Slc26a3 deletion alters pH-microclimate, mucin biosynthesis, microbiome composition and increases the TNFα expression in murine colon

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Abstract

Aim: SLC26A3 (DRA) mediates the absorption of luminal Cl− in exchange for HCO3− in the distal intestine. Its expression is lost in congenital chloride diarrhoea (CLD) and strongly decreased in the presence of intestinal inflammation. To characterize the consequences of a loss of Slc26a3 beyond disturbed electrolyte transport, colonic mucus synthesis, surface accumulation and composition, pH microclimate, microbiome composition and development of inflammation was studied in slc26a3−/− mice.

Methods: The epithelial surface pH microclimate and the surface mucus accumulation in vivo was assessed by two photon microscopy in exteriorized mid colon of anaesthetized slc26a3−/− and wt littermates. Mucus synthesis, composition and inflammatory markers were studied by qPCR and immunohistochemistry and microbiome composition by 16S rRNA sequencing.

Results: Colonic pH microclimate was significantly more acidic in slc26a3−/− and to a lesser extent in cftr−/− than wt mice. Goblet cell thecae per crypt were decreased in slc26a3−/− and increased in cftr−/− colon. Mucus accumulation in vivo was reduced, but much less so than in cftr−/− colon, which is possibly related to the different colonic fluid balance. Slc26a3−/− colonic luminal microbiome displayed strong decrease in diversity. These alterations preceded and maybe causally related to increased mucosal TNFα mRNA expression levels and leucocyte infiltration in the mid-distal colon of slc26a3−/− but not of cftr−/− mice.

Conclusions: These findings may explain the strong increase in the susceptibility of slc26a3−/− mice to DSS damage, and offer insight into the mechanisms leading to an increased incidence of intestinal inflammation in CLD patients.

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INTRODUCTION

SLC26A3 (also called “downregulated in adenoma,” DRA) is expressed in the apical membrane of the intestinal epithelium with colonic predominance. All known mutations in patients with congenital chloride diarrhoea (CLD) were located in the SLC26A3 gene, which led to the assumption that SLC26A3 may be the ileocolonic Cl−/HCO3− exchanger that had been functionally identified as being defective in CLD. Several years later followed the identification of first the murine and then the human SLC26A3 protein as a Cl−/HCO3− exchanger in heterologous expression systems. Its expression in the intestinal brush border membrane, and the fact that slc26a3−/− mice develop a disease that resembles the human CLD underlined the importance of this transporter in intestinal salt and fluid absorption. The current model based on early isotope flux studies in isolated brush border vesicles, and more recently investigations in Caco2bbe cells, and in knockout mice sees SLC26A3 as an apical Cl−/HCO3− exchanger that results in electroneutral NaCl absorption when operating in parallel with an apical Na+/H+ exchanger. Its loss shifts the balance to less fluid secretion. Studies in cellular expression systems, suggest that SLC26A3 enhances the gating of CFTR. SLC26A3 may be the CFTR-dependent HCO3− export system in the mid-distal colon, an intestinal segment which expresses no other apical Cl−/HCO3− exchanger gene with significant expression levels. Other functions of SLC26A3, for example its role in HCO3− secretion, mucus synthesis and secretion, the maintenance of pH-microclimate, microbiome composition and epithelial barrier properties are not well studied.

The composition, physical properties and function of intestinal mucus has become a major area of research, because a disturbed intestinal mucus layer has proven to be an important causative factor in human intestinal diseases such as inflammatory bowel disease and cystic fibrosis. Colonic mucins are either membrane-bound and form the glycocalyx (Muc/mouse)1,3,4,12,13,16/MUC/human)1,4,12,13,17, or are secreted and form the adherent mucus gel (Muc2/MUC2). The extracellular domains of the membrane bound mucins are also shed into the lumen, and both types of mucin have important protective and regulatory functions. The secretion, release from the membrane, and gel-forming properties of mucins depend on the ambient pH, the HCO3− concentration, and the hydration of the adjacent extracellular space, and in this context SLC26A3 may be an important player for the mucus layer properties in the colon.

SLC26A3 expression is decreased in murine and human inflamed intestinal mucosa, and we previously observed a strong reduction in alkalinisation rates in isolated mid-distal colonic mucosa from both inflamed compared to non-inflamed and in slc26a3−/− compared to wt mice. Disturbances in luminal pH, in mucin synthesis and composition, mucus layer properties and secretory rate, and the luminal microbiome have also been described in patients with inflammatory bowel disease.

The first aim of the present study was an investigation into the consequences of lost Slc26a3 expression on the colonic mucous bicarbonate “barrier,” including the measurement of the colonic surface pH-microclimate and mucus output in vivo, and on the development of spontaneous intestinal inflammation. When we observed the striking goblet cell thecae depletion in the slc26a3−/− colon, we also performed a microbiome analysis at the genera level, to obtain a first glimpse into the potential alterations of the colonic luminal microclimate to explain in part the reduced mucus content and development of mucosal inflammation. In addition, we studied the cftr−/− colon to get more insight into the function of CFTR in colonic mucus synthesis, secretion and accumulation, and in the interrelationship of Slc26a3 and CFTR function in this segment of the gut.

