm/dm
n.s.
**
Days
B
Relative Abs at 450nm
WT-CCN1 5µg
WT-CCN1 20µg
PBS
1hr
3hr
6hr
Time
C
pg of IL6/ml of serum
*
PBS
WT-CCN1
DM-CCN1
D
pg of IL6/ml of serum
Ccn1wt/wt
Ccn1dm/dm
CCN1-treated Ccn1dm/dm
*
Days

mAbs against integrin α_M or β_2 abrogated CCN1-induced *IL-6* expression, showing the requirement of $\alpha_M\beta_2$ (figure 23C). In addition, WT-CCN1, but not DM-CCN1, greatly upregulated *IL-6* mRNA and protein levels in 18Co human colonic fibroblasts (figure 23D-E). Since CCN1 and TNFα are known to be co-expressed in inflammation, we tested whether they act synergistically to regulate IL-6. Remarkably, CCN1 in combination with TNFα induced *IL-6* mRNA in 18Co cells by nearly 250-fold, or 15- and 23-fold higher than stimulation by CCN1 or TNFα alone, respectively (figure 23D). This synergistic effect was also observed by measuring IL-6 protein levels in the conditioned media of 18Co cells (figure 23E). Consistent with integrin $\alpha_6\beta_1$ being the principal CCN1 receptor in fibroblasts (37), pretreatment of 18Co cells with anti-α_6 mAb blocked CCN1-induced *IL-6* expression (figure 23F). 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 22A) and inability of DM-CCN1 to induce IL-6 *in vivo* (figure 22C).

4.5 CCN1-induced IL-6 promotes intestinal epithelial healing

To determine whether deficits in *IL-6* expression contributes to impaired healing in *Ccn1*$^{dm/dm}$ mice, we delivered five daily injections of 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 24A). 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 24B). Histological evaluation showed

Figure 23. 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 μg/ml of purified WT-CCN1 or DM-CCN1, or BSA for 24 hrs. (C) I13.35 macrophages were incubated with blocking mAbs against integrin α_M (50 μg/ml) or β_2 (50 μg/ml) 1 hr prior to treatment with WT-CCN1 for 24 hrs. *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 μg/ml of purified WT-CCN1, DM-CCN1, BSA, or 25 ng/ml of TNFα, or BSA for 24 hrs. (F) 18Co fibroblasts were incubated with blocking mAb against integrin α_6 for 1 hr, then treated with WT-CCN1 or BSA with and without TNFα for 24 hrs as above. *IL-6* mRNA was measured by qRT-PCR. Data shown as mean ± SD of triplicate experiments.

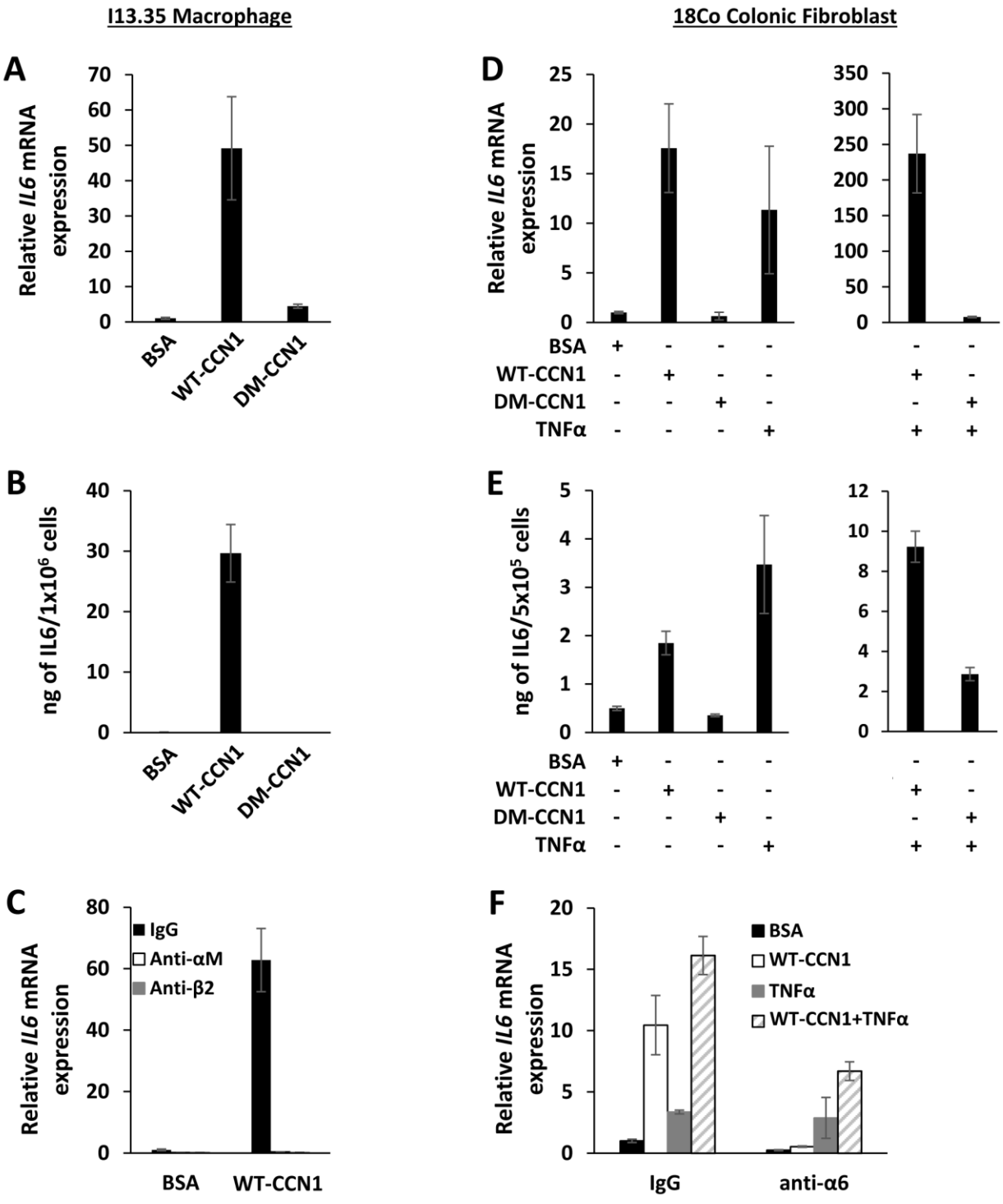
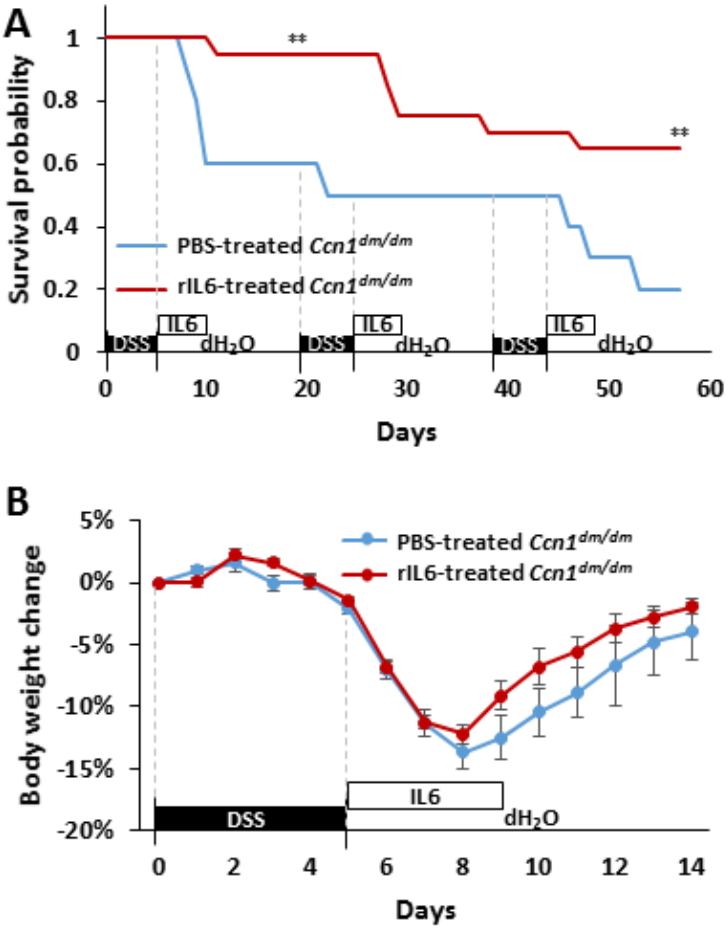

I13.35 Macrophage
18Co Colonic Fibroblast
A
Relative IL6 mRNA expression
70
60
50
40
30
20
10
0
BSA
WT-CCN1
DM-CCN1
D
Relative IL6 mRNA expression
25
20
15
10
5
0
350
300
250
200
150
100
50
0
BSA + - - - - -
WT-CCN1 - + - - + -
DM-CCN1 - - + - - +
TNFα - - - + + +
B
ng of IL6/1x10^6 cells
40
30
20
10
0
BSA
WT-CCN1
DM-CCN1
E
ng of IL6/5x10^5 cells
5
4
3
2
1
0
12
10
8
6
4
2
0
BSA + - - - - -
WT-CCN1 - + - - + -
DM-CCN1 - - + - - +
TNFα - - - + + +
C
Relative IL6 mRNA expression
80
60
40
20
0
IgG
Anti-αM
Anti-β2
BSA
WT-CCN1
F
Relative IL6 mRNA expression
20
15
10
5
0
BSA
WT-CCN1
TNFα
WT-CCN1+TNFα
IgG
anti-α6

Figure 24. Delivery of IL-6 enhances survival of *Ccn1*$^{dm/dm}$ mice. (A) *Ccn1*$^{dm/dm}$ mice (*n*=20) were injected i.p. with rIL-6 (100 ng) or PBS 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 PBS daily for 5 days after DSS feeding.


