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Clcn5 Knockout Mice Exhibit Novel Immunomodulatory Effects and Are More Susceptible to Dextran Sulfate Sodium-Induced Colitis

Philip Alex[*,†,1], **Mei Ye**[*,‡,1], **Nicholas C. Zachos**[*], **Jennifer Sipes**[*], **Thuan Nguyen**[†], **Maxim Suhodrev**[*], **Liberty Gonzales**[†], **Zubin Arora**[*], **Ting Zhang**[*], **Michael Centola**[†], **Sandra E. Guggino**[*], and **Xuhang Li**[*]

[*]Division of Gastroenterology, Department of Medicine, Johns Hopkins University, Baltimore, MD 21205

[†]Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

[‡]Department of Internal Medicine and Geriatrics, Zhongnan Hospital, Wuhan University, Wuhan, China

Abstract

Although the intracellular Cl^-/H^+ exchanger Clc-5 is expressed in apical intestinal endocytic compartments, its pathophysiological role in the gastrointestinal tract is unknown. In light of recent findings that CLC-5 is downregulated in active ulcerative colitis (UC), we tested the hypothesis that loss of CLC-5 modulates the immune response, thereby inducing susceptibility to UC. Acute dextran sulfate sodium (DSS) colitis was induced in *Clcn5* knockout (KO) and wild-type (WT) mice. Colitis, monitored by disease activity index, histological activity index, and myeloperoxidase activity were significantly elevated in DSS-induced *Clcn5* KO mice compared with those in WT mice. Comprehensive serum multiplex cytokine profiling demonstrated a heightened Th1–Th17 profile (increased TNF-α, IL-6, and IL-17) in DSS-induced *Clcn5* KO mice compared with that in WT DSS colitis mice. Interestingly, *Clcn5* KO mice maintained on a high vitamin D diet attenuated DSS-induced colitis. Immunofluorescence and Western blot analyses of colonic mucosa validated the systemic cytokine patterns and further revealed enhanced activation of the NF-κB pathway in DSS-induced *Clcn5* KO mice compared with those in WT mice. Intriguingly, high baseline levels of IL-6 and phospho-IκB were observed in *Clcn5* KO mice, suggesting a novel immunopathogenic role for the functional defects that result from the loss of Clc-5. Our studies demonstrate that the loss of Clc-5 1) exhibits IL-6–mediated immunopathogenesis, 2) significantly exacerbated DSS-induced colitis, which is influenced by dietary factors, including vitamin D, and 3) portrays distinct NF-κB–modulated Th1–Th17 immune dysregulation, implying a role for CLC-5 in the immunopathogenesis of UC.

Address correspondence and reprint requests to Dr. Xuhang Li, Division of Gastroenterology, Department of Medicine, Johns Hopkins University, 746 Ross, 720 Rut-land Avenue, Baltimore, MD 21205. xuhang@jhmi.edu.

[1]P.A. and M.Y. contributed equally to this work.

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Although characterized by distinct clinical and histopathological features, the etiology and pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel disease (IBD), have not yet been fully defined (1, 2). The mucosal immune system is the central effector of intestinal inflammation, with inflammatory mediators, primarily cytokines, playing a central role in modulating innate and adaptive immune responses in IBD (3, 4). We and others have shown from studies of human patients and animal IBD models that both CD and UC have specific mucosal-damage pathways, characterized by the dysregulation of distinct Th1 and Th2 cytokine profiles at different stages of the disease process (4–6). These studies have demonstrated UC to be a prototypic Th2-type disorder (mediated by IL-4, IL-5, and IL-10) and CD to be primarily associated with Th1–Th17-type responses mediated by TNF-α, IL-12, IFN-γ, and IL-17 (4, 6, 7).

Genes associated with IBD are generally categorized into those affecting immune response and microbial recognition and those affecting ion and water transport (8, 9). Diarrhea (altered fluid transport) is one of the most prevalent symptoms in patients with IBD (10). Impaired colonic salt and water transport in IBD occur as a result of decreased Na^+ absorption and increased Cl^- secretion and have been described to be major pathogenic factors in IBD-associated diarrhea (11, 12). The dysregulation of several membrane transporters in different models have been linked to IBD-associated diarrhea, including Na^+/K^+ ATPase (13–15), the epithelial Na^+ channel (16), Na^+/H^+ exchangers 1 and 3 (NHE1,3; in cell models only) (17, 18), and $Na^+/K^+/2Cl^-$ (13, 16). Recently, we demonstrated the coordinated downregulation of several Na^+ transporters in sigmoid mucosal biopsies of patients with active IBD and mice with experimental colitis, including that of the chloride channel CLC-5, NHE1,3 (but not NHE2), epithelial Na^+ channel, Na^+/K^+ ATPase, and Na^+/H^+ exchanger regulatory factor 1 (19).

The chloride channel CLC gene family encodes nine known isoforms in mammals, the mutations and/or disruptions of some of which have been shown to underlie human diseases and pathology, including Bartter syndrome (with or without deafness), Dent disease, lysosomal storage diseases, myotonia, blindness, male infertility, defective endocytosis, osteopetrosis, leukodystrophy, and neurodegeneration (20–25). One of these isoforms, a 746-aa protein CLC-5, encoded by the CLCN5 gene is a voltage-dependent Cl^-/H^+ exchanger (20, 26). Mutations in the CLCN5 gene are associated with X-linked renal tubulopathy of Dent disease, with functional defects in both patients and mouse that are characterized by low-m.w. proteinuria, aminoaciduria, glycosuria, phosphaturia, hypercalciuria, nephrolithiasis, and progressive renal failure (27–35). In general, CLC channels have been demonstrated to contribute to a host of biological and cellular processes, including cell migration, proliferation, and apoptosis (36). Only CLC-3 has been shown to play a critical role in TGF-β–induced apoptosis of human airway epithelial cells and has recently been shown to be involved in the recruitment and activation of immune cells in the respiratory tract (36, 37). However, the specific role of CLC-5 and/or cellular mediators that modulate important immune functions to trigger downstream signaling pathways has not yet been defined.