RESULTS

2.1 Weight development, intestinal length and stool water content

Figure 1A shows the weight development of the slc26a3−/− and wt littermates from 4 to 20 weeks of age. In contrast to the observation by Schweinfest et al., no significant weight difference was observed, probably because of the dietary supplement for the slc26a3−/− mice. Our experiments did not permit to excise and weigh the total gut, but the caecum of the slc26a3−/− mice was fluid filled, which may add some weight to the slc26a3−/− mice, obscuring a slight weight deficit in the slc26a3−/− compared to wt mice. For the majority of the data displayed, mice were grouped into three age groups, early (4-9 weeks), mid (10-15 weeks) and late (16-20 weeks). This covers the average healthy wellbeing score of 1-2 life span of the slc26a3−/− mice. With increasing age, the colonic length increased significantly in the slc26a3−/− mice compared to wt, while the small intestinal length did not differ.
**FIGURE 1** Weight development, intestinal length and stool water content in the slc26a3−/− mice and wt littermates. (A) No significant difference in the weight development in the slc26a3−/− mice compared to wt (n = 10 pairs). (B) Unchanged small intestine length. (C) Elongated colonic length. (D) Increased stool water content from early adulthood. *P < .05, ‡P < .001. Age groups: Early 4-9 wk, Mid—10-15 wk, Late—16-20 wk

**FIGURE 2** Strongly altered mucin composition and goblet cell thecae count in slc26a3−/− mid-distal colon from juvenile age. (A) Strong decrease in the staining of neutral mucins. (B,C) Decreased goblet cell (G.C) theca count in the slc26a3−/− crypts. (B) shows representative IHC, (C) shows the number of Muc2+ thecae per crypt (n = 3-5 pairs). *P < .05, †P < .01, ‡P < .001
As described before, stool water content was significantly increased from adolescence (Figure 1D).

2.2 | Mucin expression and composition, and goblet cell thecae count in the slc26a3−/− colonic mucosa

We previously reported a lack of firmly adherent mucus in the mid-distal colon of slc26a3−/− mice.19 To better understand this phenomenon, we searched for differences in mucus staining, mucin granule number, and mucin (Muc1/2/3/4/13) gene expression in slc26a3−/− and wt mice at different ages. The total goblet cell thecae number per crypt was strikingly reduced in slc26a3−/− compared to wt mid-distal colonic mucosa, and the relative staining pattern of acidic to neutral mucins was altered in all age groups (Figure 2A,B). At weaning, Muc1, Muc2, Muc3 (murine analogue of Muc17) and Muc4 mRNA expression was not significantly altered in the mid-distal colon of slc26a3−/− compared to wt mice, but was significantly different at later time points, with Muc2 and Muc3 mRNA expression significantly decreased and Muc1 and Muc4 mRNA expression increased in adult slc26a3−/− compared to wt mid-distal colon (Figure 3A-D). However, expression of Muc13 and trefoil factor 3 (Tff3) mRNA, which is also predominantly synthesized in goblet cells in murine colon,25 was not significantly changed between slc26a3−/− and wt colon (Figure 3E,F).

2.3 | Surface mucus accumulation over time

The assessment of “surface mucus accumulation over time” uses 15 µm size fluorometric beads for an in vivo assessment of mucus output and expansion. Once the beads settle on top of the mucus, the increase in distance between the epithelial surface and the fluorescent beads is assessed over time by two photon microscopy.26 No significant difference was observed in the non-stimulated state, whereas the mucus accumulation was significantly reduced above the slc26a3−/− colonic mucosa compared to the respective wt segment (Figure 4A). Figure 4B-D relate to experiments explained in the next two paragraphs.

2.4 | Surface pH-microclimate in different intestinal segments

The surface (juxtamucosal) pH was determined by overlaying the cecal, proximal and mid-distal colonic exteriorized mucosa with unbuffered saline containing 5 µmol L−1 SNARF-1 with a pH ~ 6.8. The pH was then fluorometrically assessed...
at different time points and at different distances from the epithelial surface. Figure 4C,D displays the hydrogen ion concentrations assessed directly above the epithelium in the slc26a3−/− and (D) cftr−/− mice compared to wt at 30 and 60 min after overlaying with unbuffered saline. (C and D) The bars show the hydrogen ion concentrations [H+] (n = 3-5 pairs/strain). *P < .05, †P < .01, ‡P < .001

FIGURE 5 Surface pH microclimate is Slc26a3—dependent in caecum and mid-distal colon. Fluorometric assessment of the pH microclimate at 5,10 and 15 min after stabilization measured at 200 µm distance from the epithelium above the (A) Caecum (B) mid-distal colon and (C) proximal colon in the wt and slc26a3−/− mice in vivo. The bars show the hydrogen ion concentrations [H+]. (D) pH at different distances from the caecal epithelium after overlay with an unbuffered solution of approximate pH 6.8. (n = 3-5 pairs). *P < .05, †P < .01
2.5 | Surface pH and mucus accumulation in \( cftr^{-/−} \) and \( wt \) in comparison to that in \( slc26a3^{-/−} \) and \( wt \) mid-distal colon

Because the results regarding the surface mucus accumulation were unexpected, given the previously discussed correlation between low surface pH and defective mucus secretion and expansion,\(^{13,28,29}\) we also studied the mucus accumulation in the \( cftr^{-/−} \) mid-distal colon under identical experimental conditions. A dramatic decrease in the rate of mucus accumulation was observed in the \( cftr^{-/−} \) colon compared to \( wt \) littermates (Figure 4B). The surface pH-microclimate, moreover, was also significantly more acidic than \( wt \) pH-microclimate, but less acidic than that above the \( slc26a3^{-/−} \) colon (Figure 4C,D).

