Segment-specific impact of TNF-α-induced inflammation on HCO_3^- homeostasis and epithelial barrier function in the murine intestine

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Abstract

Dysregulated epithelial ion transport and impaired epithelial permeability are central to inflammatory bowel disease pathophysiology, but data on the importance of chronic inflammation for Ileocolonic epithelial HCO_3^- output is sparse. We therefore studied the segment-specific impact of TNF-α-induced inflammation on HCO_3^- homeostasis and epithelial barrier function in the murine ileum, proximal and mid colon. The $TNF^{\Delta ARE}$ mouse model displays mild to moderate inflammation in the ileocolon with high a pro-inflammatory cytokine profile, but an intact epithelial lining. Ussing chamber experiments (HCO_3^- output, I_{sc}, ^3H-mannitol fluxes, dilution potentials, impedance spectroscopy), *in vivo* luminal perfusion experiments (HCO_3^- secretion and fluid absorption), classical histology, realtime PCR, western blot, and fluorescence immunohistochemistry were used to examine transepithelial mechanisms (ion exchangers, transporters, and channels involved in HCO_3^- transport) as well as paracellular pathways (tight junction associated proteins).

In the Ileum and mid colon, the high basal HCO_3^- secretion was dependent on luminal Cl^-, and strongly decreased in the inflamed mucosa. Cl^-/HCO_3^- exchanger DRA mRNA and protein were both down-regulated, whereas Cl^- channel CFTR, Na^+/H^+ exchanger NHE3 and epithelial Na^+ channel ENaC mRNA were not significantly changed. This indicates that the observed defect occurs due to downregulation of DRA.

In the proximal colon, DRA and PAT-1 mRNA abundance was similar in inflamed and healthy mice with extremely low levels, which is a precondition to study paracellular permeability. In this segment, where luminal alkalization rates are very low in general, HCO_3^- output was found to be significantly increased in inflamed mice. Additionally, increased permeability together with a loss of cation selectivity was observed. Tight junctional protein expression of claudin-2 and -5 was found to be upregulated while occludin was down-regulated. The observed HCO_3^- loss is likely to occur via leaky tight junctions rather than via HCO_3^- secreting ion transporters.

Taken together, TNF-α-induced inflammation has a segment-specific impact on HCO_3^- homeostasis and epithelial barrier function in the murine ileum, proximal and mid colon, which leads to an altered acid-base balance.

Keywords: intestine, inflammation, bicarbonate

Zusammenfassung

Dysregulierter epithelialer Transport und veränderte epitheliale Permeabilität sind zwei Kennzeichen entzündlicher Darmkrankheiten. Die pathophysiologischen Folgen von chronischer Entzündung auf die epitheliale HCO_3^--Homöostase sind jedoch größtenteils unbekannt. Daher untersuchten wir die segmentspezifische Bedeutung von TNF-α-induzierter Entzündung auf die HCO_3^--Homöostase und die epitheliale Barriere im murinen Ileum, proximalen und mittleren Kolon. Das verwendete Mausmodel $TNF^{\Delta ARE}$ zeigt milde bis moderate Entzündung im Ileokolon mit hohen pro-inflammatorischen Zytokinspiegeln, aber einer intakten Epithelschicht. Um sowohl transepitheliale Mechanismen (Ionen-Austauscher, Transporter und Kanäle die in den HCO_3^--Transport involviert sind) als auch die parazellulären Pfade (Schlussleistenproteine) zu untersuchen, wurden Versuche in der Ussing Kammer (HCO_3^- Transport, Kurzschlussstrom (I_{sc}), ^{3}H-Mannitolfluxe, Dilutionspotentiale, Impedanzspektroskopie), luminale Perfusions-experimente *in vivo* (HCO_3^--Sekretion und Flüssigkeitsabsorption), klassische Histologie, realtime PCR, Westernblot und Fluoreszenz-Immunhistologie durchgeführt.