Identifying changes of CLC-5 and associated modulators in IBD may lead to a better understanding of the molecular causes for IBD-associated diarrhea. Because IFN-γ inhibits intestinal transport by downregulating Na^+/K^+ ATPase and $Na^+/K^+/2Cl^-$, we and others previously suggested that IBD-associated inflammatory cytokines may play a role (6, 13). In experimental colitis models, we recently demonstrated that diarrhea was associated with significant elevation of various cytokines in colonic mucosa (6). In the acute dextran sulfate sodium (DSS) colitis model, Th1–Th2 cytokines (IL-6, IFN-γ, and IL-17) were increased, whereas in chronic colitis models IL-6 and IFN-γ (but not IL-12 p40/70 and IL-17) were elevated. Our studies also suggested that, although there are clear differences in the production of specific cytokines between DSS and trinitrobenzene sulfuric acid models of experimental colitis, these cytokine differences result in a similar clinical consequence: diarrhea (6). The cytokine network in IBD is a complex and dynamic system in which cellular and humoral cytokines, chemokines, and growth factors regulate the initiation and perpetuation of inflammation (4, 6). Given the significance of CLC-5 and that CLC-5 is downregulated in sigmoid mucosal biopsies of most patients with active UC (19), we tested the hypothesis that the loss of CLC-5 modulates the immune response, thereby inducing susceptibility to UC. Herein, we demonstrate that the loss of Clc-5 1) significantly exacerbates DSS-induced colitis, 2) is associated with elevated baseline levels of IL-6 and phospho-IκB, and 3) is influenced by dietary factors, such as vitamin D and NF-κB–mediated distinct Th1–Th17 immune dysregulation, implying the role of downregulated CLC-5 in the immunopathogenesis of UC.

Materials and Methods

Animals and diet

C57BL/6 *Clcn5* knockout (KO) mice, created by deletion of exon VI of *Clcn5* (35), and wild-type (WT) C57BL/6 age-matched adult male mice (6–8 wk old) were group-housed at Johns Hopkins Animal Facility under controlled temperature (25°C) and photoperiods (12:12 h light–dark cycle). Care and experimentation of mice were performed in accordance with institutional guidelines under protocols approved by the Institutional Animal Care and Use Committee. Mice were fed on Harlan Teklad Diet (H-diet; Harlan Laboratories, Madison, WI). In studies of dietary effects on DSS-induced colitis, subgroups of mice were also fed with NIH-31 modified open formula diet (Z-diet; Zeigler Brothers, Gardners, PA) or a vitamin D (4.18 IU/g)-supplemented H-diet (Vit D-enriched H-diet).

Abs

Abs used include: IL-6 mAb (Transduction Laboratories, Lexington, KY), IL-17 and NF-κB polyclonal Abs (pAbs) (Santa Cruz Biotechnology, Santa Cruz, CA), IL-12 p40/70 pAb (BioSource International, Camarillo, CA), phospho-IκB pAb (Abcam, Cambridge, MA), and actin pAb (Sigma-Aldrich, St. Louis, MO). Clc-5 pAb was obtained as previously described (35).

Induction of colitis

Acute colitis of C57BL/6 mice was induced by feeding mice ($n \geq 7$ mice in each group) with 2.5% (w/v) DSS (molecular mass 40 kDa; ICN Biochemicals, Aurora, OH) as described previously (6).

Evaluation of colitis

Animals were observed twice daily for weight, water/food consumption, morbidity, stool consistency, piloerection, and the presence of gross blood in feces and at the anus. Disease activity index (DAI) was calculated as described previously (6). At day 7 following induction with DSS, animals were sacrificed by CO_2 inhalation, rapidly dissected, and the entire colon was quickly excised, photographed, and gently cleared of feces with 4°C saline. Small segments of the colon taken for histopathology and immunohistochemistry were fixed in 10% normal buffered formalin as described previously (6). Sections (4 μm) were stained with H&E (Richard Allen Scientific, Kalamazoo, MI), histological scores were blindly determined with minor modifications from Obermeier et al. (38), and histological activity index (HAI) was calculated as described previously (6). The activity of the enzyme myeloperoxidase (MPO), a marker of polymorphonuclear neutrophil primary granules, was also determined in colonic mucosa as described previously (6, 39)

Isolation of colonic mucosa and extraction of proteins for SDS-PAGE and Western blot analysis

At 4°C, the mucosa was scraped from the muscle layer of the colon, and samples were snap frozen and stored at −80°C for the remaining experiments. Frozen tissue samples were homogenized in homogenization buffer (50 mM Tris-HCl [pH 7.2]) containing Na_3VO_4 and a protease-inhibitor mixture (Sigma-Aldrich) using an Omni TH homogenizer (Omni International, Marietta, GA). Following sonication, the homogenate was centrifuged at 2000 × *g* for 10 min. Supernatants were collected as total mucosal proteins, and protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Protein extraction, SDS-PAGE, and Western blots were performed as described previously (6, 19, 40).

Serum collection and biometric multiplex cytokine profiling

Blood was collected by cardiac puncture in endotoxin-free, silicone-coated tubes without additive. Blood samples were allowed to clot at room temperature for 30 min before centrifugation (2200 × *g*, 4°C, 10 min), and the serum was collected and stored at −80°C until analyzed. A multiplex sandwich immunoassay from the Bio-Plex Protein Array System (Bio-Rad), which contains fluorescently labeled microspheres conjugated with mAbs specific for 16 target cytokines, was used as described previously (6, 41). Analytes measured include IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ, TNF-α, GM-CSF, interferon-inducible protein 10, keratinocyte-derived chemokine (KC), MCP-1, monokine-induced by IFN-γ, and MIP-1α (source of Abs: Invitrogen, Carlsbad, CA).

Isolation of peritoneal macrophages and PBMCs

Peritoneal macrophages were prepared as previously described (42). Briefly, 2 ml thioglycollate broth was injected into the peritoneal cavities of mice to elicit macrophages. After 3 d, peritoneal cells were recovered by washing the peritoneal cavity with 10 ml DMEM. Peritoneal macrophages were then purified by adhesion to tissue culture plastic (4 d after initial culture). Mouse PBMCs were isolated from whole blood by Ficoll gradient. Briefly, whole blood from three mice (~2–3 ml) was mixed with an equal volume of HBSS and then overlaid onto 9 ml Ficoll. After centrifugation (with brake off) at 2500 rpm for 30 min, the PBMC fraction (buffy coat layer) was collected and diluted two times with HBSS. PBMCs were recovered by centrifugation at 1500 rpm for 10 min.