The mucus composition and goblet cell theca count was also studied in \( cftr^{-/−} \) mid-distal colon (Figure 6A). Alcian blue/periodic acid-Schiff (AB/PAS) staining showed tightly packed, enlarged mucus thecae and extra-granular mucus attached to the epithelium, in the \( cftr^{-/−} \) mid-distal colonic epithelium. There was also a difference in mucin mRNA expression compared to \( wt \) epithelium, with a significant increase in \( Muc1 \) and unaltered \( Muc2, Muc3, Muc4 \) and \( Muc13 \) (Figure 6B-F). This pattern was completely different from that observed in the \( slc26a3^{-/−} \) colon.

2.6 | Fluid absorptive and secretory capacity of the \( slc26a3^{-/−} \) and \( cftr^{-/−} \) compared to the respective \( wt \) mid-distal colon

Mucus expansion also depends on luminal hydration, and \( Ca^{2+} \) ions can be exchanged for \( Na^{+} \) ions instead of being precipitated by \( HCO_3^- \) during mucin molecule expansion.\(^{30,31}\) We therefore studied the fluid absorptive rates in \( slc26a3^{-/−} \) and \( cftr^{-/−} \) mid-distal colon in vivo and found a significant
reduction in fluid absorptive rate in the \textit{slc26a3}^{−/−}, but not in the \textit{cftr}^{−/−} colon compared to the respective \textit{wt} littermates (Figure 7A,B).

The secretory capacity of the epithelium was assessed by measuring the forskolin (FSK)-induced \( I_{sc} \) increase in the absence of luminal \( \text{Cl}^- \) (to block electrogenic \( \text{Cl}^-/\text{HCO}_3^- \) exchange in the luminal membrane). FSK-induced increase in \( I_{sc} \) was not significantly different between \textit{slc26a3}^{−/−} and \textit{wt} colonic mucosa, while no FSK-induced \( I_{sc} \)-response was elicited in \textit{cftr}^{−/−} colonic mucosa (Figure 7C,D).

2.7 | Colonic microbiome composition in the presence and absence of \textit{Slc26a3} expression

The luminal microbiome was studied in the caecum, the proximal and distal half of the colon of \textit{slc26a3}^{−/−} and \textit{wt} littermates. One set of \textit{wt} littermates were cohoused with the \textit{ko} mice and received the same diet and drinking solution, the other were kept in separate cages and received standard chow and sterilized tap water. This approach was feasible because of an excess mortality of \textit{ko} mice. All mice were sacrificed at 8-9 weeks of age, and faeces was collected immediately from the indicated segments. While the microbiome, analysed at the phyla level by 16S rRNA sequencing, was not significantly different in the \textit{wt} fed with different diets, it was significantly different between the \textit{slc26a3}^{−/−} and cohoused littermates (Figure 8A). Principal coordinates analysis revealed samples clustered by genotype for all intestinal segments. Relative abundance revealed a significant influence of genotype (Figure 9A-C), but not of diet (Figure S1A-C). An increase in the abundance of a number of phyla was observed for \textit{Bacteroidetes}, \textit{Deferribacteres}, and a decrease of others such as \textit{Actinobacteria} and \textit{Firmicutes}, and virtual loss of some phyla, such as \textit{Tenericutes} and \textit{TM7} (Figure 8A). No significant differences were observed in the different colonic segments.

2.8 | Increase in pro-inflammatory cytokine expression in \textit{slc26a3}^{−/−} mid-distal colon

The mRNA expression levels for proximal and mid-distal colon were investigated in adult mice (16-20 weeks). Both \textit{TNFα} and \textit{Il-1β} expression was significantly increased in the mid-distal colon of \textit{slc26a3}^{−/−} mice compared to \textit{wt} (Figure 9A-B).

**FIGURE 7** Almost normal anion secretory, but significantly compromised fluid absorptive capacity in \textit{slc26a3}^{−/−} mid-distal colon, while the opposite is seen in \textit{cftr}^{−/−} mid-distal colon. (A) Fluid absorptive rate in vivo in the mid-distal colon of \textit{slc26a3}^{−/−} mice during perfusion with a \( \text{CO}_2 \) buffered solution of \( \text{pH} \) 7.4. B, fluid absorptive rate in \textit{cftr}^{−/−} mid-distal colon. Differences in the \textit{wt} rates likely due to different mouse strains. Calculated \( I_{sc} \) in (C) \textit{slc26a3}^{−/−} mice, (D) \textit{cftr}^{−/−} mid colonic mucosa before and after the removal of luminal \( \text{Cl}^- \) and before and after the addition of \( 10^{-6} \text{ mol L}^-1 \) forskolin (FSK) to the serosal compartment. FSK-induced \( \Delta I_{sc} \) was almost identical to \textit{wt} in the \textit{slc26a3}^{−/−} colonic mucosa, while it was absent in the \textit{cftr}^{−/−} mucosa (n = 5). \(^{\dagger} P < .01\)
littermates (Figure 10A,B). No increase in pro-inflammatory cytokine levels were observed in the mid-distal colon of cftr−/− mice (Figure 10C,D). The mRNA expression of additional cytokines and lymphocyte marker Cd3e and neutrophil marker Ly6g were measured in all age groups, as shown in Figure 11.