Im Ileum und mittleren Kolon war die hohe basale HCO_3^- Sekretion abhängig von luminalem Cl^- und in der entzündeten Mukosa stark vermindert. Die mRNA Expression des Cl^-/HCO_3^- Austauschers DRA war signifikant verringert, wohingegen der Cl^- Kanal CFTR, der Na^+/H^+ Austauscher NHE3 und der epitheliale Na^+ Kanal ENaC unverändert exprimiert waren. Das deutet darauf hin, dass der beobachtete Defekt auf die verminderte Expression von DRA zurückzuführen ist.

Im proximalen Kolon war die mRNA von DRA und PAT-1 auf einem extrem niedrigen Level und unverändert zwischen entzündeter und gesunder Mukosa exprimiert, was eine Vorraussetzung für Messungen der parazellulären Permeabilität darstellt. In diesem Segment sind die luminalen Alkalisierungsraten generell sehr niedrig, jedoch wurde eine erhöhte HCO_3^--Abgabe in der entzündeten Maus festgestellt. Zusätzlich wurden eine erhöhte Permeabilität und ein Verlust der Kationenselektivität beobachtet. Die Schlussleistenproteine Claudin-2 und -5 waren hoch-reguliert, während Occludin herunterreguliert war. Der beobachtete HCO_3^--Verlust ist somit über undichte Schlussleisten und nicht über HCO_3^- sezernierende Ionentransporter zu erklären.

Zusammengefasst bewirkt eine TNF-α-induzierte Entzündung segmentspezifische Veränderungen der HCO_3^--Homöostase und Barrierefunktion im murinen Ileum, proximalen und mittleren Kolon, und trägt so zu einem veränderten Säure-Base-Gleichgewicht bei.

Schlagworte: Darm, Entzündung, Bikarbonat

List of Abbreviations

ARE	AU-rich regulatory element
Caco-2 cells	Human colorectal intestinal epithelial cell line
C2BBe1	Human colorectal epithelial cell line, subclone of Caco-2
CA IV	Carbonic anhydrase isoform IV
$CD8^+$ T-lymphocyte	CD8 positive cytotoxic T-lymphocyte, expressing the glycoprotein CD8 on its cells surface
CD	Crohn´s disease
CFTR	Cystic fibrosis transmembrane regulator; Cl^- channel
DRA	Downregulated in adenoma (SLC26a3); Cl^-/HCO_3^- exchanger
ENaC	Epithelial Na^+ channel
H&E	Haematoxylin and Eosin
HCO_3^-	Hydrogen carbonate, bicarbonate
IBD	Inflammatory bowel disease
I_{sc}	Short circuit current
Na^+/K^+ pump	Sodium-potassium adenosine triphosphatase
NBC	Sodium bicarbonate cotransporter
NHE1	Sodium-hydrogen exchanger 1 (SLC9a1)
NHE3	Sodium-hydrogen exchanger 3 (SLC9a3)
NKCC1	Na^+K^+ $2Cl^-$ cotransporter (SLC12a2)
PAT-1	Putative anion transporter 1 (SLC36a1)
SCFAs	Short chain fatty acids
TER	Transepithelial resistance
TJL-scoring system	The Jackson Laboratory scoring system
TNF-α	Tumor necrosis factor-α
UC	Ulcerative colitis

1. General Introduction

Inflammatory bowel disease (IBD), encompassing both Crohn´s disease (CD) and ulcerative colitis (UC), is characterized by chronic and relapsing inflammation of the gastrointestinal tract. Although it is known that genetic predisposition and dysregulated immune response (Kaser et al. 2010), environmental factors such as the gut mictobiota (Hill and Artis, 2010), impaired epithelial barrier properties (Bruewer et al. 2006) and altered epithelial ion transport (Farkas et al. 2011) play major roles in these complex diseases, the pathogenesis is still not fully uncovered. Since luminal pH control is critical for many important epithelial functions, this thesis illuminates the segment-specific impact of TNF-α (Tumor necrosis factor-α)-induced inflammation on HCO_3^- homeostasis and epithelial barrier function in a murine model of IBD.