Immunocytochemistry

Immunocytochemical detection was determined in 4-μm paraffin-embedded colonic sections as described previously (6) using Alexa Fluor 488 (Invitrogen) goat anti-mouse IgG (MCP-1 and IL-17) or Alexa Fluor 568 (Invitrogen) goat anti-mouse IgG (IL-6).

Statistical analysis

Statistical analysis (for all of the data with the exception of cytokine experiments) within and between each groups was done using a Student *t* test, and descriptive results are presented as mean ± SD. p values <0.05 were considered statistically significant.

Statistical analysis of cytokine profiles

Analyte concentrations were quantified by fitting using a calibration or standard curve. A five-parameter logistic regression analysis was performed to derive an equation that allowed concentrations of unknown samples to be predicted. Statistical differences in measured values were assessed by a Mann-Whitney *U* test. Data are presented with mean, median, upper, and lower quartile values. p values <0.05 were considered statistically significant.

Multidimensional scaling

Multidimensional scaling (MDS) is an iterative process to detect meaningful underlying dimensions to explain observed similarities or dissimilarities between the groups studied (43). This analysis uses correlational matrices to construct configurations of the data in a lower-dimensional matrix, such that the relative distances between the groups are similar to those in the higher-dimensional matrix. The degree of correspondence between the distances and the matrix input by the user is measured (inversely) by a stress function defined by $\Phi = \Sigma[d_{ij} - f(\delta_{ij})]^2$, where d_{ij} is the Euclidean distance and δ_{ij} is the observed distance. The proximities and distances are then represented on a two-dimensional Shepard diagram scatter plot, which facilitates visualization and the interpretation of patterns. All of the statistical analyses for MDS were performed with R software, version 2.8 (44).

Results

Clcn5 KO mice exhibit increased susceptibility to DSS-induced colitis

We recently demonstrated the coordinated downregulation of several transporter proteins, including CLC-5, in the sigmoid colon of patients with active IBD, suggesting its potential contribution to IBD-associated diarrhea (19). To investigate whether the loss of Clc-5 would induce susceptibility to colitis, we subjected *Clcn5* KO mice to the well-established DSS chemical model of mucosal inflammation. Oral DSS administration for 7 d induced more significant acute colitis in *Clcn5* KO mice when compared with that in WT mice ($n = 7$ mice per group) (Fig. 1). This was characterized by a significant increase in DAI scores ($p = 0.034$), as demonstrated by greater weight loss, earlier appearance of diarrhea/loose feces, and fecal blood in DSS-induced *Clcn5* KO mice when compared with those in DSS-induced WT mice, as early as 5 d following induction with DSS (Fig. 1*A*). Morphological examination at day 5 and day 7 of induction revealed significant reduction in colon length and increased loose bloody stools in DSS-induced *Clcn5* KO mice when compared with those in DSS-induced WT mice (Fig. 1*B*). Histological examination of acute DSS colitis in *Clcn5* KO mice was characterized by greater loss of architecture, fewer goblet cells, and increased inflammatory cell infiltration at the areas of lesions when compared with those in DSS-induced WT mice (Fig. 1*C*). This was also demonstrated by the significant increase in HAI colitis scores for DSS-induced *Clcn5* KO mice relative to those for DSS-induced WT mice ($p = 0.039$) (Fig. 1*D*). Neutrophil infiltration, as demonstrated by increased MPO activity, was also significantly elevated in *Clcn5* KO mice induced with DSS colitis when compared with that in DSS-induced WT mice, indicative of an increased susceptibility to DSS-induced colitis ($p = 0.028$). The colons of *Clcn5* KO mice uninduced with DSS colitis appeared normal and not different from those of control WT mice (Fig. 1). Our data therefore demonstrate that the loss of Clc-5 significantly exacerbates DSS-induced colitis.

Unique systemic immune profiles in *Clcn5* KO mice at both basal conditions and when induced with DSS colitis

It is not known whether Clc-5 modulates the immune response to trigger distinct signaling inflammatory pathways. To analyze the influence of the cytokine patterns in the increased susceptibility of *Clcn5* KO mice to DSS, we performed systemic multiplex serum cytokine profiling in DSS-induced *Clcn5* KO and WT mice. The levels of 16 systemic cytokines covering a broad spectrum of immune and inflammatory mechanisms were measured in parallel following induction of colitis. Acute DSS-induced *Clcn5* KO mice demonstrated a cellular, cytotoxic, and chemotactic profile with significant elevated levels of IL-12 and IL-17 ($p < 0.05$), as well as IFN-γ, KC, MIP-1α, and MCP-1 ($p < 0.001$), when compared with those in DSS-induced WT mice (Fig. 2*A*, 2*B*). This predominantly chemotactic cellular immune profile is distinct and is unlike any of our prior observations from several autoimmune conditions (6, 41, 45–47), suggesting the unique immunomodulatory role of Clc-5 in DSS colitis. Interestingly, the baseline levels of IL-6 were significantly elevated in *Clcn5* KO mice when compared with those in WT mice (Fig. 2*C*), suggesting the likelihood that IL-6 may function as a proinflammatory mediator in *Clcn5* KO mice.

MDS analyses reveal unique categorical cytokine networks in *Clcn5* KO mice

Complementary multivariate analytical methods provide a vivid picture of the biological significance of the immune profile network. MDS provides a means of identifying correlational configurations of statistically significant cytokines and allows for a visual representation of the pattern of proximities within the groups studied (43). As depicted in Fig. 3, MDS analysis of the cytokine patterns in DSS-induced *Clcn5* KO mice identified a strong positive cluster between IL-12 and IL-17 ($r = 0.742$, $p = 0.041$), whereas those clusters were weaker in DSS-induced WT mice ($r = 0.558$, $p = 0.057$) (Fig. 3*A*). DSS-induced *Clcn5* KO mice also showed a significantly tight positive cluster among KC, MIP-1α, and MCP-1 ($r = 0.713$, $p = 0.027$), whereas those clusters were absent in DSS-induced WT mice (Fig. 3*A*). However, DSS-induced WT mice showed a strong positive correlation between IL-12 and IFN-γ ($r = 0.758$, $p = 0.03$), one that was not present in DSS-induced *Clcn5* KO mice (Fig. 3*B*). Interestingly, even at basal conditions (uninduced) *Clcn5* KO mice showed a strong positive correlation between IL-6 and IL-10 ($r = 0.722$, $p = 0.039$), which was absent among all other groups studied (Fig. 3*C*). These unique representations provide a visual inspection of similarities and differences between cytokine changes among the group, indicating the intricate but distinct immune network associated with Clc-5 expression.