2.9 | Altered mucus barrier precedes the development of intestinal inflammation

In order to understand the causal relationship between the changes in mucus granule depletion and the onset of intestinal inflammation, we studied the mucosal infiltration by inflammatory cells and Lipocalin-2 (Lcn-2) concentration in the stool. Both the expression of the neutrophil markers and pro-inflammatory cytokines, as well as the Lcn-2 concentration in the stool, was not significantly different between slc26a3−/− and cohoused wt littermates at young age. However, all these parameters were significantly increased in slc26a3−/− mice at 16-20 weeks (Figure 11A-D). This suggests that the mucus depletion precedes the development of mucosal inflammation.

3 | DISCUSSION

The present investigation sheds further light on the functional role of colonic Slc26a3, and on the consequences of Slc26a3 dysfunction for colonic epithelial health. Slc26a3 deletion resulted in a sustained decrease in the colonic surface pH microclimate. Both mucus synthesis and the goblet cell theca count was altered, and mucin composition was changed from an early age, with a decrease in the secreted Muc2 and the membrane-resident Muc3, both of which are important for mucosal protection. The microbiome composition showed profound differences between slc26a3−/− and cohoused wt littermates. The changes in mucus composition and abundance, and the altered microbiome, preceded the onset of increased pro-inflammatory cytokine expression in slc26a3−/− colonic mucosa and the increase in the concentration of the inflammatory marker lipocalin in the stool of these mice, and may play a causative role. The results also show that the dramatic alterations seen in mucus homeostasis in the slc26a3−/− colon are of a completely different molecular nature than those observed in the absence of CFTR expression, despite low surface HCO3− availability in both slc26a3−/− and cftr−/− colon. This may be due to the very different colonic fluid dynamics in the two models.

Early studies of patients with CLD suggested a defect in an intestinal Cl− transport mechanism, most likely a Cl−/HCO3− exchanger.32,33 This was confirmed more than two decades later, when all previously observed mutations found in patients with CLD were found in the Slc26a3 gene.1 A Slc26a3 knockout mouse was created, which displayed the key features of human CLD, namely a strong increase in Cl− and water content in the stool. The mice also displayed strongly delayed weight gain, hyperaldosteronism, and colonic crypt hyperplasia.6 After the description of the strong increase in the incidence of both acute and chronic intestinal inflammation in a cohort of CLD patients,34,35 the slc26a3−/− mouse model was studied for potential insights into the pathophysiology of CLD-associated intestinal inflammation, and decreased HCO3− secretory rates, an absent colonic adherent mucus layer, and a strong increase in DSS-induced lethal intestinal damage was reported.19

The current study was aimed at discovering the molecular reasons for the weakened colonic epithelial barrier in the slc26a3−/− colon, and to search for signs of spontaneous colonic inflammation. The study focused on the mid-distal colon, which is the site of highest Slc26a3 expression and function in the murine intestine, but the caecum and proximal colon was also studied for selected questions.18,24,27
In contrast to the first report in the slc26a3−/− strain, the slc26a3−/− mice did not develop a severe weight deficit (Figure 1A), but they did have loose stools from birth, developed colonic elongation, and a high rate of anal prolapse and/or sore tail root, which are typical sequelae of chronic diarrhoea in mice irrespective of the underlying pathomechanism. When these complications developed, the mice were sacrificed. The colonic tissue from mice sacrificed because of genotype-related illness and those sacrificed for experimental reasons were studied independently. However, the spontaneous development of intestinal inflammation was not related to, or secondary to, the development of anal prolapse, but an independent feature of the genotype. (Figure S2A-E).

Because one as yet unexplained feature of our previous study had been the lack of firmly adherent mucus, we further investigated the mucus homoeostasis in the mid-distal colon of the slc26a3−/− and wt littermates. The relationship of acid/neutral mucins as judged by AB/PAS staining was shifted to acidic mucins and the goblet cell theca count was dramatically decreased from early age (Figure 2A,B). A decrease in Muc2 (secretory) and Muc3 (membrane-resident) mRNA was observed, which was significant from juvenile age onward throughout the observation period (Figure 3B,C). At higher age, a significant increase in Muc1 mRNA expression was observed, but the overall expression levels for Muc1 were much lower than for the other two mucin genes. To assess the possibility of a compensatory mechanism by other mucin proteins, we also looked at Muc4 and Muc13, two other cell surface mucins and found that while Muc13 remained unchanged, there was a significant increase in Muc4 at the mid age group (Figure 3D,E). Expression of Tff3 mRNA was not significantly decreased (Figure 3F), suggesting that the defect may not be in a shift of epithelial lineage determination, since Tff3 is also a goblet cell-expressed gene. Possibly, the low surface pH or other luminal factors such as microbial components that have easier access to the epithelial surface because of the lack of firmly adherent mucus cause an accelerated release of the mucus granules from the slc26a3−/− goblet cells, preventing the build-up of large apical thecae, as seen in the cftr−/− goblet cells. An elegant way to differentiate some of the above named causal relationships would be raising the slc26a3−/− mice in a germ-free environment.

