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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*

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 disease (3–6). 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 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 clcn5 KO mice suggest 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–mediated Th1–Th17 immune dysregulation, implying a role for Clc-5 in the immunopathogenesis of UC. *The Journal of Immunology,* 2010, 184: 3988–3996.

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 Clcn5, NHE1.3 (but not NHE2), epithelial Na⁺ channel, Na⁺/K⁺ ATPase, and Na⁺/H⁺ exchanger regulatory factor 1 (19).

The chloride channel Clcn5 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

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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; ILC, inducible lineage-committed; KO, knockout; m, lamina muscularis mucosae; MDS, multidimensional scaling; MPO, myeloperoxidase; NHE1,3 (but not NHE2), epithelial Na⁺ channel, Na⁺/K⁺ ATPase, and Na⁺/H⁺ exchanger regulatory factor 1 (19).

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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.
protein CLC-5, encoded by the CLCN5 gene is a voltage-de-
dependent Cl−/H+ exchanger (20, 26). Mutations in the CLCN5
gene are associated with X-linked renal tubulopathy of Dent dis-
 ease, with functional defects in both patients and mouse that are
characterized by low-m.w. proteinuria, aminoaciduria, glycosuria,
phosphaturia, hypercalciuria, nephrolithiasis, and progressive re-
nal failure (27–35). In general, CLC channels have been demon-
strated 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 have
recently demonstrated that diarrhea was associated with significant
increase 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) are increased, whereas in chronic co-
litis 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 sulfonic 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 dy-
namic system in which cellular and humoral cytokines, chemok-
ines, 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 sus-
ceptibility 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 An-
imal 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 (D-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 = 7 mice in
each group) with 2.5% (w/v) DSS (molecular mass 40 kDa; ICN Bio-
chemicals, 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 de-
scribed previously (6). At day 7 following induction with DSS, animals were
sacrificed by CO2 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 immunohisto-
chemistry were fixed in 10% normal buffered formalin as described pre-
viously (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. (36), and histological ac-
tivity 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

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 ex-
periments. Frozen tissue samples were homogenized in homogenization
buffer (50 mM Tris-HCl [pH 7.2]) containing Na2VO4 and a protease
inhibitor mixture (Sigma-Aldrich) using an Omni TH homogenizer
(Omni International, Marietta, GA). Following sonication, the homoge-
nate was centrifuged at 2000 × g for 10 min. Supernatants were collected
to as total mucosal proteins, and protein concentrations were measured
using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Protein ex-
traction, 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
vials without additive. Blood samples were allowed to clot at room tem-
perature 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 included IL-2, IL-4, IL-5, IL-6, IL-10, IL-12,
IL-17, IFN-γ, TNF-α, GM-CSF, interferon-inducible protein 10, ker-
atinocyte-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
elicited. After 3 d, peritoneal cells were recovered by washing

Immunocytochemistry

Immunocytochemical detection was performed in 4-μm paraffin-embed-
ded 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) was performed using the Student t test, and descriptive results are presented as mean ± SD. p values <0.05 were considered statistically significant.

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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. 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 (versely) by a stress function defined by $\Phi = \sum (d_{ij} - f(d_{ij}))^2$, where $d_{ij}$ is the Euclidean distance and $f(d_{ij})$ 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. 1A). 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. 1B). 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. 1C). 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. 1D). 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 uninuded 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. 2A, 2B). 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. 2C), 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. 3A). 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. 3A). 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. 3B). 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. 3C). 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. 4A, 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-kB–mediated immunomodulatory profile. DSS-induced Clcn5 KO colitis had significantly higher NF-kB and phospho-IkB protein expression in the colon than that in WT DSS-induced colitis, suggesting the role of the NF-kB proinflammatory pathway in the distinct immune regulation associated with Clc-5 expression (Fig. 4B). Intriguingly, the baseline levels of phospho-IkB (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. 4C, 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 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.
Fig. 1A, 1B). 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 \textit{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 \textit{Clcn5} KO mice 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-\gamma when compared with those in DSS-induced \textit{Clcn5} KO mice. C, Interestingly, uninduced \textit{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 \textit{Clcn5} KO mice.

**FIGURE 3.** Unique categorical cytokine networks identified in \textit{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 \textit{Clcn5} KO mice. A, Two strong positive clusters, 1) between IL-12 and IL-17 and 2) among MPO, KC, MIP-1\beta, and MCP-1, were identified in DSS-induced \textit{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-\gamma when compared with those in DSS-induced \textit{Clcn5} KO mice. C, Interestingly, uninduced \textit{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 \textit{Clcn5} KO mice.

**FIGURE 4.** Correlation of cytokine profiles in colon with that of systemic levels in \textit{Clcn5} KO mice. Western blot and immunofluorescence analyses of cytokines from mucosal scrapings and tissue of colon in DSS-induced and uninduced WT and \textit{Clcn5} KO mice. A, WB show significantly higher IL-6, IL-12 p40/70, and IL-17 protein expression in acute DSS-induced \textit{Clcn5} KO colon when compared with that in WT DSS-induced colon. B, WB show significantly higher levels of colonic NF-\kappaB and phospho-I\kappaB protein expression in acute DSS-induced \textit{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 \textit{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.
Clen5 KO mice relative to that in WT mice, suggesting the novel finding that IL-6 may contribute to an immunopathogenic role (Supplemental Fig. 1A). 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. 1B). 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.