Alteration of cytokine profiles in colon validate and correlate with that of systemic cytokines

To determine whether the observed systemic cytokine profiles in acute experimental colitis of *Clcn5* KO mice correlated with that of local levels seen within the colonic mucosa, immunoblots and immunofluorescence analysis of colons from DSS mice were performed. Proteins were extracted from mucosa scraped from freshly excised colons, and samples were analyzed by SDS-PAGE Western blots using primary Abs for IL-6, IL-12 p40/70, and IL-17. As shown in Fig. 4*A*, acute DSS-induced *Clcn5* KO colitis had significantly higher IL-6, IL-12 p40/70, and IL-17 protein expression in the colon than that in WT DSS-induced colitis, suggesting similar patterns of changes to those observed in the systemic levels, thereby validating the correlation of the systemic immune response to that observed in local tissue.

Acute experimental colitis of *Clcn5* KO mice is characterized by an NF-κB–mediated immunomodulatory profile. DSS-induced *Clcn5* KO colitis had significantly higher NF-κB and phospho-IκB protein expression in the colon than that in WT DSS-induced colitis, suggesting the role of the NF-κB proinflammatory pathway in the distinct immune regulation associated with Clc-5 expression (Fig. 4*B*). Intriguingly, the baseline levels of phospho-IκB (no DSS treatment) were also significantly elevated in *Clcn5* KO mice compared with those in WT mice, suggesting the likelihood that phospho-IκB, along with IL-6, may function as a proinflammatory mediator in *Clcn5* KO mice.

Changes in mucosal cytokine levels are consistent with systemic levels as observed by immunofluorescence. As shown in Fig. 4*C*, IL-6 and MCP-1 were elevated in *Clcn5* KO mice with DSS-induced colitis, predominantly associated with lamina propria infiltrating mononuclear cells, demonstrating the immune modulatory potential of inflammatory

infiltrates and further validating the observed systemic cytokine profiles with that of local levels seen within tissue.

Elevated levels of IL-6 protein expression identified in the kidney, but not the colon, of *Clcn5* KO mice

Although *Clcn5* KO mice had significantly high systemic IL-6 levels relative to those of WT controls, the colonic mucosa from *Clcn5* KO mice did not exhibit elevated levels of IL-6 protein expression in Western blots compared with those of WT controls (Supplemental Fig. 1*A*, 1*B*). Because mutations in the CLCN5 gene are associated with functional defects characterized by nephrolithiasis and progressive renal failure (27, 29, 31–33, 48–50), we investigated the protein expression of IL-6 in the kidney cortex of *Clcn5* KO mice and WT mice. SDS-PAGE and Western blot studies demonstrated a significant increase in IL-6 protein expression in the kidney cortex of *Clcn5* KO mice relative to that in WT mice, suggesting the novel finding that IL-6 may contribute to an immunopathogenic role (Supplemental Fig. 1*A*). To further define the expression and localization of renal IL-6 expression in *Clcn5* KO mice, we performed immunofluorescence analyses on frozen sections of kidney cortex of WT and *Clcn5* KO mice. Immunofluorescence analyses showed that IL-6 was significantly elevated in the kidney cortex of *Clcn5* KO mice compared with that in WT mice (Supplemental Fig. 1*B*). In WT kidney, a low level of IL-6 expression was localized to the tubular basement membrane. However, in the *Clcn5* KO kidney, there was increased expression of IL-6 in the basement membrane and upregulation in the interstitium, surrounding renal tubules, as well as epithelial cells of the proximal tubules, which was not observed in WT kidney sections. Our data demonstrate, for the first time, the involvement of novel immunomodulatory effects in a mouse model of Dent disease.

Clc-5 is highly expressed in the macrophages and weakly expressed in the colonic mucosa of WT mice but not in PBMCs

Because the immune profiles of *Clcn5* KO mice are distinctly different from those of WT mice both under the basal condition (baseline level) and in DSS-induced colitis, we speculate that Clc-5 may be expressed in the immune cells, where it could directly affect the function of these cells. Peritoneal macrophages and PBMCs, including lymphocytes and monocytes, were isolated from both WT and *Clcn5* KO mice. Surprisingly, Clc-5 was highly expressed in the macrophages (comparable to that in kidney), but not in PBMCs (Supplemental Fig. 2). Low levels of Clc-5 expression were also observed in the colonic mucosa of WT mice. These data provide the first evidence that Clc-5 is expressed in immune cells.

Protective effect of diet and vitamin D on *Clcn5* KO DSS-induced colitis

A case-control study to evaluate the etiological role of dietary factors in UC identified dietary patterns that were associated with an increased risk to develop UC (51). To evaluate the influence of diet on the development of experiment colitis in *Clcn5* KO mice, we subjected a group of mice (n = 5 per group) to a separate diet: Z-diet (Supplemental Fig. 3). The diet was initiated from birth (in both mother and F1 generation used in these experiments) and contains greater amounts of vitamin D, vitamin B_{12}, selenium, and choline