Surprisingly, the mucus accumulation above the surface cells in vivo was not significantly slower in the basal state in slc26a3−/− compared to wt mice (Figure 4A). This seemed counterintuitive given the decrease in mucus granule count and Muc2/3 gene expression in the age group in which we studied mucus accumulation (>12 wk/age). However, the mucus accumulation assessment in vivo is a measurement of a layer build-up that is viscous enough to prevent the sinking of 15 µm spheres onto the epithelial surface and, therefore, a rough reflection of mucus secretory rate in vivo, but gives no information about the physiochemical properties of the mucus layer. The height of the accumulated mucus is therefore different from other measurements of colonic mucus layers, such as the firmly adherent mucus layer assessed by microelectrode measurements after gentle suction for loose mucus. However, the height of the accumulated mucus at the beginning of the experiment after gentle suction, and after 30 minutes, observed in a previous report by us using the same technique, was similar to the thickness of adherent mucus layer observed by microelectrode measurements. As detailed below, fluid movements from and into the epithelium are likely additional parameters that determine the dimensions of the mucus layer in vivo. In addition, the lack of perfusion during the observation time will allow mucus hydration and expansion in this experimental model.

As previous investigations highlighted the importance of the luminal HCO₃⁻ availability close to the goblet cells, we also studied the surface pH-microclimate above the

**FIGURE 9** Significant variance in the microbiome between wt and slc26a3−/− mice. Principal coordinate analysis (PCoA) in (A) caecum. Axis 1 explaining 28.9% of the variance; axis 2, 18.8%; axis 3, 17.4%. (B) Proximal colon. Axis 1 explaining 32.6% of the variance; axis 2, 25.0%; axis 3, 16.7% and (C) Distal colon. Axis 1 explaining 25.6% of the variance; axis 2, 19.3%; axis 3, 13.3%. Further results of the microbiome analysis are given in the supplementary files.
**FIGURE 10** Increased mRNA expression of pro-inflammatory cytokines in the *slc26a3*−/− mid-distal colon. Significantly increased (A) TNFα and (B) Il-1β mRNA expression in mid-distal colonic mucosa of adult (16-22 wk of age) *slc26a3*−/− mice and wt littermates. C and D, TNFα and Il-1β mRNA expression in the mid-distal colon of *cftr*−/− and wt mice were not significantly different. Different TNFα mRNA expression in the wt of both strains are likely strain-related. *P < .05

**FIGURE 11** Gradual development of spontaneous inflammation in *slc26a3*−/− mid-distal colon. A, Lipocalin-2 assessment in the stool, (B) mRNA expression of *Ly6g* a marker for neutrophils and granulocytes, (C) *Cld3e* and (D) Il-13 all point to a gradual development of a mild colonic inflammation in full adulthood. *P < .05, †P < .01, ‡P < .001
colonic epithelium of slc26a3−/− and wt mice. The surface pH-microclimate was very acidic in the slc26a3−/− caecum and mid-distal colon, the colonic segments with the highest Slc26a3 expression and function in wt mice. In the murine proximal colon, where Slc26a3 expression and function is low, no difference in surface pH was observed between slc26a3−/− and wt colon.

The same parameters (surface mucus accumulation and surface pH-microclimate) were assessed in the cftr−/− mid-distal colon under identical experimental conditions. The mucus accumulation above the epithelium was dramatically decreased in cftr−/− compared to wt mice, although the surface pH was significantly higher than above the slc26a3−/− epithelium at 60 minutes observation time. As mucus hydration also depends on epithelial fluid balance, we studied the Cl− secretory and the fluid absorptive capacity of the mid-distal colonic epithelium in both mouse models. The Cl− secretory capacity was studied by assessing the FSK-induced ΔIsc calc. in Cl− and HCO3− free luminal bath conditions, to normalize pathological intracellular anion concentrations in cftr−/− epithelial cells, to inhibit the action of potentially electrogenic Slc26a3 transport on transepithelial voltage, and to be able to measure bicarbonate output. Under these experimental circumstances, FSK is able to elicit a robust Isc response in both slc26a3+/+, slc26a3−/− and cftr+/−, but zero Isc response in cftr−/− mid-distal colonic mucosa. This demonstrates that slc26a3−/− colonic mucosa is able to mount a cAMP-dependent fluid secretory response in the crypts, whereas the cftr−/− mucosa is not. In addition, the slc26a3−/− mid-distal colon has a fluid absorptive deficit (Figure 7A), whereas the cftr−/− colon does not. Thus, crypt luminal hydration is severely compromised in cftr−/− colon, but not in slc26a3−/− colon. This may explain the strong difference in mucus accumulation.

The fluid absorptive capacity was significantly reduced in slc26a3+/+ but not in cftr−/− mid-distal colon. In these experiments, we used a CO2-buffered luminal perfusate with a pH of 7.4, in order to allow the mouse to make maximal use of its upregulated colonic NHE3 protein as a mechanism for sodium and fluid absorption. We had previously reported that if perfused with unbuffered saline, the mid-distal colon of anaesthetized slc26a3+/+ mice does not absorb fluid at all, which is likely due to the fact that the perfusate quickly assimilates to the pH of the pH-microclimate, which is inhibitory to NHE3. Indeed, the mid-distal colon was able to absorb fluid in the present study, but the absorptive rate was only approx. 50% of wt rate. Therefore, the slc26a3−/− mid-distal colon is characterized by normal fluid secretory, but reduced absorptive capacity, whereas the cftr−/− mid-distal colon absorbs normally but cannot secrete fluid. Both share a defect in luminal alkalinisation, albeit of different molecular nature. Therefore, the dramatically lower surface mucus accumulation in cftr−/− vs slc26a3−/− colon is likely due to the completely different fluid dynamics in the colonic crypts. It is accompanied with a higher intracellular mucus accumulation in and at the goblet cell thecae in cftr−/− vs slc26a3−/− colon. The mucus secretory defect in cftr−/− goblet cells at the cryptal base has been visualized by time-lapse microscopy, and was associated both with a higher intracellular and a higher mucus granule pH in the goblet cells. Slc26a3−/− deficient enterocytes also have a higher pHi than WT enterocytes, but mucus expulsion in goblet cells in slc26a3−/− crypts have not yet been visualized, and it is not known whether the surface-predominant SLC26A3 is also expressed in goblet cells and what the outcome on mucus secretion may be.

