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J Immunol published online 24 February 2010
http://www.jimmunol.org/content/early/2010/02/24/jimmunol.0901657

Supplementary Material http://www.jimmunol.org/content/suppl/2010/02/24/jimmunol.0901657.DC1

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

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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 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, 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 CLC N5 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 recently
demonstrated that diarrhea was associated with significant eleva-
tion 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 co-
ilitis models IL-6 and IFN-γ (but not IL-12 p40/p70 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, chemo-
kines, 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 suscep-
tibility 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-IskB, and 3) is
influenced by dietary factors, such as vitamin D and NF-kB-
mediated distinct Th1–Th17 immune dysregulation, implying the
role of downregulated CLC-5 in the immunopathogenesis of UC.

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
described previously (6). At day 7 following induction with DSS, animals were
sacrificed by CO₂ inhalation, rapidly dissected, and the entire colon was
easily removed. Sections (4 μm) of the colon were stained with H&E (Richard
Allen Scientific, Kalama zoo, MI), histological scores were blindly determined
with minor modifications from Obermeier et al. (38), and histological ac-
tivity index (HAI) was calculated as described previously (6). The activity of
the enzyme myeloperoxidase (MPO), a marker of polymorphonuclear neu-
rophil primary granules, was also determined in colonic mucosa as de-
scribed 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 ex-
periments. Frozen tissue samples were homogenized in homogenization
buffer (50 mM Tris-HCl [pH 7.2]) containing Na₂VO₃ and a protease-
inhibitor mixture (Sigma-Aldrich) using an Omni TH homogenizer
(Chrontech, Santa Cruz, CA), IL-12 p40/p70 pAb (BioSource International, Camarillo,
CA), phospho-IskB pAb (Abcam, Cambridge, MA), and actin pAb
(Sigma-Aldrich, St. Louis, MO). Clc-5 pAb was obtained as previously
described (35).

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 (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-kB polyclonal Abs (pAbs) (Santa Cruz Biotechnology, Santa
Cruz, CA), IL-12 p40/p70 pAb (BioSource International, Camarillo,
CA), phospho-IskB pAb (Abcam, Cambridge, MA), and actin pAb
(Sigma-Aldrich, St. Louis, MO). Clc-5 pAb was obtained as previously
described (35).

Isolation of peritoneal macrophages and PBMCs
Peritoneal macrophages were prepared as previously described (42). Briefly,
2 ml thioglycolate 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 adherence 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 twice with HBSS. PBMCs were recovered by centrifugation
at 1500 rpm for 10 min.

Immunocytochemistry
Immunocytochemical detection was determined 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) 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 \( \Phi = \sum (d_{ij} - f(\delta_{ij}))^2 \), where \( d_{ij} \) is the Euclidean distance and \( f(\delta_{ij}) \) 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 DSS treatment (Fig. 1A). Acute DSS-induced colitis of 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 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. 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-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. 4B). 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. 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.
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.

**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.
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 Deng disease.

Clec-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 Clec-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, Clec-5 was highly expressed in the macrophages (comparable to that in kidney), but not in PBMCs (Supplemental Fig. 2). Low levels of Clec-5 expression were also observed in the colonic mucosa of WT mice. These data provide the first evidence that Clec-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 factors identified dietary patterns that were associated with the etiology of UC, we performed a case-control study to evaluate the etiological role of dietary factors in UC identified dietary patterns that were associated with the etiology of UC. In so doing, we present here the first evidence that Clec-5 may be expressed in immune cells.

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 in several Na"+ transporters in sigmoid mucosal biopsies of patients with active IB 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 Deng 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 Clec-5 would induce susceptibility to UC. In so doing, we present here the first evidence that the loss of Clec-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 Clec-5 function. Clec-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 Clec-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 Clec-5 may be involved in Nhe3 trafficking. Lack of Clec-5 causes alkalization of the early and perhaps recycling endosomes (56) but not late endosomes. Because Clec-5 acts to provide the neutralizing negative charge for the proton moved by the H-ATPase that acidifies this compartment, the lack of Clec-5 increases the pH in the endosomal compartment. The lack of Clec-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 Clec-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 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 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 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 hypocalcemia 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 implicate 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 immune response of the host (72, 73). We observed attenuation of DSS colitis in genetically or immunologically compromised subjects. 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 immunoregulatory 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-kB-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.

Disclosures

The authors have no financial conflicts of interest.

References