A
Survival probability
1
0.8
0.6
0.4
0.2
0
**
**
PBS-treated Ccn1dm/dm
rIL6-treated Ccn1dm/dm
IL6
DSS
dH2O
IL6
DSS
dH2O
IL6
DSS
dH2O
0
10
20
30
40
50
60
Days
B
Body weight change
5%
0%
-5%
-10%
-15%
-20%
PBS-treated Ccn1dm/dm
rIL6-treated Ccn1dm/dm
DSS
IL6
dH2O
0
2
4
6
8
10
12
14
Days

that rIL-6 treatment accelerated mucosal restitution in *Ccn1*$^{dm/dm}$ mice compared to vehicle controls, although improvements in histological scores still lagged behind wild type mice (figures 16, 25). 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 wild type, *Ccn1*$^{dm/dm}$, rIL-6 treated- and CCN1-treated-*Ccn1*$^{dm/dm}$ mice stained with antibodies against PCNA, a proliferation marker. There were significantly fewer proliferating cells in *Ccn1*$^{dm/dm}$ mice compared to wild type mice (p<0.01), and treatment with rIL-6 or WT-CCN1 partially or completely restored cell proliferation, respectively, to wild type levels (figure 26). Furthermore, PCNA-positive cells co-localized with E-cadherin, an epithelial cell marker, in wild type 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 BSA, rIL-6, or EGF (epidermal growth factor) as a positive control and stained for Ki67. Treatment of YAMC cells with rIL-6 increased Ki67-positive cells in a dose-dependent manner and to the same extent as EGF, a known mitogen for IECs, when both were applied at 100 ng/ml (figure 27). Pretreatment of cells with anti-IL-6Rα antibody 1-hr prior to rIL-6 addition blocked the increase in Ki67 positive cells, indicating that rIL-6 acts directly through its specific receptor to enhance IEC proliferation.

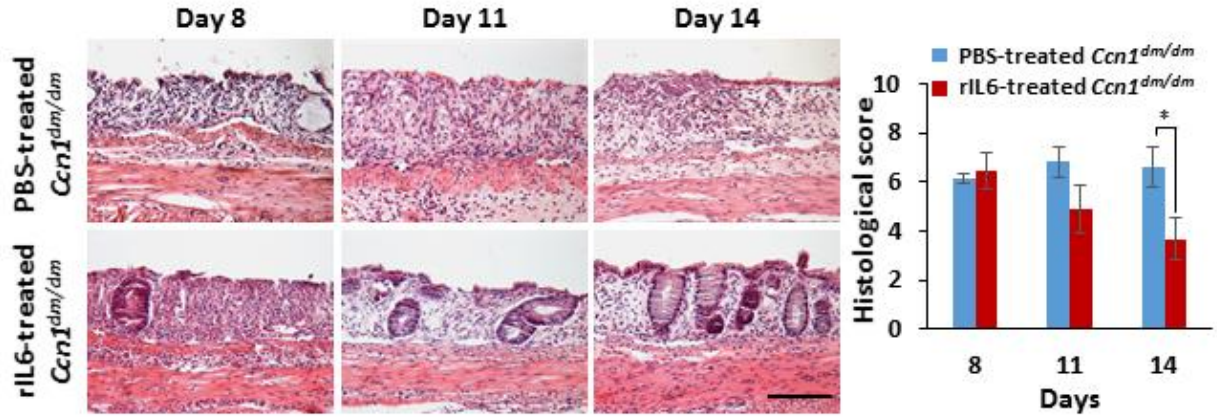


Figure 25. Treatment with IL-6 promotes mucosal repair. Mucosal damage in mice treated with 100 ng of rIL-6 or PBS daily for 5 days after DSS feeding was evaluated by H&E staining of paraffin-embedded sections of the distal colon. Histological scores are shown on the right (*n*=3-4; **p*<0.05). Scale bar=100 µm.

Figure 26. Restoration of IEC proliferation in *Ccn1*$^{dm/dm}$ mice. 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 (green) and E-cadherin (red) was detected by immunofluorescence microscopy to assess IEC proliferation. DAPI was used for counterstaining. Quantitation of PCNA staining is shown below (*n*=3-4; **p*<0.05, ***p*<0.01). Scale bar=50 µm.

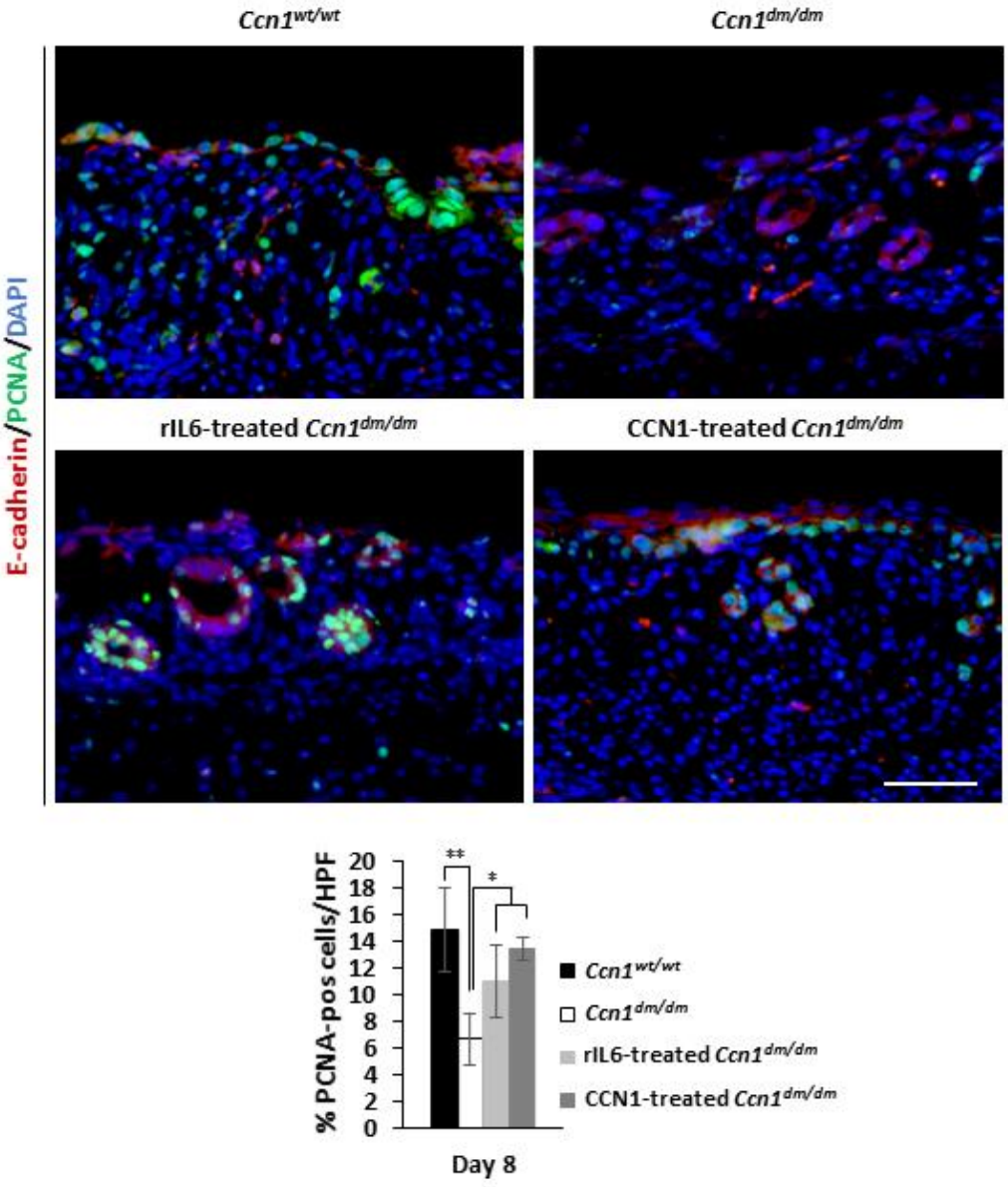
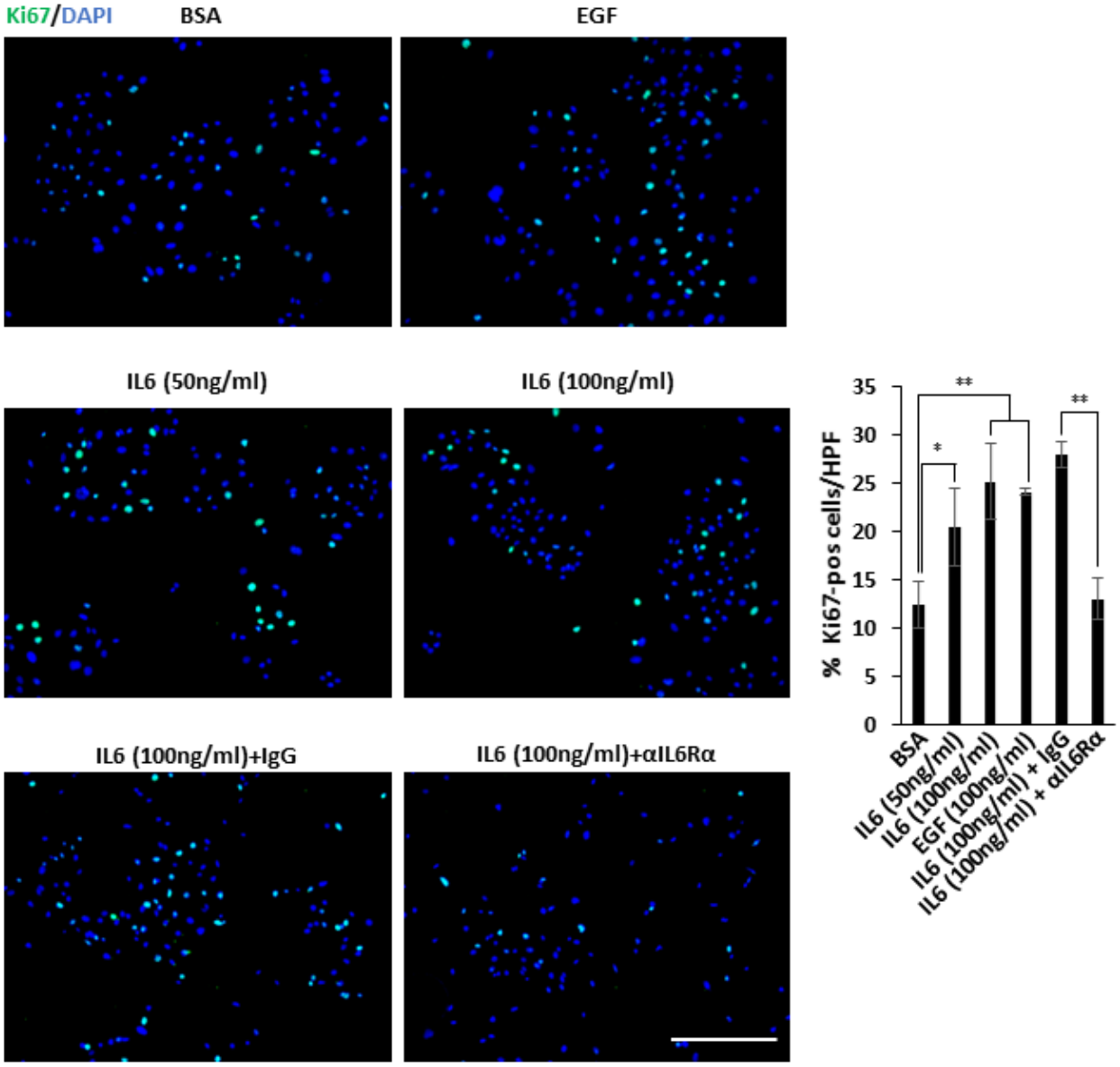