While the slc26a3−/− mid-distal colonic mucosa develops inflammation, the cftr−/− mucosa does not. The absence of an adherent mucus layer and the decreased expression of both secreted and membrane-resistant mucins at early age in the slc26a3−/− but not the cftr−/− colon may therefore be a causative factor in the development of inflammation. Another factor may be an altered microbiome. In the absence of toxic or ischemic damage, the development of inflammation is likely pathogen-dependent. Since “dysbiosis,” defined as microbiome changes in comparison to the normal cohort, is considered an important component in the pathophysiology of intestinal inflammation, we were curious about potential alterations in the microbiome in the slc26a3−/− colon. In order to control for the different diets between the conventionally raised, and mice raised on the special diet that is required to obtain normal weight in slc26a3−/− mice, we cohoused slc26a3−/− and wt littermates fed on the special diet, and raised an additional cohort of littermates from the same litters (who have excess wt mice) on standard chow. Surprisingly, the different diets did not result in significant differences in the cecal and colonic microbiome composition in wt littermates, but the cohoused slc26a3−/− and wt littermates (both on special diet) had very large differences in cecal and colonic microbiome (with no significant differences in the two intestinal segments). A decreased abundance of Firmicutes was observed in the slc26a3−/− colon. Reduced diversity and decrease in Firmicutes is also a signature frequently observed in IBD patients. A decrease in Firmicutes phyla was observed in the nhe3 deficient mice, which developed a spontaneous bacterially-mediated distal colitis. Beyond the reduction in diversity and decrease in the Firmicutes, reports on the microbiome in mouse models and patients with intestinal inflammation are not uniform at this stage. It was not the goal of the present study to establish a causative relationship between the altered microbiome and the inflammatory changes observed in the mid-distal colon of slc26a3−/− mice at this stage, but such studies will hopefully be conducted in the future.

In summary, the absence of Slc26a3 expression results in altered barrier properties of the murine colonic mucosa.
that occur early in life and precede the onset of inflammation. A remarkably low surface pH-microclimate, an altered mucin composition, the previously described lack of adherent mucus layer, and a strongly reduced microbiome diversity may all be causative factors associated with the gradual onset of distal colonic inflammation observed in these mice. It is quite likely that similar alterations are present in the CLD patients’ colon and may be related to their high incidence of colonic inflammation. Further studies are needed to establish both the reasons for the microbiome changes, and their pathophysiological significance.

4 | MATERIALS AND METHODS

4.1 | Animals

Slc26a3−/− mouse generation has been described previously by the group of scientists that made the mice available to us, and were bred and maintained at Hannover Medical School under standard temperature and light conditions, and were allowed free access to food and water. Both the slc26a3+/+(wt) and the slc26a3−/− (ko) mice were routinely fed a special diet (Low-fat, energy-rich, Altromin C0197) along with the normal chow and a half maximal Pedialyte drinking solution (69.38 mmol L−1 Glucose, 17.45 mmol L−1 Sodium Chloride, 1.46 mmol L−1 Sodium Citrate and 3.14 mmol L−1 Potassium Citrate) to prevent dehydration and enable increased survival post weaning. All mice in the experiments were age-matched and used between 4-26 weeks of age. The wild-type and knockout littermates were monitored daily. The cftr−/− strain was generated in the laboratory of Martin Evans. The breeding and maintenance of the cftr−/− and its wt littermates were performed as previously described.

and again, both cftr−/− and wt littermates were fed identical diets and cohoused whenever possible (fighting males were separated). All experiments involving animals were approved by the Hannover Medical School Committee on investigations involving animals and an independent committee assembled by the local authorities. The study and all the methods performed conforms with good publishing practice in physiology.

4.2 | Stool water content

Stool water content was measured weekly. Mice were placed in clean cages with no bedding and closely observed. Pellets or, in the case of the slc26a-l- mice, the pasty stool, were immediately gathered after defecation. The wet weight (W) was obtained by weighing the pellet immediately post collection and were dried overnight at 80°C. The dry weight (D) was then measured and the stool water content was calculated using the following formula, wherein E stands for the weight for the empty tube.

\[
\text{Stool water content (\%)} = \frac{W - D}{W - E} \times 100
\]

Once calculated, the data were represented as an average of total 4 weeks for each time point (4-7 weeks, 8-11 weeks, 12-15 weeks and 16-20 weeks).