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 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 Clcn5 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 Clcn5 function. Clcn5 is an intracellular 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 Clcn5 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 Clcn5 may be involved in Nhe3 trafficking. Lack of Clcn5 causes alkalization of the early and perhaps recycling endosomes (56) but not late endosomes. Because Clcn5 acts to provide the neutralizing negative charge for the proton moved by the H-ATPase that acidifies this compartment, the lack of Clcn5 increases the pH in the endosomal compartment. The lack of Clcn5, 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 Clcn5, 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 the disease process. We have previously shown that acute experimental DSS-induced colitis was represented by a distinct set of CLC-5 KO mice. Further examination also identified levels of IL-6 in proximal tubules, 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 NF-κB 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 baseline 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 osteoelastic 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 multifaceted 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 hypercalcemia and nephropathiasis 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 B12, and selenium (and low iodine, vitamin K1, vitamin B2, vitamin B6, and vitamin B7, 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 down-regulation 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 pathophysiological 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 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.

References
gene which is expressed in kidney and is a candidate for Dent’s disease (an X-linked hereditary nephrolithiasis). Hum. Mol. Genet. 3: 2053–2059.
SUPPLEMENTAL DATA

Fig. S1. Clc-5 is highly expressed in the macrophages and weakly expressed in the colonic mucosa of WT mice, but not in peripheral blood monocytes (PBMCs). Peritoneal macrophages (MΦ) and peripheral blood monocytes (PBMCs), were isolated from both WT and Clcn5 KO mice (6-8 weeks old). Equal amount of proteins (60 μg per lane) from purified macrophages and PBMCs were separated by SDS-PAGE and blotted for Clc-5 using anti-Clc-5 polyclonal antibody (1:1000 dilution) (A). Lane 1, PBMCs isolated from WT mice; lane 2, empty lane (no sample was loaded); lane 3, MΦ isolated from WT mice; lanes 4, 5, 10, and 11, colonic mucosa isolated from WT mice; lanes 6 & 7, colonic mucosa isolated from Clcn5 KO mice (a negative control, in which no Clc-5 is expressed); lanes 8 & 9, total extracts from kidney cortex of WT mice (as positive controls for Clc-5). Clc-5 was highly expressed in the macrophages (compare lane 3 with lanes 8 & 9; ~1/3 of that in kidney), but not in PBMCs (lane 1). Colonic mucosa of WT mice express a low level of Clc-5 expression, which is more visible after prolonged exposure [compare lanes 10 & 11 (long exposure) to lanes 4 & 5 (short exposure) of the same blot]. The same blot was also probed for actin as a sample loading control (B). Representative of at least three independent experiments was shown.

Fig. S2. Elevated levels of IL-6 protein expression were observed in the kidney, but not the colon, of Clcn5 KO mice. Protein expression of IL-6 in the kidney cortex of Clcn5 KO and WT mice was analyzed by SDS-PAGE/Western blot and immunofluorescence, as
described in Fig. S2. (A) Significant increase in IL-6 protein expression in the kidney cortex of Clcn5 KO relative to WT mice, suggesting the novel finding that IL-6 may contribute to an immunopathogenic role. Statistical analysis of the expression of IL-6 by normalization to Actin is also shown. (B) Immunofluorescence analysis of expression and localization of renal IL-6 expression using frozen sections of kidney cortex of WT and Clcn5 KO mice. IL-6 was significantly elevated in the kidney cortex of Clcn5 KO mice compared to WT. In WT kidney, 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, upregulation in the interstitium, surrounding renal tubules, as well as in epithelial cells of the proximal tubules, which was not observed in WT kidney sections. Representatives of at least 3 independent experiments are shown in A and B. Statistical significance is denoted by ** for p values less than 0.001.

Fig S3. Major differences in Z-diet when compared to the H-diet. A group of mice were subjected to a separate NIH-31 Modified Open Formula Diet, denoted Z-diet from birth to evaluate the influence of diet on the development of experiment colitis. As shown, this diet contains greater amounts of Vit-D, Vit B12, selenium, and choline and lower amounts of Iodine, Vit-K3, Inositol, Pantothenic Acid, Vit B2, Vit B6, Vit B7 and Vit E (among others) when compared to the H-Diet (Harlan Teklad Diet), which is the regular diet used at our mouse facility.
Fig S1

(A) Renal

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- IL-6
- Actin

![Graph showing bar chart with WT and Clcn5 KO groups for Renal, with IL-6 and Actin levels, and a statistically significant difference indicated by **.](image)

(B) Renal

WT vs Clcn5 KO

![Images showing immunofluorescence staining for Renal tissue in WT and Clcn5 KO groups, with IL-6 and Actin labeled in red and blue respectively.](image)
Fig S2

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Actin

Long exposure

Clcn5
Fig S3

Differences in Z-Diet when compared to H-Diet

**Increases in Z-Diet relative to H-Diet**

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**Decreases in Z-Diet relative to H-Diet**

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