(all >1.5-fold) and lower amounts of iodine, vitamin K_3, inositol, pantothenic acid, vitamin B_2, vitamin B_6, vitamin B_7, and vitamin E (all <1.5-fold) when compared with the H-**d**iet, which is the regular diet used at our mouse facility (Supplemental Fig. 3). Interestingly, a significant increase in colitis, as demonstrated by DAI scores, was observed in DSS-induced *Clcn5* KO mice maintained on H-diet, when compared with that of *Clcn5* KO DSS-induced mice on the Z-diet ($p = 0.047$) (Fig. 5*A*). Among the differences in dietary components between H-diet and Z-diet, vitamin D has been reported to play a role in preventing or ameliorating the IBD in the colitis model of IL-10 KO mice (52). Because Z-diet contains 2-fold higher level of vitamin D than that in the H-diet (Supplemental Fig. 1), we hypothesized that the high level of vitamin D in the Z-diet might be an important reason why Z-diet exhibited protective effect on colitis of *Clcn5* KO mice. Because we did not observe the beneficial effect of Z-diet on DSS colitis of WT mice, we decided to test the effect of vitamin D supplement on DSS colitis in *Clcn5* KO mice by giving the mice Vit D-enriched H-diet, which was specially formulated H-diet containing a higher level of vitamin D (4.18 IU/g), which is the same as that found in the Z-diet. As shown in Fig. 5*B* and 5*C*, DSS-treated *Clcn5* KO mice on Vit D-enriched H-diet for 4 wk exhibited a more significant reduction of disease activity than those on regular H-diet. Interestingly, this effect depends on the duration of the diet change: *Clcn5* KO mice on Vit D-enriched H-diet for 1 wk exhibited only marginal improvement of DSS colitis compared with that of the mice on the same diet for 4 wk (Fig. 5*B*). There was no significant difference in colitis DAI (and MPO scores, data not shown) of DSS-induced WT mice between the mice fed with the H-diet and the mice fed the Z-diet (Fig. 5*A*). This suggests that the loss of Clc-5 significantly exacerbates DSS-induced colitis and is influenced by the dietary factors, such as vitamin D. Further quantitative studies are warranted to more fully investigate whether the loss of Clc-5 is a true risk factor in the development of UC.

Discussion

Diarrhea continues to be a frequent symptom in UC patients, the underlying mechanisms of which depend on various factors, including, but not limited to, the location, extent, and severity of the inflammation, altered motility, associated infections, and iatrogenic factors (10, 53). Furthermore, diarrhea in UC is associated with fluid and electrolyte imbalance, indicative of impaired gastrointestinal epithelial ion transport (54). Recently, we demonstrated the coordinated downregulation of CLC-5 among several Na^+ transporters in sigmoid mucosal biopsies of patients with active IBD and mice with experimental colitis (19). However, the specific role of CLC-5 in UC has not yet been defined.

Previous studies have demonstrated that the Guggino *Clcn5* KO mice develop classical signs of Dent disease with fluid and electrolyte imbalance manifested by polyuria, low-m.w. proteinuria, aminoaciduria, glycosuria, and hypercalciuria (28–30, 35). Although fluid, sodium, and chloride measurements have not yet been performed on the stool from these mice, *Clcn5* KO mice afford an ideal model to investigate whether the loss of CLC-5 would induce susceptibility to UC. In so doing, we present here the first evidence that the loss of Clc-5 significantly exacerbates both clinical and histopathological signs of DSS-induced UC-like colitis. It is important to infer the mechanisms of the phenotypic abnormalities in intestinal mucosa as a result of the lack of Clc-5 function. Clc-5 is an intracellularly

localized Cl^-/H^+ exchanger that has been suggested to play an important role in regulation of protein trafficking in epithelial cells (54). Our recent studies have shown that, in mouse mucosa, both Nhe3 and Clc-5 comigrated in the same endosomal pool isolated by OptiPrep gradient fractionation (data not shown). Because trafficking of Nhe3 between plasma membrane and endosomal compartments is a major regulatory mechanism of Nhe3 (55), our data suggest that Clc-5 may be involved in Nhe3 trafficking. Lack of Clc-5 causes alkalinization of the early and perhaps recycling endosmes (56) but not late endosomes. Because Clc-5 acts to provide the neutralizing negative charge for the proton moved by the H-ATPase that acidifies this compartment, the lack of Clc-5 increases the pH in the endosmal compartment. The lack of Clc-5, and consequent alkaline endosomal pH, causes trafficking defects in the apical membrane proteins Na/phosphate cotransporter 2a (Npt2a), Nhe3, and megalin (55). In proximal tubules devoid of Clc-5, a significantly reduced apical membrane expression of megalin (57) results in low-m.w. proteinuria. Furthermore, both Npt2a and Nhe3 are mislocalized to a subapical compartment in the proximal tubule of *Clcn5* KO mice. The reduced apical membrane localization of Npt2a and Nhe3 decreases transport activity and thus results in hyperphosphaturia and sodium loss, respectively. By analogy, we expect that the same physiological outcome would be present in the intestine where Nhe3 is well known to traffic on and off of the plasma membrane (58). The idea of less surface Nhe3 caused by defective Nhe3 trafficking in the *Clcn5* KO mice would be compatible with other mouse KO studies that have shown the lack of colonic Nhe3 or its regulatory factor Na^+/H^+ exchanger regulatory factor 1 (data not shown) causes diarrhea and susceptibility to IBD (59). Given these considerations and the studies reported herein, our data further substantiate the hypothesis that IBD-associated diarrhea manifests as a result of the coordinated downregulation of multiple Na^+ transporters and related regulatory proteins, including NHE3 and CLC-5.

Our studies herein also ascertained that the exacerbated colitis from the loss of Clc-5 was influenced by specific immune-mediated dysregulation, implying the immunopathogenic role of downregulated CLC-5 in UC. The activation of the innate immune system has been shown to provide the source of cytokines, which includes IL-12, that then trigger the adaptive $CD4^+$ T cellular and humoral immune response in IBD (2). Costimulated and activated T cells then secrete a distinct set of cytokines that perpetuate the disease process. In UC, there are specific mucosal-damage pathways characterized by dysregulated cytokine profiles at different stages of the disease process (2). We have previously shown that acute experimental DSS-induced colitis was represented by a distinct set of Th1–Th17 cytokine profiles that involved altered expression of TNF-α, IL-6, IL-17, and KC (6). Our current studies of experimental colitis using *Clcn5* KO mice demonstrated a further exacerbation of the Th1–Th17 immune response, characterized by elevated levels of TNF-α, IL-6, and IL-17, suggesting the immune-mediated pathophysiological role of downregulated CLC-5 in UC.

The inflammatory response to an antigenic stimulus in UC is primarily manifested by the recruitment of distinct chemokines characteristic for the activation that function as critical players in the regulation of the immune response (6, 60). Our profiles identified a significant positive cluster among KC, MIP-1β, and MCP-1 as active chemokines in the signaling

network of the acute experimental colitis in *Clcn5* KO mice. It is likely that these distinct chemokines drive the initial acute innate immune response, which is consistent with the finding that MPO levels, a marker of innate neutrophil activity, were significantly elevated in the colonic tissues of acute experimental colitis in *Clcn5* KO mice. Chemokines involved in the recruitment of $CD4^+$ T cells are also expressed in colonic epithelial cells, and it has been shown that in patients with UC colonic enterocytes are the major source for neutrophil-directing chemokines, such as MCP-1 (61, 62), which is in agreement with our observations.