4.3 | Histology and immunohistochemistry

Colonic tissues were harvested and fixed in 4% paraformaldehyde. About 3 µm paraffin-embedded sections were stained for the different mucins with AB/PAS or by anti-Muc2 immunohistochemistry as described below. Heat-mediated antigen retrieval was performed at 95°C for 20 minutes on the rehydrated 3 µm paraffin sections using 10 mmol L−2 sodium citrate buffer, pH 6, followed by quenching with 50 mmol L−1 NH4Cl for 15 minutes at room temperature. Unspecific binding was blocked with 4% goat serum for 1 hour at room temperature. The sections were then incubated with Mucin2 (Rabbit polyclonal H-300, Santacruz #sc-15334) the primary antibody overnight at 4°C. Sections were then incubated with the corresponding secondary antibody (Invitrogen #A11008) for 1 hour at room temperature. The nuclei were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride. The immunostained sections were mounted using Fluorogel with Tris Buffer and visualized under the Olympus FluoView™ FV1000 confocal microscope. The goblet cell thecae were counted as the total number of Mucin2+ stained thecae/crypt.

4.4 | Quantitative PCR protocol

Gene expression levels of a variety of pro-inflammatory cytokines and mucins were analysed in the mid-distal colon by qPCR, using ribosomal protein S9 (RPS9) as reference gene. RNA extraction, cDNA transcription and qPCR analysis were performed as per manufacturer’s instructions. Briefly, the total RNA was extracted using RNeasy® Mini Kit (Qiagen GmbH) and the quality was assessed using QIAxcel RNA QC Kit v2.0 (Qiagen GmbH). 1 µg RNA was then reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen GmbH). cDNA was diluted 1:40 with DNase free water and 4 µL of the dilution was used as a template for PCR. Each reaction additionally contained 5 µL 2X qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems) and an appropriate amount of primers (Table S1).
4.5 | Quantification of faecal lipocalin (Lcn-2) by ELISA

Faecal lipocalin levels were measured in the stool samples of the Slc26a3+/+ and Slc26a3−/− mice as described earlier with minor modifications. Briefly, stool samples from Slc26a3+/+ and Slc26a3−/− mice were collected weekly from 5 weeks until 20 weeks of age and were frozen at −80°C until further use. The faecal samples were reconstituted in PBS (100 mg mL−1) and vortexed to get a homogeneous faecal suspension. Lcn-2 levels were measured in the homogeneous suspension using Duoset murine Lcn-2 ELISA kit (R&D Systems #DY1857) as per manufacturer’s instructions.

4.6 | In vivo colonic epithelial surface pH measurement

Measurement of the surface pH microclimate was performed as described previously for the small intestine, with modifications. Briefly, the mid-distal colon of the anaesthetized, blood-gas- and arterial blood pressure-controlled mouse was exteriorized with an intact blood supply and mounted on a custom-made perfusion. After stabilization, the chamber was perfused with unbuffered saline, pH ~ 6.8, containing free 5 µmol L−1 carboxylic acid SNARF-1 (Molecular Probes), and the perfusion was stopped before images were collected. SNARF-1F was excited at 780 nm, and the emission was collected at 580 nm (523-605 nm) and 640 nm (610-700 nm), using a 2-photon laser scanning microscope with an upright Leica TCS SP2 confocal microscope with a 20X water immersion objective and a MaiTai Ti:sapphire-pulsed laser (Spectra-Physics, Darmstadt, Hessen, Germany), 15 µm, Crimson Fluorescent Microspheres (Darmstadt, Hessen, Germany), 15 µm, Crimson Fluorescent Microspheres (Darmstadt, Hessen, Germany), 15 µm, Crimson Fluorescent Microspheres (Darmstadt, Hessen, Germany). The ratio of 640/580 was converted into the pH using an in vitro calibration curve as described before.42,49 The pH measurements were taken every 5 minutes for 1 hour near epithelial surface and at the distance of every 100 µm up to 900 µm above the surface in the mucus layer. The pH values in the pH gradient are represented as proton concentrations [H+] in the bargraphs (Figures 4C,D, 5A-C), and in pH-values in the pH gradient (Figure 5D). The mice were between 13 and 26 weeks of age, because pilot experiments had shown that in particular the slc26a3−/− mice are less stable at younger age during the operative procedure. The older mice had a lower death rate during this procedure, and had a higher frequency of systemic blood pressure in the range of the wt mice during the experiment.

4.7 | In vivo surface mucus layer accumulation

Measurement of the surface mucus layer accumulation was performed as previously described by Singh et al26 Briefly, post-surgery the exposed mucosal surface was covered with 1 mL of unbuffered saline, pH=6.8, after gentle suction of the pre-existing mucus layer. For the measurement of the basal (60 minutes; post-surgery) and forskolin-(FSK) stimulated (80 minutes saline + 30 minutes 100 µmol L−1 FSK) mucus accumulation, the fluorescent polystyrene beads (1.0 × 10^6 beads mL−1; FluoSpheres® Polystyrene Microspheres (Darmstadt, Hessen, Germany), 15 µm, Crimson Fluorescent 625/645 Invitrogen, F-8839) were sprinkled on the top of the saline layer and given five minute time to settle down at the end of the time point. The distance between the beads and the surface of mucosa was measured for each bead in the Z-axis. For each set of the data, 3-5 mice were used and 9-30 beads from each experiment were accessed for the mucus thickness measurement. Further details and optical illustrations of the technique can be found in Singh et al26

4.8 | In vivo mid-distal colonic fluid absorption

The in vivo fluid transport experiments were performed as previously described. Briefly, single pass perfusion of a mid-distal segment was performed in tracheally intubed, isoflurane-anesthetized mouse (1.8%-2.2%), ventilated with a Univentor minivent (TYPE845) which supplied 25% O₂ + 75% air and mixed with 5% isoflurane by an anaesthetic machine. The respiration rate and TV (tidal volume) were regulated depending on the weight of mice according to the recommendation from the Operating Instructions for the Mouse Ventilator MiniVent Type 845. Catheters were placed in the carotis for measuring the blood pressure and infuse Na₂CO₃ to counteract the development of metabolic acidosis during surgery, and in the femoral vein for infusing electrolyte solution to compensate for fluid loss. In the systemically alkalotic slc26a3−/− mice, no base equivalents were infused. After the operation, blood was taken from the carotid catheter to measure hematocrit and perform blood gas analysis. Net colonic fluid movement was assessed gravimetrically as described.