Ccn1wt/wt
Ccn1dm/dm
E-cadherin/PCNA/DAPI
rIL6-treated Ccn1dm/dm
CCN1-treated Ccn1dm/dm
% PCNA-pos cells/HPF
20
18
16
14
12
10
8
6
4
2
0
**
*
Ccn1wt/wt
Ccn1dm/dm
rIL6-treated Ccn1dm/dm
CCN1-treated Ccn1dm/dm
Day 8

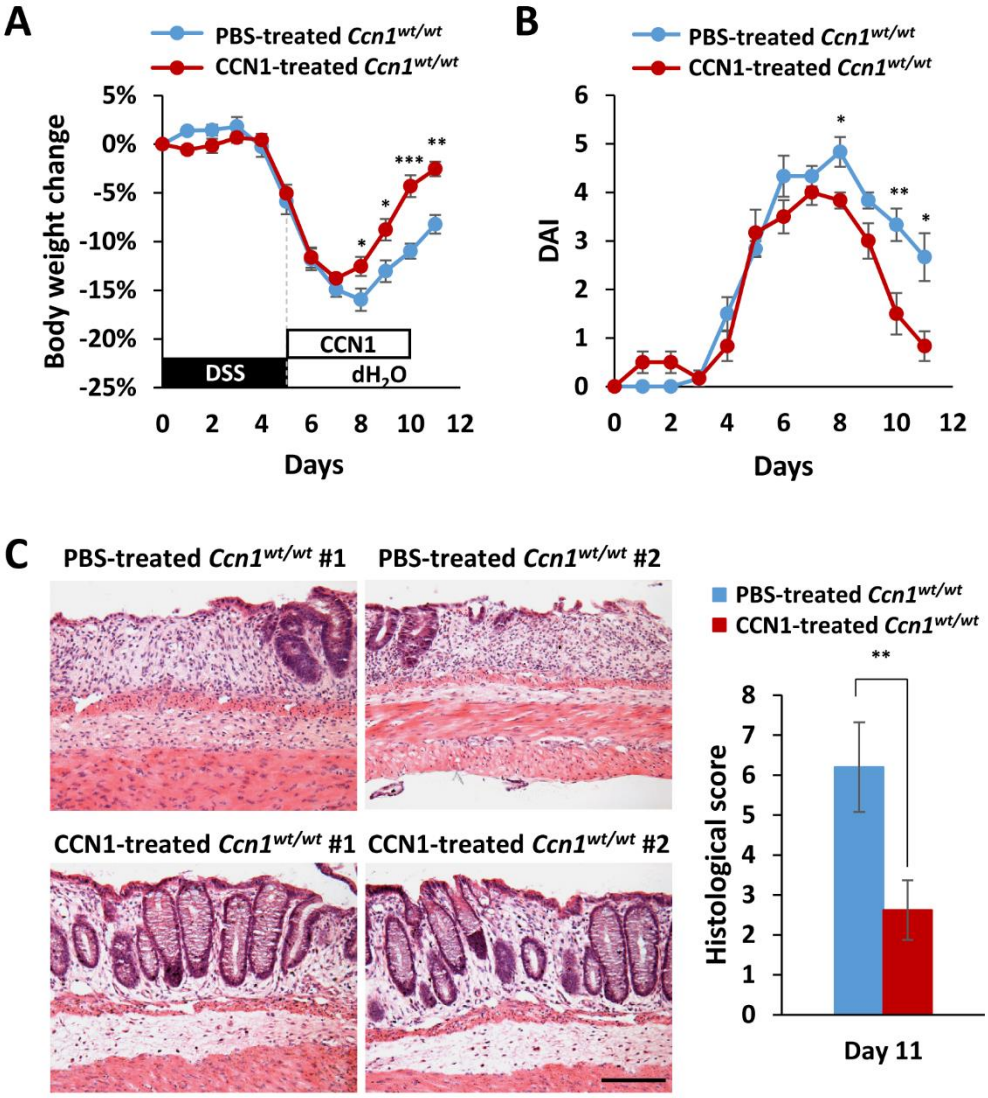
Figure 27. IL-6 induces IEC proliferation. YAMC cells were treated with BSA, IL-6 (50 or 100 ng/ml), or EGF (100 ng/ml) for 24 hrs and stained for Ki67 by immunofluorescence. Where indicated, YAMC cells were pre-incubated with blocking polyclonal antibodies for IL-6 receptor α (IL-6Rα) or IgG for 1 hr before IL-6 addition. Quantitation of Ki67-positive cells normalized to DAPI is shown on the right. Data are shown as mean ± SD; *p<0.05, **p<0.01. Scale bar=300 μm.


Ki67/DAPI
BSA
EGF
IL6 (50ng/ml)
IL6 (100ng/ml)
IL6 (100ng/ml)+IgG
IL6 (100ng/ml)+αIL6Rα
% Ki67-pos cells/HPF
0
5
10
15
20
25
30
35
*
**
**
BSA
IL6 (50ng/ml)
IL6 (100ng/ml)
EGF (100ng/ml)
IL6 (100ng/ml) + IgG
IL6 (100ng/ml) + αIL6Rα

4.6 CCN1 promotes recovery and mucosal healing in DSS-challenged wild type 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 µg i.p.) into wild type animals with colitis induced by 5% DSS. WT-CCN1 protein accelerated body weight recovery and attenuated DAI from days 8-11 in wild type mice with colitis (figure 28A-B). Histology on the distal colon showed significantly fewer areas of ulceration and higher number of organized crypts compared to PBS-treated controls ($p<0.01$)(figure 28C). Thus, CCN1 therapy enhances recovery and promotes mucosal healing in colitis even in WT mice with fully functional CCN1.

Figure 28. 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 PBS or 10 μg 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 ± SEM; n=6; *p<0.05, **p<0.01, ***p<0.001. Scale bar=100 μm.


A
PBS-treated Ccn1wt/wt
CCN1-treated Ccn1wt/wt
Body weight change
5%
0%
-5%
-10%
-15%
-20%
-25%
DSS
CCN1
dH2O
0 2 4 6 8 10 12
Days
B
PBS-treated Ccn1wt/wt
CCN1-treated Ccn1wt/wt
DAI
0 1 2 3 4 5 6
0 2 4 6 8 10 12
Days
C
PBS-treated Ccn1wt/wt #1
PBS-treated Ccn1wt/wt #2
CCN1-treated Ccn1wt/wt #1
CCN1-treated Ccn1wt/wt #2
PBS-treated Ccn1wt/wt
CCN1-treated Ccn1wt/wt
Histological score
0 1 2 3 4 5 6 7 8
Day 11

5. CCN1 SUPPRESSES COLITIS-ASSOCIATED CARCINOGENESIS

5.1 Background

Colorectal cancer (CRC) is a significant contributor of morbidity and mortality worldwide with highest incidence rates in developed countries (89). In the United States, CRC affects both sexes equally and accounts for nearly 150,000 new cases of cancer every year as the third most common cancer diagnosis (89;90). Prognosis is closely associated with the stage of disease at diagnosis, inciting advances in screening programs and improvements in diagnostic techniques (91). Despite these efforts, CRC remains as the second leading cause of death among cancers with approximately 50,000 deaths every year (90). Therefore, a better understanding of CRC is needed to develop new prevention methods and therapeutic agents.

Diagnosis of IBD is a major risk factor for developing CRC (62). Duration and severity of inflammation as well as proportion of tissue affected by the disease all influence CRC risk in IBD patients (92). Mechanistically, chronic inflammation functions to drive all aspects of tumorigenesis. First, it mediates tumor initiation through production of reactive oxygen and nitrogen species, which directly damages DNA and increases oncogenic stress (93). Second, it enhances tumor promotion through cytokines and growth factors that support survival and proliferation of premalignant cells (94). Lastly, it modulates tumor progression by inducing matrix altering enzymes, chemokines, and angiogenic factors through regulation of NF-κB to influence tumor invasion and metastasis (95). Hence, factors that can regulate both inflammation and tissue homeostasis during acute and chronic injury are promising therapeutic targets for inflammation-associated cancers.

CCN1 expression is also linked to many cancers (29). In general, CCN1 promotes the growth of established tumor cells *in vitro* and in xenografts, however, its role in tumor initiation has not been reported to date (22;29). The pro-tumorigenic activity of CCN1 may be in large part due to the potent angiogenic activity, which can increase tumor vasculature and promote tumor growth (48;49;96). Furthermore, CCN1 can directly enhance cancer cell proliferation, survival, invasion and metastasis independent of its angiogenic function (97;98). Consistent with these findings, CCN1 expression correlated with tumor stage and poor prognosis in various human cancers including colorectal cancer (99-101). In contrast, CCN1 has also demonstrated the ability to suppress tumorigenesis. For example, in non-small-cell lung cancer, hepatocellular carcinoma, endometrial adenocarcinoma, and melanoma, forced expression of CCN1 reduced tumor growth, increased cancer cell apoptosis, or both (22). These observation may be explained by the ability of CCN1 to induce apoptosis and senescence during inflammation and during tissue repair (36;54). Consistent with the proposal that CCN1 negatively regulates tumorigenesis, here, we present the first report of CCN1 effects in a carcinogenesis model, and show that CCN1 suppresses colitis-associated cancer.

5.2 *Ccn1*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ mice suffer increased tumor burden in colitis-associated tumorigenesis

To evaluate the role of CCN1 in colitis-associated cancer, we treated wild type, *Ccn1*$^{d125a/d125a}$, and *Ccn1*$^{dm/dm}$ mice with azoxymethane (AOM) followed by three cycles of 2.5% (w/v) DSS (figure 29A). We observed that more *Ccn1*$^{d125a/d125a}$ (100%) and *Ccn1*$^{dm/dm}$ mice (87.5%) bore tumors compared to wild type (50%), showing higher tumor incidence (p<0.05)(figure 29B). *Ccn1*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ mice also exhibited more tumors with a

median of 8 and 5.5 tumors per mouse, respectively, compared to 0.5 in wild type (p<0.002), although the sizes of the tumors were comparable (figure 29B-D). Notably, the tumor incidence between *Ccn1*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ mice were not statistically different. Similar to our findings from DSS-induced colitis experiments, *Ccn1*$^{d125a/d125a}$ mice showed significantly greater body weight loss, whereas *Ccn1*$^{dm/dm}$ mice demonstrated a slower body weight recovery, after each cycle of DSS challenge (figure 30), consistent with exacerbated colitis or impaired healing in *Ccn1*$^{d125a/d125a}$ mice and *Ccn1*$^{dm/dm}$ mice, respectively. Cytokine profiling revealed that tumors from *Ccn1*$^{dm/dm}$ and *Ccn1*$^{d125a/d125a}$ mice, in general, expressed elevated levels pro-tumorigenic cytokines. For example, *Ccn1*$^{dm/dm}$ tumors showed increases in expression of *IL-6, IL-17, CXCL1,* and *ARG-1*, whereas levels of *TNFα, IL-1B, IL-17, CXCL1,* and *ARG-1* were increased in *Ccn1*$^{d125a/d125a}$ tumors (figure 31). This suggests that CCN1 may be a regulator of cytokine expression within the local tumor environment. Collectively, these findings indicate that CCN1 suppresses colitis-associated tumorigenesis.