The mechanisms of the innate–adaptive interface in IBD have been well-demonstrated to primarily involve the TLR pathway, which generates inflammatory factors modulated by the NF-κB pathway (63). This is initiated by the stimulation of intermediate kinases, leading to phosphorylation of the inhibitor of B kinase and subsequent release of NF-κB, which then translocates to the nucleus, where it activates transcription of proinflammatory genes (63). Our studies in experimental colitis using *Clcn5* KO mice also show increased activation of phospho-IκB and increased dissociation of NF-κB, suggesting the role of the TLR–NF-κB pathway mediated inflammatory mechanisms in the acute experimental colitis of *Clcn5* KO mice.

IL-6 plays an important role in differentiation and growth of hematopoietic progenitor cells and lymphocytes and in the generation of the Th17 immune population (64). IL-6 contributes to increased T cell survival, which then accumulates in the lamina propria, leading to perpetuation of inflammation (65). Elevated proinflammatory levels of IL-6 have been identified in several chronic inflammatory conditions, including UC (1, 64), which is consistent with the increased systemic levels of IL-6 in acute experimental colitis of *Clcn5* KO mice. Interestingly, we identified elevated baseline systemic levels of IL-6 in *Clcn5* KO mice when compared with those in WT mice. We also demonstrated that *Clcn5* KO mice exhibit elevated basal activation of IκB in colon, indicating the possibility that increased IκB and IL-6 activation at basal conditions may sensitize the *Clcn5* KO mice to DSS-induced colitis. Further examination also identified elevated levels of IL-6 in proximal tubules, suggesting the likelihood that IL-6 may function as a proinflammatory mediator in *Clcn5* KO mice. IL-6 has been previously demonstrated to enhance osteoclastogenesis by the induction of the NF-κB ligand in osteoblastic cells (66) and has also shown to be the central pathogenic player in arthritis. Given the multifacted role of IL-6, our findings therefore suggest that the increased production of IL-6 under basal conditions in the kidney may explain the development of both hypercalciuria and nephrolithiasis in *Clcn5* KO mice, although a direct correlation has not been established (67, 68). Moreover, elevated IL-6 is involved in defective bone mineralization, which may also explain the sporadic presentation of rickets/osteomalacia in patients with Dent disease (27, 51, 69–71).

Why does the lack of Clc-5 expression lead to alteration of both basal and DSS-induced immunological responses? Our preliminary study demonstrates that Clc-5, a Cl^-/H^+ exchanger previously known to be expressed only in renal and intestinal epithelial cells (20, 28–30), is highly expressed in macrophages. This finding implicates an interesting possibility that Clc-5 may play an important and previously unrecognized role in regulating innate immunity. It is therefore necessary to further study this potentially novel function of Clc-5 in macrophages.

Diet is also suspected to influence the severity of IBD symptoms by influencing the microbial flora and directly modulating the mucosal immune response of the host(72,73). We observed attenuation of DSS colitis in *Clcn5* KO mice maintained on a separate diet (Z-diet) containing high vitamin D, vitamin B_{12}, and selenium (and low iodine, vitamin K_3, vitamin B_2, vitamin B_6, and vitamin B_7, among others), when compared with those maintained on a regular diet (H-diet). Because this was specifically not observed in the WT mice fed with the Z-diet, our data suggest that genetic predisposition appears to provide the substrate for such effects, implying the potential of a double-hit mechanism in the immunopathogenesis of UC, a disease contributed by both genetic and environmental factors. This therefore implies that diet can impact the severity of UC symptoms, particularly in patients genetically predisposed to either mutations or downregulation of CLC5. Among the dietary factors that may be involved in modulation of DSS colitis, we identified vitamin D as a key factor contributing to the protective effect of DSS-induced colitis on *Clcn5* KO mice. vitamin D, recognized for a long time to play a significant immunomodulatory role, exerts a marked inhibitory effect on both adaptive and innate immune systems (74). Vitamin D has been reported to play a role in preventing or ameliorating IBD in the colitis model of IL-10 KO mice (53, 75). Furthermore, it was demonstrated that vitamin D receptor-deficient mice were more sensitive to DSS-induced colitis, implicating a beneficial effect of dietary vitamin D in the therapy of colitis (76). Our present data provide additional strong evidence that supports a protective role of vitamin D in IBD, particularly in genetically or immunologically compromised subjects.

Because the recurrent manifestation of diarrhea can have a significant impact on fluid and electrolyte status and given the varied pathophysiologic mechanisms of diarrhea in IBD and the heterogeneous nature of the disease, individually tailored treatments are essential for effective management (10, 53). Our studies demonstrate that the loss of Clc-5 both 1) exhibits IL-6–mediated immunopathogenesis and 2) significantly exacerbates DSS-induced colitis mediated by NF-κB-modulated Th1–Th17 immune dysregulation, which is influenced by dietary factors, particularly dietary vitamin D. These studies imply the role of downregulated CLC-5 in the immunopathogenesis of UC with a better understanding of the molecular causes for UC-associated diarrhea and provide rational strategies to pursue new therapeutic targets for the management of diarrhea in UC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

CD	Crohn's disease
DAI	disease activity index
DSS	dextran sulfate sodium
e	epithelial disruption
HAI	histological activity index
H-diet	Harlan Teklad Diet
i	inflammatory infiltrate
IBD	inflammatory bowel disease
KC	keratinocyte-derived chemokine
KO	knockout
m	lamina muscularis mucosae
MDS	multidimensional scaling
MPO	myeloperoxidase
NHE	Na^+/H^+ exchanger
Npt2a	Na/phosphate cotransporter 2a
pAb	polyclonal Ab
s	submucosal edema
UC	ulcerative colitis
Vit D-enriched H-diet	vitamin D-supplemented H-diet
WT	wild-type
Z-diet	NIH-31 modified open formula diet