The gastrointestinal barrier, consisting of epithelial cells and the connecting tight junctions that provide contact between neighbouring cells, separates the body from the luminal environment which is potentially loaded with noxious pathogens and harmful molecules. At the same time, water, nutrients, ions and solutes have to cross the epithelium to maintain homeostasis of the internal milieu. The permeability is mediated via two major routes: the transepithelial/ transcellular pathway which is predominantly regulated by selective transporters, exchangers and channels for specific ions, electrolytes, sugars and short chain fatty acids (SCFAs), and the paracellular pathway, which leads through the space between the cells and is regulated by the tight junctional complex (see figure 1).

1.1 pH /HCO_3^- homeostasis and epithelial ion transport mechanisms

pH homeostasis is one of the major tasks of an organism, since protein and enzyme function, the structure of the cell and the permeability of membranes are challenged by a changing pH. Intestinal surface and luminal pH control, with HCO_3^- being one of the major protagonists (Binder 2005), is critical for many aspects of the epithelial defence and transport function, including the rheological properties of the mucus layer and thus the opportunity for bacterial penetration (Swidsinski et al. 2007) as well as the luminal bacterial composition, colonization and proliferation (Duncan et al. 2009). A large number of transporters, channels and exchangers maintain the required pH in the intestine.

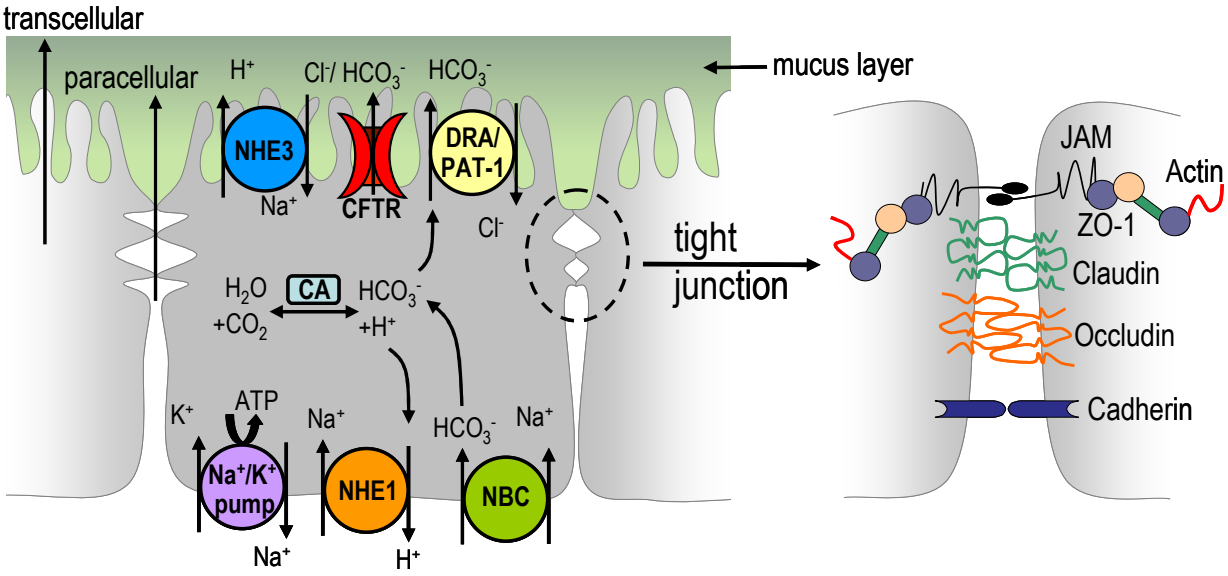

Figure 1: Structure of the ileocolonic epithelial lining with the mucus layer and possible transcellular and paracellular pathways for HCO_3^-. The electrolyte transporters CFTR, NHE3 and DRA are located in the apical membrane, whereas the Na^+/K^+ pump, NHE1 and NBC are found on the basolateral side. They are expressed in different amounts and patterns along the ileocolon. Additionally, HCO_3^- can be generated by carbonic anhydrases (CA, left). The complex structure of tight junctions with some of the composing proteins is schematically shown on the right.