Figure 29. *Ccn1*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ mice suffer increased tumor incidence and multiplicity in colitis-associated carcinogenesis. (A) Schematic of AOM/DSS-induced carcinogenesis. (B) Tumor incidence and numbers in wild type (*n*=18), *Ccn1*$^{dm/dm}$ (*n*=16), and *Ccn1*$^{d125a/d125a}$ (*n*=8) mice were counted. Wild type mice (50%) had lower tumor incidence than *Ccn1*$^{dm/dm}$ (87.5%), and *Ccn1*$^{d125a/d125a}$ mice (100%) ($p<0.05$, Fisher's exact test), and lower tumor multiplicity ($p<0.002$, t-test). Red horizontal bars indicate the median. (C) Tumor size was measured from H&E sections on QI Imaging ImagePro (*n*>20). (D) Representative colons at experimental endpoint. Scale bar=1 cm.

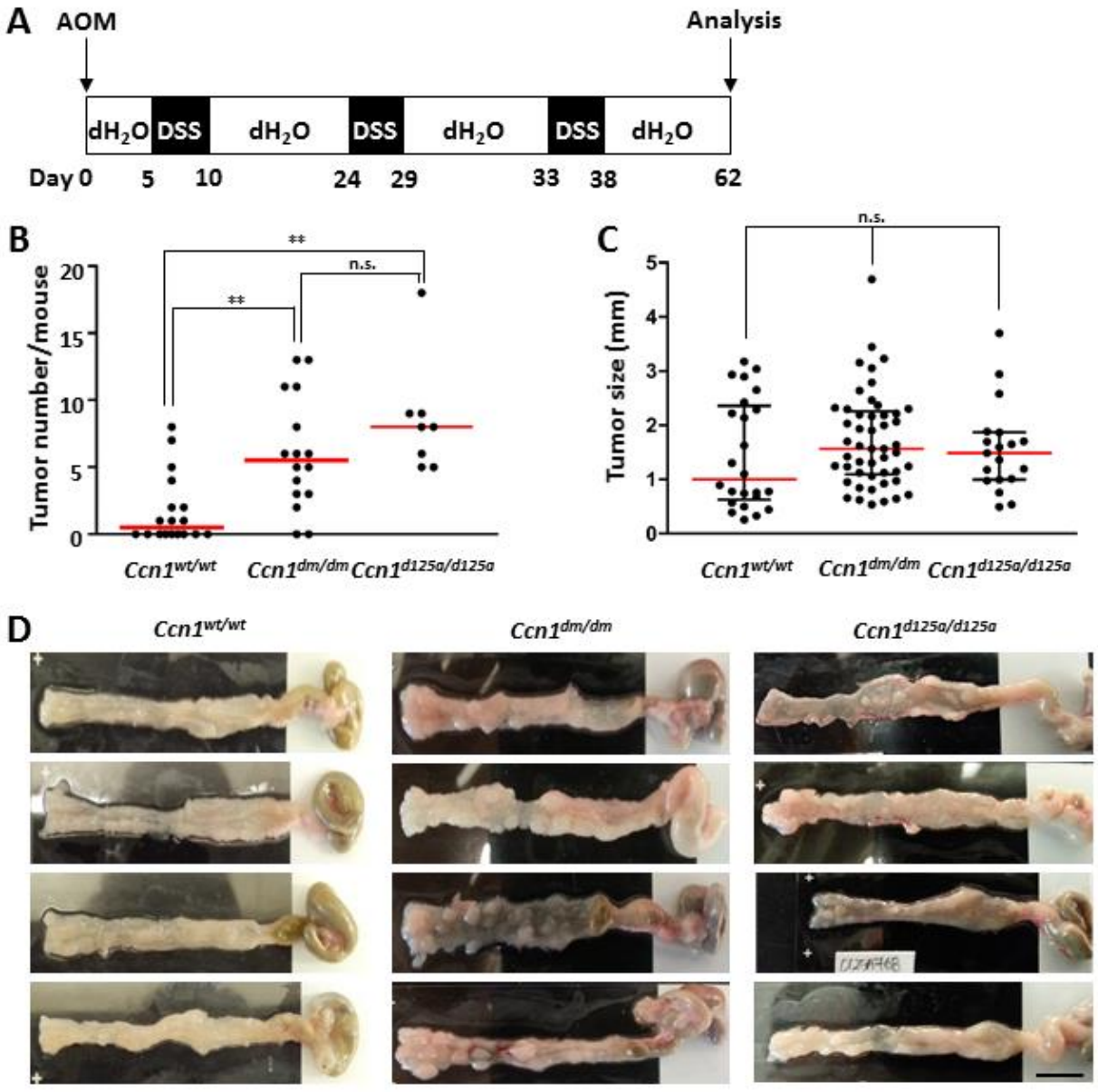

A
AOM
Analysis
dH2O
DSS
dH2O
DSS
dH2O
DSS
dH2O
Day 0
5
10
24
29
33
38
62
B
Tumor number/mouse
20
15
10
5
0
**
**
n.s.
Ccn1wt/wt
Ccn1dm/dm
Ccn1d125a/d125a
C
Tumor size (mm)
5
4
3
2
1
0
n.s.
Ccn1wt/wt
Ccn1dm/dm
Ccn1d125a/d125a
D
Ccn1wt/wt
Ccn1dm/dm
Ccn1d125a/d125a

Figure 30. *Ccn1*$^{d125a/d125a}$ mice showed most severe weight loss while *Ccn1*$^{dm/dm}$ mice exhibited slowed body weight recovery with each round of DSS feeding. Body weight changes were recorded daily. Data are shown as mean ± SEM; *p>0.05.

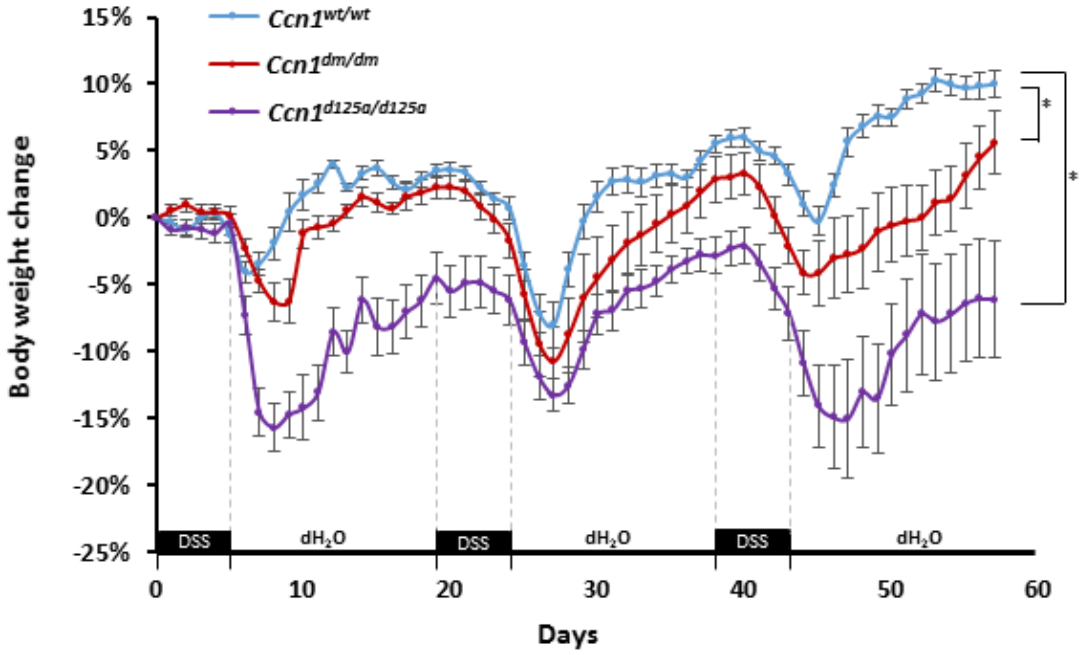
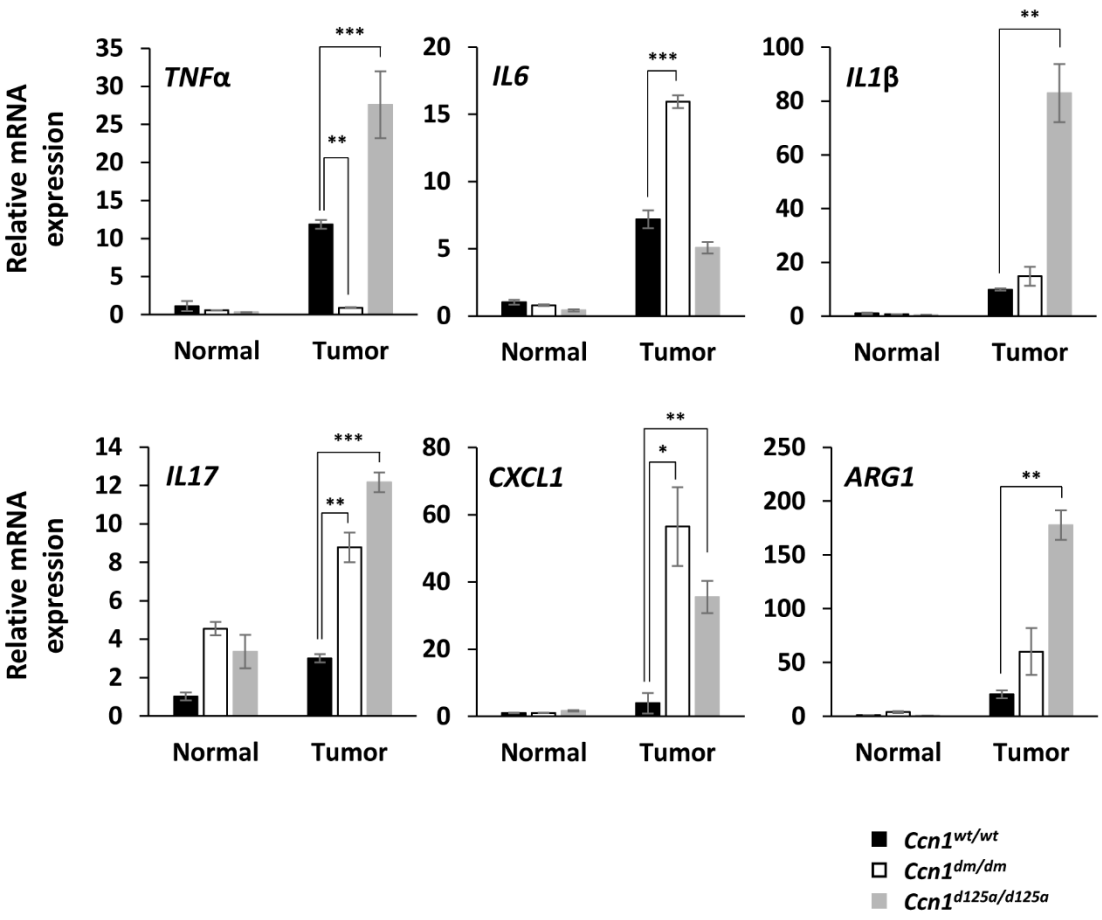