4.9 | Short circuit (Isc) measurements in isolated chambered mid-distal colonic mucosa

Ussing chamber experiments were performed in the open-circuit mode similarly as described previously,18 with intermittent current pulses (200 µA every 60 seconds) to record the electrical resistance (Rt) and to calculate short-circuit current (Isc). In brief, the mid-distal colon of slc26a3−/−, cftr−/− and respective wt mice were stripped off their external serosal muscle layers and mounted in Ussing chambers with an exposed area of 0.625 cm². Neural activity and prostaglandin
generation were blocked with tetrodotoxin (1 µmol L⁻¹, serosal) and indomethacin (3 µmol L⁻¹, serosal). Transepithelial Isc was calculated as [µEq cm⁻² tissue surface area]. The serosal solution contained (in mmol L⁻¹) 108 NaCl, 25 NaHCO₃, 3 KCl, 1.3 MgSO₄, 2 CaCl₂, 2.25 KH₂PO₄, 8.9 glucose, and 10 sodium pyruvate, and was gassed with 95% O₂/5% CO₂ (pH7.4). The mucosal solution (154 mmol L⁻¹ NaCl or 154 mmol L⁻¹ Na-gluconate) was gassed with 100% O₂.

Basal parameters were first measured under a luminal Cl⁻ present condition (ie, 154 mmol L⁻¹ NaCl) for 20 minutes, then luminal Cl⁻ was removed by replacing luminal NaCl with isotonic Na-gluconate solution and the basal bicarbonate secretory rate under Cl⁻ free condition (ie, 154 mmol L⁻¹ Na-gluconate) was measured for 20 minutes. Forskolin was then added to the serosal solution with an end concentration of 10 µmol L⁻¹.

4.10  |  Microbiome sampling and processing

The microbiota of slc26a3+/+ and slc26a3⁻/⁻ caecum, proximal colon and distal colon samples was analysed as described previously, with modifications. Prior to DNA extraction, the bacteria were inactivated at 80°C for 15 minutes in the lysozyme buffer (as described in the QIAamp Mini and Blood Mini Kit protocol (Qiagen). Samples were disrupted using a FastPrep-24 machine (MP Biomedical) and equal amounts of 0.1 and 0.5 mm zirconia beads, with three 40 seconds grinding steps at speed 6.0 with intermittent storage on ice. For enzymatic lysis, samples were incubated with proteinase K (Qiagen) and lysozyme (Sigma) at 37°C overnight, followed by renewed disruption in the FastPrep-24 machine as described above. Post removal of cell debris by centrifugation at 16 060 × g for 2 minutes, DNA was extracted from the supernatant following the QIAamp Mini Tissue protocol (Qiagen) starting at the RNase A step. DNA was eluted from the QIAamp columns with 100 µL AE Buffer.

4.11  |  Partial amplification and deep sequencing of 16S rRNA genes

16S ribosomal DNA gene fragments were PCR-amplified using 454 FLX Titanium fusion primers including the universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 541R (5'-WTTACCGCGGCTGCTGG-3') as template-specific parts. Amplicons were separated from small fragments by gel electrophoresis on 1% agarose gels, and 500-700 bp fragments were extracted using the QIAquick gel extraction kit (Qiagen). Amplicon concentrations were determined using the Quant-iT PicoGreen dsDNA Kit (Invitrogen) on a QuantiFluor single-Tube Fluorometer (Promega). Samples were pooled at 25 ng each to include nine different MID-labelled samples per pool. Pools were further processed following the emPCR Method Manual—Lib-L SV (January 2010 version, modified according to the Long Fragment Protocol TCB No. 2011-001 and the Guidelines for Amplicon Experimental Design as of March 2012, Roche 454), and sequenced from the 3' end on a 454 Genome Sequencer FLX+ (Roche) using Titanium chemistry ( Sequencing Method Manual for GS FLX+ instruments and XLR70 Kits, August 2012 version, Roche 454). Raw data were processed using the GS Run Browser Software 2.8 (Roche).

4.12  |  Sequence processing and bioinformatic analysis

Data analysis were performed as described previously, based on Mothur, version 1.28.0. The detailed procedure is provided in the supplementary methods.

4.13  |  Statistics

Statistical analysis was performed using GraphPad Prism Version 8.00. Unless otherwise indicated, comparisons of two groups which passed the Shapiro-Wilke normality test were compared with the two-tailed Student t test. In case one or both groups did not pass the normality test, analysis was performed using the non-parametric Mann-Whitney U test. For multiple comparisons, a standard two-way ANOVA was used. All results were expressed as the mean ± SEM. Statistical analysis for the microbiome analysis was performed as described in detail in the supplementary methods.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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