All segments from duodenum to distal colon have varying and specialized mechanisms for both absorbing and secreting acid and base equivalents. Key pathways including the Cl^- channel CFTR, the cation exchanger NHE3 (Na^+/H^+) as well as DRA and PAT-1 (Cl^-/HCO_3^-), the Na^+/K^+ pump which provides the driving force, the Na^+/H^+ exchanger NHE1 and the Na^+/HCO_3^- cotransporter NBC are in charge of the intestinal acid-base balance. These transporters are expressed in a highly segment-specific manner (see figure 1).

HCO_3^- is actively secreted in most parts of the intestine, with the highest secretion found in the duodenum. Here, it is the most important defense mechanism against acidic discharge from the stomach (Flemström et al. 2002). Along the whole intestine, the epithelium is covered with a mucus layer composed of mucins, phospholipids, electrolytes, water and a large amount of HCO_3^- that keeps the immediate vicinity of the surface epithelium at neutral pH (Flemström and Kivilaakso 1983). The mucus layer serves as a protection against potentially infectious agents from the lumen and ensures a smooth passage of digested food. Additionally, Garcia et al. (2009) reported that mucin requires CFTR-dependent HCO_3^--secretion in order to be released and expanded properly. The loss of the intestinal mucus layer is sufficient to lead to injury and barrier dysfunction of the otherwise intact intestine (Sharpe et al. 2010).

In the proximal large intestine, SCFAs are generated mainly from nonabsorbed carbohydrates by colonic bacterial metabolism and form the main luminal anions in colonic fluid (Binder et al. 2005). They provide the main nutrition for enterocytes and are absorbed via nonionic diffusion driven by pH or proton gradient or actively e.g. via a SCFA/HCO_3^- exchanger (Ruppin et al. 1980, Mascolo et al. 1991), which to this date is not molecularly identified (Binder 2010). In this particular segment, the functional expression of DRA is almost negligible, as Talbot and Lytle (2010) showed in the rodent colon. They also correlated mucosal surface pH with the apical expression of DRA and NHE3 along the ileocolon and found a matching low pH in the proximal colon that rose towards the mid colon.

Although evidence for dysregulated electrolyte transport was found some time ago (Harris et a. 1972, Hawker et al. 1980), only more recently the molecular identification of ion transporters helped to understand their complex involvement in inflammation (Nemeth et al. 2002; Seidler et al. 2006; Laubitz et al. 2008). It is now well established that cytokines have a direct impact on the expression and function of several ion transport proteins. In the rat colon, Markossian and Kreydiyyeh (2005) showed that TNF-α inhibited net water and chloride absorption, down-regulated the basolateral $Na^+/K^+/2Cl^-$ cotransporter NKCC1 and reduced the protein expression and activity of the Na^+/K^+ pump. While the epithelial barrier function was not altered, the electrogenic sodium absorption via ENaC was found to be strongly impaired in human macroscopically noninflamed CD colon because of reduced γ-ENaC transcription (Zeissig et al. 2008). Additionally, this group reported that TNF-α exposure to rat distal colon led to the same effect. Farkas et al. 2011 found a moderate dysfunction of NHE3, but a major decrease of the Cl^-/HCO_3^- exchange in biopsies from ulcerative colitis patients, associated with a downregulation of DRA.