Ccn1wt/wt
Ccn1dm/dm
Ccn1d125a/d125a
Body weight change
15%
10%
5%
0%
-5%
-10%
-15%
-20%
-25%
DSS
dH2O
DSS
dH2O
DSS
dH2O
0
10
20
30
40
50
60
Days
*
*

Figure 31. Cytokines expression in the tumors of AOM/DSS-challenged wild type, *Ccn1*$^{dm/dm}$, and *Ccn1*$^{d125a/d125a}$ mice. *TNF*α, *IL-6*, *IL-1B*, *IL-17*, *CXCL1*, and *ARG-1* gene expression was measured from isolated RNA of tumor-containing colonic tissue of AOM/DSS-challenged wild type, *Ccn1*$^{dm/dm}$, *Ccn1*$^{d125a/d125a}$ mice by qRT-PCR. Data are represented as mean ± SD; *n*=3-4.


Relative mRNA expression
TNFα
IL6
IL1β
IL17
CXCL1
ARG1
Normal
Tumor
Ccn1wt/wt
Ccn1dm/dm
Ccn1d125a/d125a

6. CCN1 IS A NOVEL REGULATOR OF NEUTROPHIL MOBILIZATION

6.1 Background

Neutrophils are the primary responders in acute inflammation, especially during bacterial and fungal infections (102). Despite this important function, approximately only 1-2% of the total neutrophil population is found in circulation under basal conditions in mice; the rest reside in the hematopoietic and storage compartments of the bone marrow, liver, and spleen (103). Therefore, during inflammatory situations, there must be a systemic effort to mobilize the neutrophils from the bone marrow into the blood so that they can be directed to the site of inflammation in the periphery. This event requires exquisite regulation as an inadequate number of neutrophils, or neutropenia, will dramatically increase the susceptibility of the host to pathogens. Alternatively, an excessive number of neutrophils, or neutrophilia, is associated with non-specific tissue destruction (104). Therefore, maintaining neutrophil homeostasis in the blood is critical for the host.

Neutrophil homeostasis is maintained by a balance of granulopoiesis in the bone marrow, neutrophil release from the bone marrow, and neutrophil clearance from the circulation (105). Granulopoiesis refers to the production of neutrophils, eosinophils, and basophils in the bone marrow originating from hematopoietic stem cells (HSCs) that divide asymmetrically and undergo differentiation through a series of lineage specific progenitors in a hierarchal fashion. Of the granulocytes, neutrophils have the shortest half-life of 6-8 hours in the circulation because they are programmed to undergo spontaneously apoptosis (105). Therefore, even under basal conditions, granulopoiesis is needed to constantly replenish the aging and lost neutrophils; it is estimated that 1-2 x 10^{11} neutrophils are generated each day in

a normal adult human (106). The kinetics of this process is largely driven by hematopoietic growth factors and cytokines, which allows for rapid amplification and dampening of neutrophil production during initiation and resolution of inflammation, respectively. Granulocyte-colony stimulating factor (G-CSF) is a critical hematopoietic growth factor for granulopoiesis as demonstrated by G-CSF null mice that exhibit severe neutropenia (107). However, G-CSF is not absolutely required for granulopoiesis or terminal differentiation of neutrophils as these mice maintain 25% residual granulopoiesis and develop fully mature neutrophils (107). Indeed, other hematopoietic growth factors and cytokines, including GM-CSF, IL-3, and IL-6, have been shown to independently accelerate granulopoiesis during inflammation to ensure that a robust army of leukocytes is present to protect the host from pathogens (109-112). These growth factors and cytokines drive granulopoiesis through CCAAT Enhancer binding protein-β (C/EBPβ), a second member of the C/EBP family of transcription factors (113). Not surprisingly, G-CSF also possesses neutrophil mobilizing activity in addition to its ability to enhance proliferation and survival (102), providing a link between granulopoiesis and neutrophil release. Coordinating these two processes is necessary for increasing the number of neutrophils in the systemic circulation where they can be directed to the site of infection.

The concentration of G-CSF under basal conditions is less than 30 pg/ml in humans but can increase 100-fold during infection, sepsis, and trauma (108), suggesting that G-CSF plays significant roles during inflammation. The signals necessary to induce neutrophil process are generated at the site of inflammation in the form of pro-inflammatory cytokines (TNFα, IL-1, IL-17) and chemokines (MIP-2, KC/CXCL1, leukotriene B4 and C5a) (105). Importantly, G-CSF is also expressed during inflammation (102). In normal conditions, neutrophils are retained in the

bone marrow due to the constitutive expression of CXCR4 on neutrophils and its ligand, SDF1α (CXCL12) (114). Specifically, CXCR4 is the gate that retains mature neutrophils in the bone marrow as its deletion shifts the pool of mature neutrophils from the bone marrow to the blood without affecting the lifespan of neutrophils (114). This constitutive function of CXCR4 is defective in patients with WHIM syndrome who suffer from neutropenia due to increased neutrophil retention and homing to the bone marrow as a result of an autosomal dominant mutation in *CXCR4* (106). Mechanistically, G-CSF down-regulates both CXCR4 and SDF1α and cooperates with pro-mobilizing CXC chemokines, KC and MIP-2, in the bone marrow to promote neutrophil release (115-117). In a thioglycollate-induced peritonitis model, it was shown that neutralization of G-CSF or CXC chemokines KC and MIP-2 with blocking antibodies inhibited the rise in the number of neutrophils in the blood by 70% (118). Furthermore, when G-CSF was co-injected with KC, there was a synergistic mobilization of neutrophils, suggesting that both factors are critical for efficient neutrophil release during inflammation (118). Therefore, the current model of acute mobilization of neutrophils from the bone marrow into the blood during inflammation involves interference of CXCR4/SDF-1α retention signals by G-CSF to facilitate CXC chemokine-induced migration of neutrophils across the bone marrow sinusoidal endothelium.

Expression of *CCN1*, as an immediate-inducible gene, is elevated in a broad spectrum of chronic inflammatory pathologies including IBD, rheumatoid arthritis, and atherosclerosis (22). Recently, it was found that CCN1 levels are elevated in the serum of patients with sepsis or giant cell myocarditis, a severe form of myocardial inflammation (119). Bacterial and viral infections, cytokines such as IL-1 and IL-17, and inflammatory lipid-mediators including prostaglandins E_2 and F_2 and sphinogosine-1-phosphate can also induce *CCN1* expression (28).

Conversely, CCN1 can stimulate expression pro-inflammatory cytokines in various cell types, including macrophages in which CCN1 upregulates a panel of cytokines that are characteristic of M1 macrophages (28;40). These findings suggest that CCN1 may serve a key role in initiating and establishing inflammation.

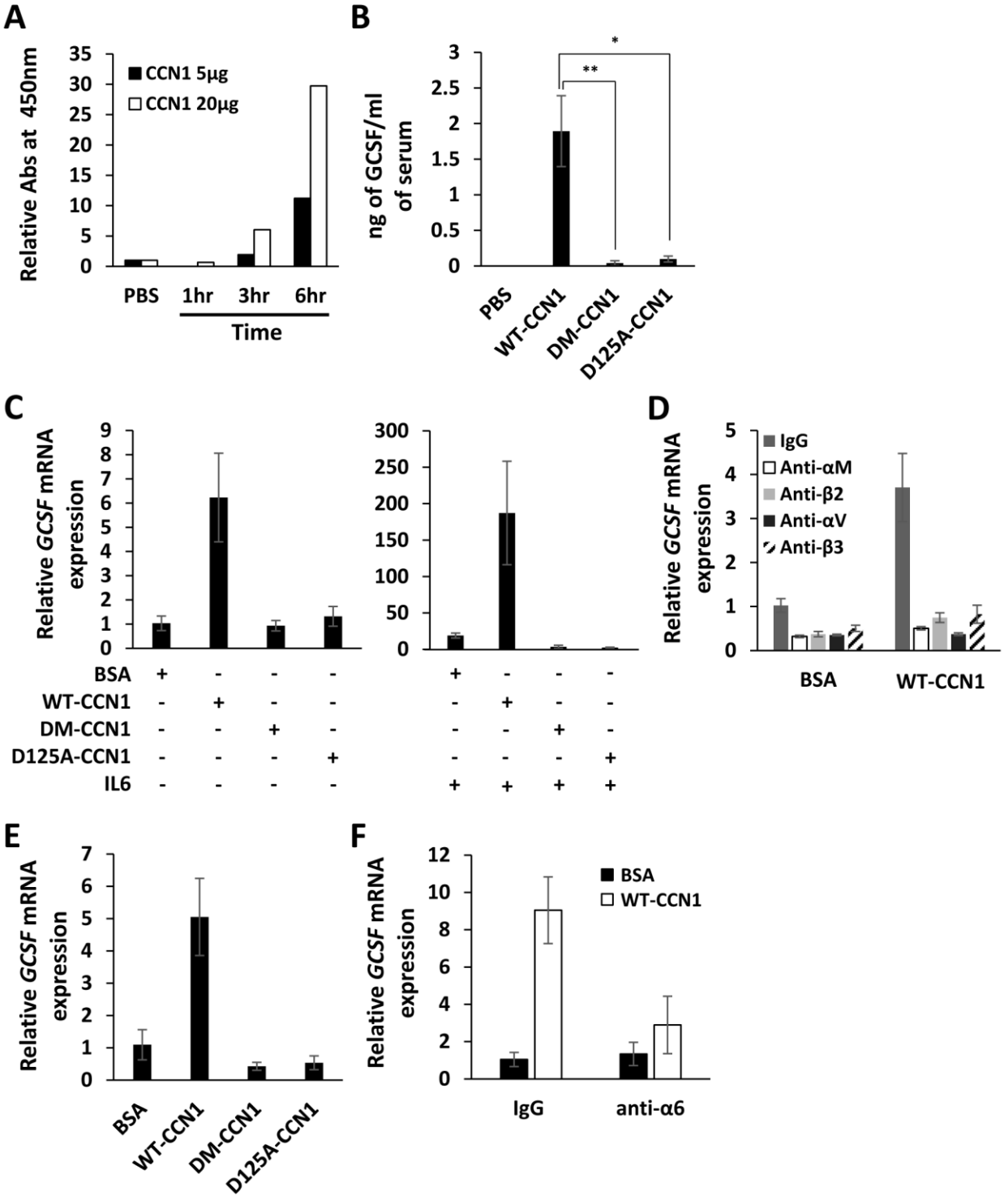
Here we show that CCN1 induces neutrophil mobilization through integrin mediated induction of *G-CSF* expression. Moreover, administration of CCN1 protein stimulated neutrophil mobilization *in vivo*. Our findings reveal a novel function of CCN1 as a potent regulator of neutrophil homeostasis.