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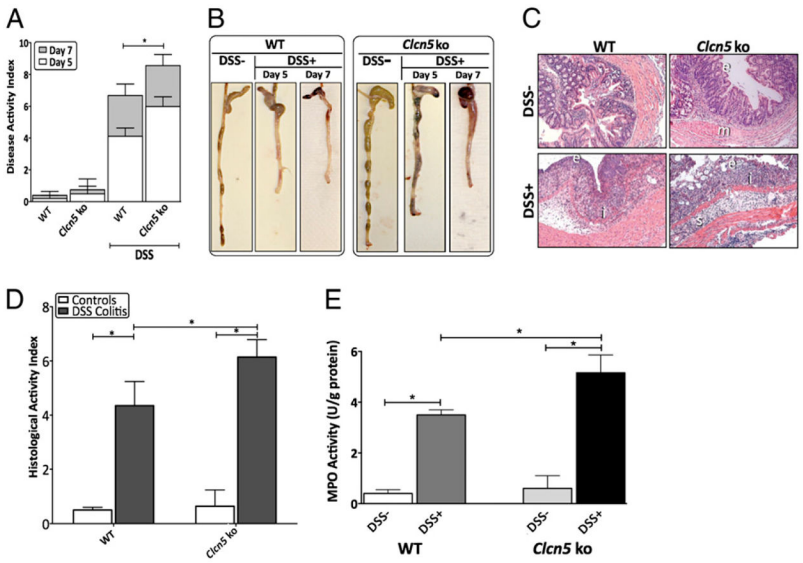

FIGURE 1.

Administration of DSS demonstrated increased susceptibility to experimental colitis in *Clcn5* KO mice. Results presented are representative of seven mice from each group. *A*, DAI was scored from WT and *Clcn5* KO mice for weight loss, stool consistency, and bleeding. *B*, Clinical assessment of acute DSS colitis in WT and *Clcn5* KO mice. *C*, Histological analysis of acute DSS colitis in WT and *Clcn5* KO mice by H&E-stained colonic sections (original magnification ×20). *D*, For detailed histological analysis, colonic sections of WT and *Clcn5* KO mice were scored in a blinded fashion as described in *Materials and Methods. E*, MPO activities in colon from WT and *Clcn5* KO mice were determined as described in *Materials and Methods. Clcn5* KO mice induced with colitis demonstrated statistically significant elevations in DAI, HAI, and MPO relative to those of WT mice, demonstrating increased susceptibility to acute experimental colitis. *$p < 0.05$. e, epithelial disruption; i, inflammatory infiltrate; m, lamina muscularis mucosae; s, submucosal edema.

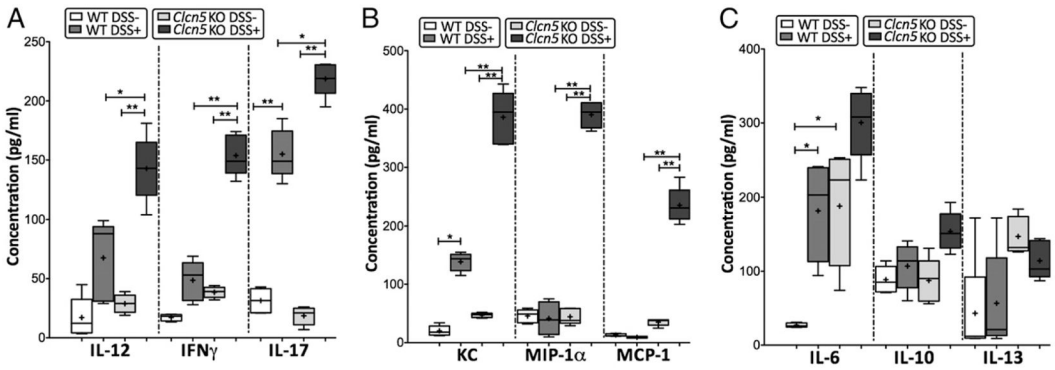

FIGURE 2.

Distinct cellular cytotoxic and chemotactic patterns identified in *Clcn5* KO mice induced with acute experimental colitis. Levels of 16 cytokines were measured simultaneously using a biometric multiplex assay from serum of WT and *Clcn5* KO mice with and without acute DSS colitis. Data is presented with mean (+), median (−), and upper and lower quartile values. *$p < 0.05$; **$p < 0.001$. *A* and *C*, Cytokine pattern in acute colitis of *Clcn5* KO mice is represented by a Th1–Th17-polarized (IL-17, IL-12, and IFN-γ) and a strong chemotactic pattern (MIP-1α, KC, and MCP-1) when compared with that of acute colitis in WT mice. *B*, No significant differences were observed in levels of humoral cytokines and other cellular cytokines and chemokines between WT and *Clcn5* KO mice. Of particular significance are the statistically increased levels of IL-6 in untreated *Clcn5* KO mice. At least seven mice were used in each group.

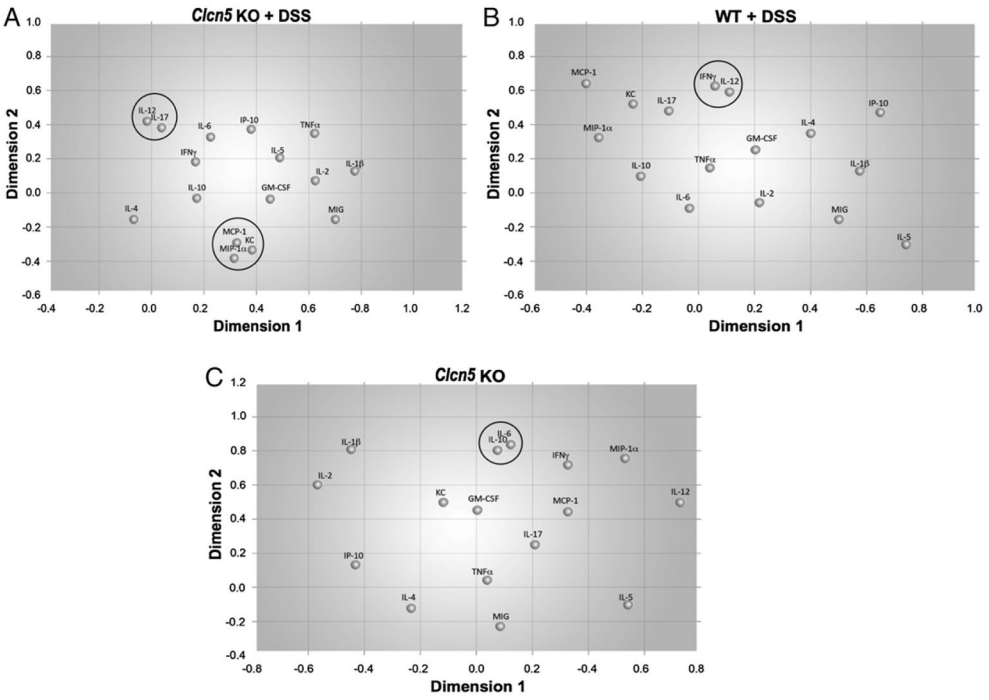

FIGURE 3.