6.2 CCN1 induces G-CSF *in vivo* and *in vitro*

To determine the ability of CCN1 to induce G-CSF *in vivo*, we injected purified WT-CCN1 protein or PBS i.p. into wild type mice, and G-CSF levels in the serum were measured by ELISA. We found that WT-CCN1 increases G-CSF protein level in the serum in a time- and dosage-dependent manner (figure 32A). Furthermore, this effect was specific for mice injected with WT-CCN1, as injection of DM- or D125A-CCN1 mutant proteins or PBS did not induce significant G-CSF accumulation in the serum (figure 32B), suggesting that CCN1 requires both $\alpha_6\beta_1/\alpha_M\beta_2$ and $\alpha_v\beta_3$ binding sites to induce G-CSF.

To identify the cellular sources of CCN1-induced *G-CSF*, we first tested CCN1 function in macrophages, as CCN1 was shown previously to regulate pro-inflammatory genes in macrophages through its interaction with integrin $\alpha_M\beta_2$ to activate NF-κB (40). We found that WT-CCN1 added to I13.35 macrophages in culture induced *G-CSF* gene expression by 6-fold compared to BSA controls, whereas addition of DM- or D125A-CCN1 mutant proteins did not (figure 32C). Given that CCN1 can induce *IL-6 in vivo* and *in vitro* in macrophages (figures 22B-C,

Figure 32. WT-CCN1, but not DM- or D125A-CCN1, induces *G-CSF in vivo* and *in vitro*. (A) G-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 μg), or (B) 24 hours after i.p. injection of 10 μg of WT-, DM-, or D125A-CCN1 protein, or PBS (*n*=4-6). **p*>0.05, ***p*>0.01. (C) *G-CSF* mRNA was measured by qRT-PCR in serum-starved (overnight) I13.35 macrophages treated with 5 μg/ml of purified WT-, DM-, or D125-CCN1, or BSA with and without rIL-6 (50 ng/ml) for 24 hrs. (D) I13.35 macrophages were incubated with blocking mAbs (50 μg/ml) against integrin α_M, β_2, α_V, or β_3 1 hr prior to treatment with WT-CCN1 for 24 hrs. *G-CSF* mRNA was measured by qRT-PCR. (E) *G-CSF* mRNA was measured by qRT-PCR in serum-starved (overnight) 1077Sk fibroblasts treated with 5 μg/ml of purified WT-, DM-, or D125-CCN1, or BSA for 24 hrs. (F) 1077Sk fibroblasts were incubated with blocking mAbs (100 μg/ml) against integrin α_6 1 hr prior to treatment with WT-CCN1 for 24 hrs. *G-CSF* mRNA was measured by qRT-PCR. Data shown as mean ± SD of triplicate experiments.


A
Relative Abs at 450nm
CCN1 5µg
CCN1 20µg
PBS
1hr
3hr
6hr
Time
B
ng of GCSF/ml of serum
*
**
PBS
WT-CCN1
DM-CCN1
D125A-CCN1
C
Relative GCSF mRNA expression
BSA
WT-CCN1
DM-CCN1
D125A-CCN1
IL6
D
Relative GCSF mRNA expression
IgG
Anti-αM
Anti-β2
Anti-αV
Anti-β3
BSA
WT-CCN1
E
Relative GCSF mRNA expression
BSA
WT-CCN1
DM-CCN1
D125A-CCN1
F
Relative GCSF mRNA expression
BSA
WT-CCN1
IgG
anti-α6

23A-B), we tested whether WT-CCN1 can act synergistically with IL-6 to regulate *G-CSF*. When I13.35 cells were treated with CCN1 in combination with IL-6, G-CSF expression was induced nearly 190-fold, or 30- and 10-fold higher than treatment with CCN1 or IL-6 alone, respectively (figure 32C). No synergism was observed when the cells were treated with DM- or D125A-CCN1 protein and IL-6, suggesting that the binding of CCN1 on integrins $\alpha_M\beta_2$ and $\alpha_v\beta_3$ are critical for synergism with IL-6 to potentiate *G-CSF* expression. Moreover, pre-incubation of macrophages with monoclonal antibody against integrin α_M, β_2, α_v, or β_3 eliminated the ability of CCN1 to upregulate *G-CSF* expression (figure 32D). Thus, consistent with our *in vivo* results, CCN1 regulates *G-CSF* in an integrin $\alpha_M\beta_2$- and $\alpha_v\beta_3$-dependent manner in macrophages. We also tested the ability of CCN1 to regulate *G-CSF* in fibroblasts as it was demonstrated that CCN1 induces expression of selective cytokines in fibroblasts (54). Similar to macrophages, 1077SK fibroblasts treated with WT-CCN1 protein, but not DM- or D125A-CCN1, in culture induced *G-CSF e*xpression by 5-fold compared to BSA controls (figure 32E). Integrin $\alpha_6\beta_1$ is the principal CCN1 receptor in fibroblasts (37). To confirm the involvement of integrin $\alpha_6\beta_1$, 1077SK fibroblasts were pre-incubated with monoclonal antibody against integrin α_6 for 1 hour prior to treatment with BSA or CCN1. The presence of anti-α_6 antibody abrogated CCN1-induced *G-CSF* expression (figure 32F), indicating that binding of CCN1 to integrin α_6 is necessary for its induction of *G-CSF* in fibroblasts. Taken together, these results indicate that CCN1 is a regulator of *G-CSF.*

6.3 CCN1 induces neutrophilia in a dose-dependent manner

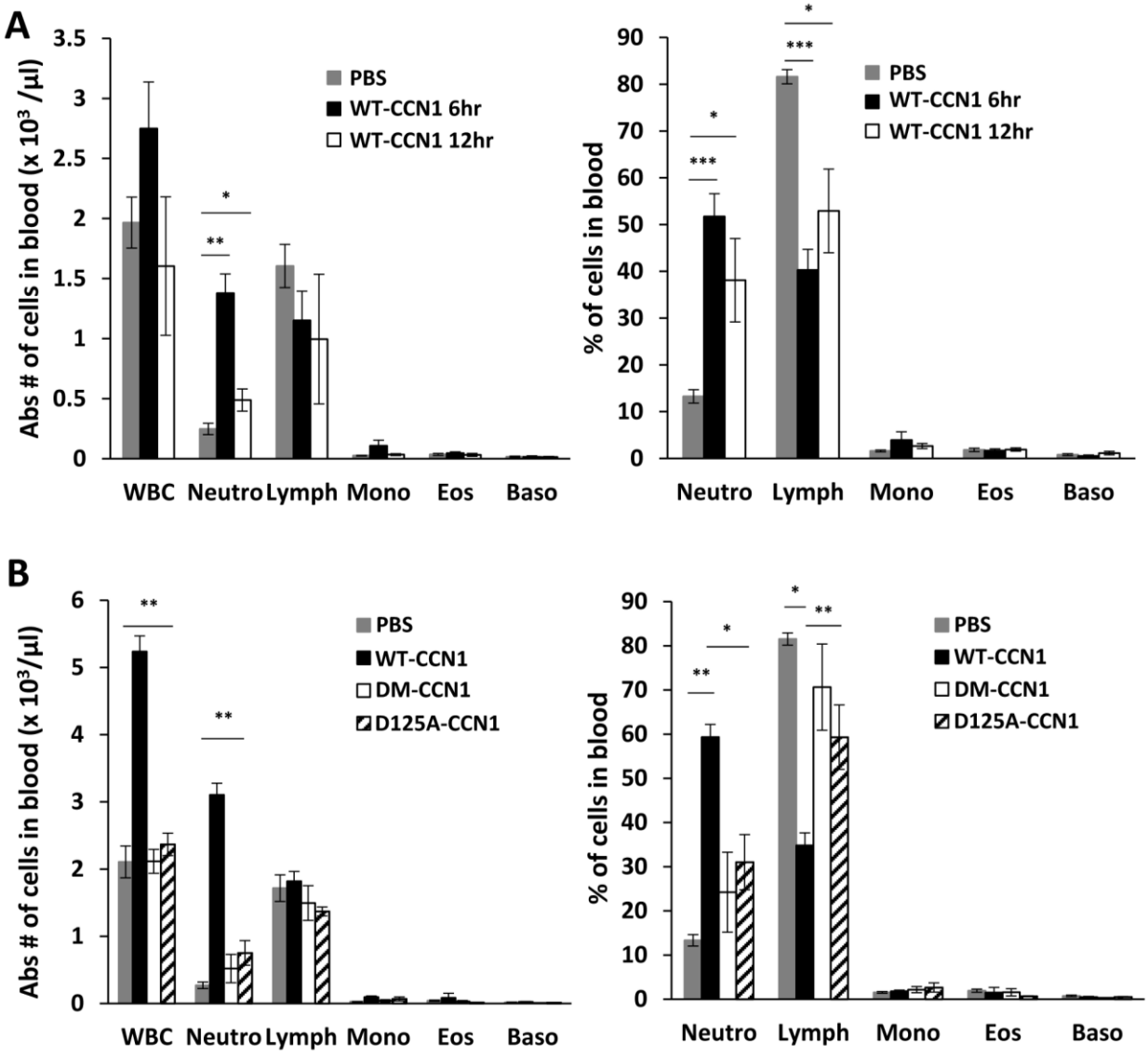
G-CSF is a prominent cytokine in regulating neutrophil homeostasis and mobilization (102). Therefore, we postulated that CCN1 may induce mobilization of neutrophils into the

blood from the bone marrow through its induction of G-CSF. Indeed, the number and percentage of neutrophils in the blood were increased to 1.4 x 10^3 cells per µl of blood and 52%, respectively, at 6 hours after mice were injected i.p. with 5 µg of WT-CCN1 compared to only 0.2 x 10^3 cells per µl of blood and 13% in PBS-treated controls (figure 33A). The number and percentage of neutrophils remained statistically elevated at 12 hours post-injection, albeit less than at 6 hours after WT-CCN1 treatment (figure 33A). Of note, there was a reduction in the percentage, but not the number, of lymphocytes in the blood after WT-CCN1 injection at 6 and 12 hours. We also examined if WT-CCN1 can induce clinical neutrophilia defined as >2.0 x 10^3 per ul of blood in mice with multiple injections of WT-CCN1. We discovered that when mice were given a single injection of WT-CCN1 daily for 5 consecutive days, it dramatically increased the number of neutrophils in the blood to 3.1 x 10^3 per ul of blood, whereas treatment with DM- or D125A-CCN1 protein or PBS did not (figure 33B). Accordingly, nearly 60% of white blood cells in the blood were neutrophils in WT-CCN1-treated animals. These findings show that CCN1 induces neutrophilia in a dose-dependent manner through it binding sites for integrins $\alpha_6\beta_1/\alpha_M\beta_2$ and $\alpha_v\beta_3$.

6.4 CCN1-induced neutrophil mobilization is mediated by independent effects of IL-6 and G-CSF

Since CCN1 and IL-6 worked synergistically to potentiate G-CSF expression in macrophages (figure 32C), we postulated that this mechanism may be critical for CCN1-induced G-CSF expression and the subsequent mobilization of neutrophils *in vivo*. To test this hypothesis, we co-injected wild type mice with WT-CCN1 and monoclonal antibody against IL-6 (anti-IL6) or isotype control, counted the number of WBCs in the blood, and measured

Figure 33. WT-CCN1, but not DM- or D125A-CCN1, induces neutrophilia. (A) Complete blood count in wild type mice at indicated times after a single injection i.p. of purified WT-CCN1 protein (5 μg) or PBS. Percentage of white blood cells in the blood shown on the right (*n*=5-10). (B) Complete blood count in wild type mice given daily injections i.p. of WT-, DM-, or D125A-CCN1, or PBS for 5 consecutive days. Percentage of white blood cells in the blood shown on the right (*n*=4-10). Data are represented as mean ± SEM; **p*<0.05, ***p*<0.01, ****p*<0.001.


A
Abs # of cells in blood (x 10³ /µl)
PBS
WT-CCN1 6hr
WT-CCN1 12hr
WBC
Neutro
Lymph
Mono
Eos
Baso
% of cells in blood
B
Abs # of cells in blood (x 10³/µl)
WT-CCN1
DM-CCN1
D125A-CCN1

G-CSF protein in the serum. We observed that although the co-administration of WT-CCN1 and anti-IL6 antibody showed fewer neutrophils in the blood compared to the isotype control, there was no difference in accumulation of G-CSF protein in either treatment groups (figure 34). This suggests that CCN1-induced IL-6 and G-CSF are working independently of each other to stimulate neutrophil mobilization in the blood after delivery of exogenous CCN1.

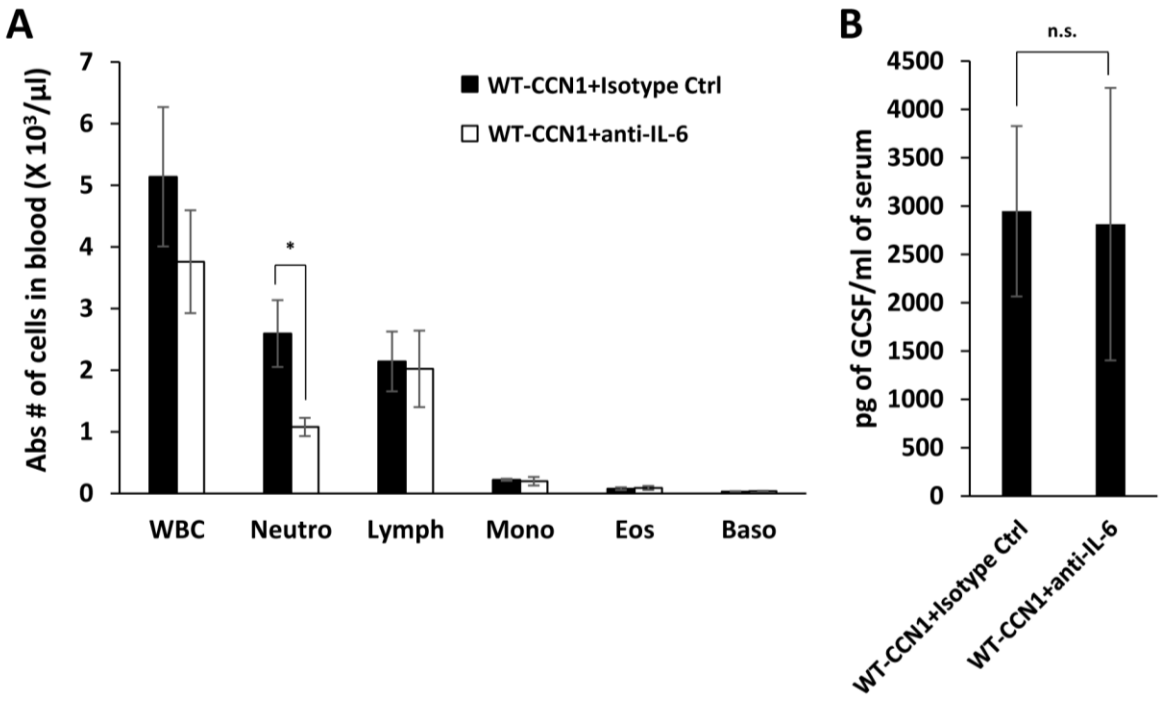


Figure 34. Co-injection of monoclonal mAb against IL-6 (anti-IL6) and WT-CCN1 lowers the number of circulating neutrophils, but not CCN1-induced G-CSF protein levels. (A) Complete blood count and (B) G-CSF protein measured by ELISA in wild type mice at 6 hours after a single injection i.p. of purified WT-CCN1 protein (10 µg) with anti-IL6 (100 µg) or isotype IgG control. Data are represented as mean ± SEM; *n*=4; n.s., not significant; *p<0.05.

7. DISCUSSION

7.1 Protective functions of CCN1 in intestinal inflammation

CCN1 is a matricellular protein that regulates diverse cellular functions through distinct integrins in various cell types (22). Although *CCN1* deregulation has been observed in many inflammation-related pathologies, different aspects of CCN1 activities may dominate depending on the context (29). For example, in skin wound healing and chronic inflammatory hepatic injuries, CCN1 restricts fibrosis by acting through $\alpha_6\beta_1$ to induce cellular senescence in myofibroblasts (54;57). In experimental hepatitis induced by concanavalin A, anti-CD95 antibody, or alcohol gavage, CCN1 exacerbates hepatocyte apoptosis by acting in synergy with TNFα and FasL (36;55). Additionally, a role for CCN1 in bone fracture repair has been attributed to its angiogenic activity (56).

We report here that CCN1 serves multiple protective functions in murine colitis, consistent with the theme that CCN1 mediates diverse functions through different binding sites on distinct integrins. *Ccn1*$^{d125a/d125a}$ mice suffer exacerbated and prolonged inflammation and tissue damage in colitis, resulting in increased mortality. Enhanced sensitivity to DSS toxicity is, in part, due to a defective mucus layers as *Ccn1*$^{d125a/d125a}$ mice showed fewer goblet cells in the crypts and a decrease in expression of *MUC2*, the major mucin glycoprotein in the outer and inner mucus layer that has been shown to be critical for colonic protection in murine colitis (78). Subsequently, *Ccn1*$^{d125a/d125a}$ mice showed skewing toward a pro-inflammatory state coupled with dramatic down-regulation of anti-inflammatory cytokines, including *IL-10* and *IL-22*. Lastly, chronic inflammation driven by a deregulated IL23/IL17/GCSF axis was observed. Although the precise point of action by CCN1 in this series of events remains unknown, these

findings indicate that CCN1 protects IECs and ameliorates intestinal inflammation in a $\alpha_v\beta_3$-dependent manner. Further studies are required to determine a causal link between the D125A mutation in CCN1 and a deficit in *MUC2* production in the large intestine. Given that CCN1 is known to regulate cell behavior as a cell adhesion substrate for integrin $\alpha_v\beta_3$, one likely explanation is that CCN1 may be indirectly enhancing MUC2 synthesis by promoting proliferation and/or differentiation of goblet cells in the intestinal crypt. Another possible scenario is that the deficit of IL10 expression increases the susceptibility of *Ccn1*$^{d125a/d125a}$ mice to DSS-induced colitis as *IL10*-deficient mice also demonstrate impaired MUC2 synthesis, leading to chronic inflammation in an infectious model of colitis (120). Measuring *Muc2* expression level in *Ccn1*$^{d125a/d125a}$ mice supplemented with recombinant IL-10 may help reveal the underlying cause.

Interestingly, *IL10*-deficient mice develop IL23/IL17-dependent chronic intestinal inflammation (121). Remarkably, *Ccn1*$^{d125a/d125a}$ mice mimic this chronic inflammatory phenotype of *IL10*-deficient mice. However, the precise mechanism by which the IL23/IL17/GCSF pathway becomes deregulated remains unknown. Notably, α_v-deficient mice develop spontaneous colitis, in part, due to the defective phagocytic function of macrophages and dendritic cells (73). In a separate study, impairment in phagocytosis of apoptotic cells was associated with deregulation of the IL23/IL17/GCSF axis (122). This line of evidence suggests that *Ccn1*$^{d125a/d125a}$ mice may suffer from impaired phagocytosis, and consequently, fail to upregulate *IL-10* and resolve inflammation, leading to the activation of the IL23/IL17/GCSF axis. Therefore, future study is needed to determine if CCN1 regulates *IL10* expression through the phagocytic function of macrophages and dendritic cells in intestinal inflammation.

7.2 Mucosal healing function of CCN1

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 (81;123). Although anti-TNFα 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 (124;125). In this report, we provide the first evidence that CCN1 promotes mucosal healing in colitis. Administration of exogenous CCN1 can accelerate mucosal healing from colitis in both wild type and *Ccn1* mutant mice, underscoring a therapeutic potential for CCN1 in IBD.