Unique categorical cytokine networks identified in *Clcn5* KO mice induced with acute experimental DSS colitis. MDS analysis was used to generate dimensions that can interpret statistically significant differences between cytokine networks in DSS-induced WT and *Clcn5* KO mice. *A*, Two strong positive clusters, 1) between IL-12 and IL-17 and 2) among MPO, KC, MIP-1β, and MCP-1, were identified in DSS-induced *Clcn5* KO mice, when compared with those in DSS-induced WT mice (marked by circles). *B*, DSS-induced WT mice showed a strong positive correlation between IL-12 and IFN-γ when compared with those in DSS-induced *Clcn5* KO mice. *C*, Interestingly, uninduced *Clcn5* KO mice showed a strong positive correlation between IL-6 and IL-10 ($r = 0.722$, $p = 0.039$), which was absent among all of the other groups studied. These unique representations provide a visual inspection of similarities and differences between cytokine changes among the group, indicating the intricate but distinct immune network in *Clcn5* KO mice.

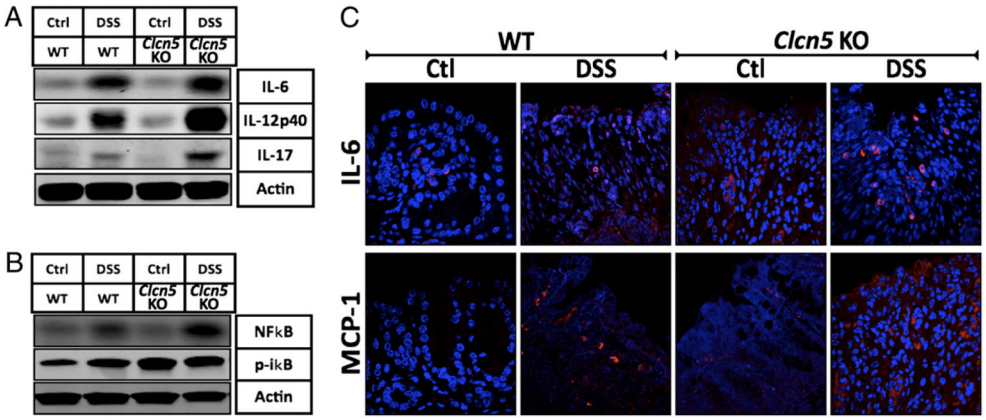

FIGURE 4.

Correlation of cytokine profiles in colon with that of systemic levels in *Clcn5* KO mice. Western blot and immunofluorescence analyses of cytokines from mucosal scrapings and tissue of colon in DSS-induced and uninduced WT and *Clcn5* KO mice. *A*, WB show significantly higher IL-6, IL-12 p40/70, and IL-17 protein expression in acute DSS-induced *Clcn5* KO colon when compared with that in WT DSS-induced colon. *B*, WB show significantly higher levels of colonic NF-κB and phospho-IκB protein expression in acute DSS-induced *Clcn5* KO colitis when compared with those in WT DSS-induced colitis. *C*, IF shows higher IL-6 and MCP-1 protein in lamina propria of acute DSS-induced *Clcn5* KO colitis when compared with those in WT DSS-induced colitis. These data validate the observed systemic cytokine profiles with those of local levels seen within tissue. Representatives of at least three independent experiments are shown in *A* and *B*.

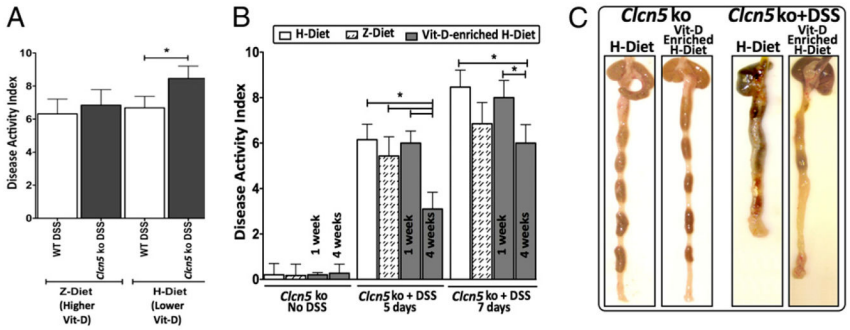

FIGURE 5.

Protective effect of vitamin D supplement on DSS-induced colitis in *Clcn5* KO mice. *A*, *Clcn5* KO mice maintained on the H-diet exhibited a significant increase in DSS-induced colitis, as demonstrated by DAI scores, when compared with *Clcn5* KO DSS-induced mice on the Z-diet ($p = 0.047$). There is no significant difference in colitis DAI and MPO scores of DSS-induced WT mice between the mice fed with the H-diet and the mice fed with the Z-diet. *Clcn5* KO mice (6–7 wk old) were fed either continuously on regular H-diet or on Vit D (4.18 IU/g)-enriched H-diet for 1 or 4 wk. A total of 2.5% DSS was given in drinking water for 7 d to induce colitis. Disease activity was monitored daily by DAI as described in Fig. 1. *B*, DAI score 5 and 7 d after DSS nduction: Mice fed on Vit D-enriched H-diet exhibited significantly less disease activity than those fed on regular H-diet, whereas 1 wk Vit D-enriched H-diet improved DSS colitis only marginally. *C*, Morphology of the colons in *Clcn5* KO mice treated with DSS for 7 d: mice on regular H-diet show severe colitis characterized by shortened colon and dark-colored blood in the stool throughout the colon and cecum when compared with that of control (no DSS). Furthermore, mice on Vit D-enriched H-diet for 4 wk exhibited marked improvement of colitis when compared with those on regular H-diet alone, including normal appearance of stool pellets in the colon (although less stool pellets in general), much less blood in the colon, and less colon shortening. *$p < 0.05$.