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 (figures 24-27). 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 NFκB, and both receptor systems have been implicated in the pathogenesis of IBD (11;126;127). Thus, it is not surprising that *IL-6* expression in the initiation phase (day 5) of colitis is independent of CCN1 (figure 22A). Remarkably, CCN1 exerts exquisite control on *IL-6*, and *IL-6* expression in the repair phase (day 8) is largely, but

transiently, 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 induces *IL-6* in macrophages through integrin $\alpha_M\beta_2$ and in fibroblasts through $\alpha_6\beta_1$ (figure 23). These activities are abrogated in DM-CCN1, consistent with impaired *IL-6* expression in *Ccn1*$^{dm/dm}$ mice. Interestingly, CCN1 synergizes with TNFα in fibroblasts to induce a high level of *IL-6* expression (figure 23D-E). This unexpected finding suggests that fibroblasts are an important source of IL-6 in inflammatory conditions where CCN1 and TNFα are co-expressed. CCN1 may also induce the expression of genes other than *IL-6* or provide additional functions that promote mucosal repair, since delivery of CCN1 fully rescued defects in *Ccn1*$^{dm/dm}$ mice, whereas IL-6 was only partially effective (figures 27-28). 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 (42). However, 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 (65;68), and a pilot clinical trial showed symptomatic improvements in CD patients treated with anti-IL-6R antibodies (88). Thus, IL-6 has been identified as a therapeutic target for IBD and clinical trials to evaluate the efficacy of anti-IL6 therapies are in progress (81;84;85). However, IL-6 is also important for intestinal mucosal homeostasis by promoting IEC proliferation and survival, and regulating intestinal barrier function (128-131). Existing results and findings presented herein are consistent with the interpretation that IL-6 plays 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 (132;133), but suffered impaired mucosal healing during recovery (86;134). These findings suggest that anti-IL-6 therapy may reduce IL-6-dependent inflammation, but may also impede mucosal healing. Therefore, selective induction of *IL-6* in mucosal healing, potentially through the CCN1/IL-6 axis, may enhance the therapeutic value of targeting IL-6 in IBD.

7.3 Tumor suppressing activities of CCN1 in colitis-driven carcinogenesis

Long-term UC patients are at increased risk for developing colorectal cancer (62). Notably, the duration, severity of inflammation, and extent of disease in UC patients have been identified as primary determinants for assessing the risk of colonic neoplasia (92). In addition, IL-6 has been identified as an important contributor to the development of colitis-associated cancer (128;129;135), and therefore it is of concern whether CCN1-induced *IL-6* may enhance colitis-associated tumorigenesis. *CCN1* expression is deregulated in numerous human cancers, and in many cases forced expression of *CCN1* in established tumor cells promotes tumor growth in xenografts, potentially through its angiogenic activity (29). However, the role of CCN1 in tumor initiation and early stages of tumor promotion has not been reported in any model of carcinogenesis to date. Our results show that both tumor incidence and tumor numbers are significantly increased in $Ccn1^{d125a/d125a}$ and $Ccn1^{dm/dm}$ mice in AOM/DSS-induced cancer, indicating that CCN1 suppresses colitis-associated tumorigenesis (figure 29).

The association between inflammation and neoplasia has strengthened based on the finding that inflammatory processes can enhance all three critical aspects of tumorigenesis: tumor initiation, promotion, and progression (93-95). In CRC, IBD has been identified as an

independent risk factor based on the severity, duration, and extent of the disease (90;92) The data presented in this study suggest that CCN1 may have multiple anti-tumorigenic functions mediated through its different integrin binding sites on distinct integrins, and requires the full protein to be intact to suppress colitis-driven cancer in mice. For example, increase in severity and extent of inflammation may be the driving force of tumorigenesis in the AOM/DSS-challenged *Ccn1*$^{d125a/d125a}$ mice (figures 3-5, 8, 30), whereas in *Ccn1*$^{dm/dm}$ mice, the tumor suppressive mechanism may stem from its ability to promote mucosal healing and thereby reduce inflammatory damage (figures 15-18, 30), or its ability to induce apoptosis and senescence in certain contexts (38;54). Although the precise role of CCN1 tumor suppression is currently unknown, our results indicate that CCN1 not only functions to dampen inflammation and accelerate mucosal healing in colitis, but also to suppress inflammation-associated tumorigenesis. Furthermore, we show that CCN1 protects against colitis-driven carcinogenesis despite its ability to induce *IL-6* expression in the injured colon. These findings suggest that CCN1 exerts exquisite control on *IL-6* expression to promote repair without enhancing tumorigenesis. Therefore, activation of the CCN1/IL-6 axis as a treatment strategy for IBD may not pose an increased cancer risk, but may instead reduce the risk of colitis-associated cancer. These postulations clearly merit further investigation.

7.4 Exogenous CCN1 regulates G-CSF and neutrophil mobilization

In this study, we uncover a novel function of CCN1 as an inducer of G-CSF. Moreover, delivery of exogenous CCN1 stimulated neutrophilia in mice. These findings identify a specific role of CCN1 in acute inflammation and provide a potential role for the accumulation of CCN1 levels in the serum of patients with sepsis or inflammatory myopathies (119).

Neutrophils are a critical arm of the host's primary defenses against microorganisms (102). Despite their indispensable function, neutrophils are very short-lived (105). Therefore, the number of neutrophils needs to be constantly replenished to ensure adequate protection from bacteria or fungi. In addition, a large number of mature and functional neutrophils reside in the bone marrow during basal conditions, and thus, need to be efficiently mobilized into the systemic circulation upon sepsis or injury at a distant site. Our findings here suggest that delivery of exogenous CCN1 can induce neutrophilia in mice (figure 33). Whether this function of CCN1 can boost immunity during local infection or sepsis requires further investigation.

Interestingly, we failed to observe any effects of endogenous CCN1 on neutrophil homeostasis as *Ccn1*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ mice did not exhibit any perturbations in neutrophil homeostasis under normal conditions (data not shown). Furthermore, contrary to expectation, both *Ccn1*$^{d125a/d125a}$ and *Ccn1*$^{dm/dm}$ mice had elevated neutrophil counts in the blood compared wild type during DSS-induced colitis (data not shown), indicating that endogenous CCN1 may not have the same function as exogenous CCN1. However, it is possible that the serum levels of CCN1 in DSS-challenged mice never reached the concentration needed to influence neutrophil mobilization, or that presence of other cytokines that are known to regulate G-CSF may have masked the effect of CCN1 on neutrophil mobilization. Further studies examining the granulopoiesis of wild type and mutant mice in normal and inflammatory conditions may provide additional insight into the working mechanism of CCN1 in neutrophil homeostasis.

Induction of *G-CSF* by CCN1 requires binding to both integrin $\alpha_M\beta_2$ and $\alpha_v\beta_3$ in macrophages and $\alpha_6\beta_1$ in fibroblasts (figure 32C-F). Consistently, neither DM-CCN1 nor D125A-

CCN1 injections into C57Bl/6 mice stimulates *G-CSF* expression or neutrophil mobilization (figure 33B). Notably, CCN1 synergizes with IL-6 in macrophages to induce a high level of *G-CSF* expression. However, the synergistic interaction between CCN1 and IL-6 was not observed *in vivo* (figure 34A). Interestingly, blockade of IL-6 signaling upon WT-CCN1 injection in the mice decreased the number of neutrophils in the blood without affecting G-CSF protein levels in the serum (figure 34B), indicating that CCN1-induced neutrophilia is most likely due to the sum of independent contributions of IL-6 and G-CSF on neutrophil mobilization. The ability of CCN1 to drive *G-CSF* expression in macrophages may depend on activation of NF-kB as it was demonstrated previously that induction of the pro-inflammatory genetic program in macrophages required integrin $\alpha_M\beta_2$-mediated activation of NF-kB (40). Although the exact mechanism by which CCN1 induces *G-CSF* remains unknown, our results reveal that exogenous CCN1 is a powerful inducer of neutrophil mobilization. Whether CCN1 can be used therapeutically to accelerate granulopoiesis or aid in re-establishment of neutrophil homeostasis in neutropenia remains open to study.

7.5 The role of CCN1 as a modulator of inflammation and inflammation-associated pathologies

The findings described in this thesis add another line of evidence that the matricellular protein CCN1 is emerging as an important regulator of inflammation, tissue repair, and tumorigenesis. Moreover, it revealed that CCN1 may help to coordinate a systemic inflammatory response by mobilizing neutrophils. As demonstrated, CCN1 directs specific aspects of complex physiologic processes in various pathology through its ability to exert

precise regulatory control in numerous cell types by acting as a ligand for distinct integrins and cell surface HSPG.

Remarkably, CCN1 serves critical protective functions in the three key steps of disease progression in murine colitis: acute and chronic inflammation, tissue repair, and inflammation-driven cancer (figure 35). First, CCN1 ameliorates inflammatory damage by regulating goblet cells and *MUC2* expression, as well as key inflammatory cytokines that are associated with the development of chronic intestinal inflammation. Second, CCN1 is critical for mucosal healing and recovery from colitis, in part through integrin-mediated induction of *IL-6* during the repair phase of disease progression. This study also underscores the importance of IL-6 in effective mucosal healing, which suggests that further optimization of treatment regimens targeting IL-6 is needed. Lastly, CCN1 demonstrates tumor suppressive activity in colitis-associated carcinogenesis.

In addition to its well-established functions at the local sites of inflammation, CCN1 may have systemic effects by regulating neutrophils (figure 35). Exogenous CCN1 increases serum G-CSF levels, a potent cytokine that stimulates neutrophil mobilization, and, concomitantly, drives neutrophilia. Again, the induction of *G-CSF* in macrophages and fibroblasts by CCN1 is integrin-dependent.

In conclusion, CCN1 is a critical modulator of inflammation and mucosal homeostasis, a suppressor of inflammation-driven cancer, and a regulator of neutrophil mobilization. These findings suggest a therapeutic potential for activating CCN1 in various inflammation-associated pathologies.

Figure 35. Functions of CCN1 in murine colitis and neutrophil homeostasis.
CCN1 modulates key steps in disease progression of intestinal inflammation, tissue repair, and inflammation-driven tumorigenesis through multiple functions mediated by different binding sites for distinct integrins (above solid line). CCN1 regulates neutrophil mobilization by inducing GCSF expression in macrophages and fibroblasts through various integrins (below solid line).

Context	Activity		Function
Initiation of colitis:	Promotes IEC survival Maintains MUC2 production		Attenuates injury and inflammation
Intestinal tissue repair:		IL6-dependent promotion of IEC proliferation	Promotes mucosal healing
Colitis-associated carcinogenesis	???	???	Reduces tumor burden
Treatment with exogenous CCN1:	Induces GCSF (macrophage)	$\alpha_6\beta_1$: induces GCSF (fibroblast) $\alpha_M\beta_2$: induces GCSF (macrophage)	Drives neutrophilia

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