1.2 Inflammation-associated epithelial barrier function

Concerning the paracellular pathway, meanwhile more than 40 proteins that are part of or are associated with tight junctions have been identified (Schneeberger and Lynch, 2004). Among them, the family of claudins, named from the Latin word *claudere* for “to close”, have emerged to be the crucial proteins defining the tight junction properties and their selectivity.

Although the group of barrier builders is proportionally bigger including claudin-1, -3, -4, -5, -8, -11, -14 and -19, there are claudins mediating permeability like claudin-2 and -10. Claudin-2

forms paracellular pores which are selective for small cations and water but almost impermeable for anions or uncharged solutes of any size (Amasheh et al. 2002, Rosenthal et al. 2010). While claudin-5 seems to be a sealing claudin without selectivity (Amasheh et al. 2005), claudin-8 has been reported to form a barrier to cations, including protons, ammonium and bicarbonate ions (Yu et al. 2003, Angelow et al. 2006).

Occludin was firstly thought of as not required for the formation of tight junction strands since Saitou et al. (1998) showed that occludin-deficient embryonic stem cells still can differentiate into polarized epithelial cells bearing tight junctions. In occludin knockout mice, the tight junctions themselves did not appear to be affected morphologically, and the barrier function of the intestinal epithelium was normal as far as examined electrophysiologically (Saitou et al. 2000). However, ongoing research revealed an important, yet not fully understood regulatory contribution of occludin concerning barrier permeability. Al-Sadi et al. (2011) showed that occludin depletion in intestinal epithelial cells leads to a selective or preferential increase in macromolecule flux, suggesting that occludin plays a crucial role in the maintenance of tight junction barrier.

This highly selective and thoroughly regulated barrier is known to be weakened or disrupted in pathophysiological situations like inflammation, indicated by an increase in paracellular permeability and a decrease in transcellular electrical resistance. Pro-inflammatory cytokines like TNF-α and interferon-γ are known to contribute to a disturbed barrier function. In cell culture experiments using Caco-2 cells, a human intestinal epithelial cell line, Marano et al. (1998) showed that as little as 5ng/ml TNF-α applied for only 5 minutes was sufficient to reduce the transepithelial resistance (TER) for 40-50% and to decrease the short circuit current (I_{sc}) for 30% after 24 hours.

In animal models of IBD, intestinal tissue expression of occludin was found to be markedly decreased (Fries et al. 1999, Gassler et al. 2001). In patients with mild to moderate Crohn's disease, Zeissig et al. (2007) showed an upregulation of pore-forming claudin-2 and downregulation and redistribution of the sealing claudin-5 and -8 as well as a downregulated expression of occludin. Furthermore, reduced and discontinuous tight junction strands were demonstrated. According to the authors, this leads to a pronounced barrier dysfunction in the inflamed intestine.

1.3 The $TNF^{\Delta ARE}$ mouse model

The mouse model used in this study was originally established in the laboratory of George Kollias (Kontoyiannis et al. 1999). A genetic deletion of the Tumor necrosis factor alpha (TNF-α) AU-rich regulatory element (ARE) leads to an inaccurate posttranscriptional degradation and therefore to an overproduction of TNF-α. This pro-inflammatory cytokine is predominantly produced by macrophages, but also by other cell types like lymphocytes, natural killer cells, myeloid cells, adipocytes and intestinal epithelial cells. Homozygous $TNF^{\Delta ARE/\Delta ARE}$ and heterozygous $TNF^{+/\Delta ARE}$ mice develop a $CD8^+$ T-lymphocyte-dependent chronic inflammatory arthritis and chronic inflammation along the intestine. A dys-/ upregulation of TNF-α was shown to be present in a large number of diseases including Alzheimer's disease, cancer and IBD. Several studies proved a multiple fold higher amount of TNF-α in patients suffering from Ulcerative colitis as well as Crohn´s disease (MacDonald et al. 1990; Segain et al. 2000). The importance of this cytokine is underlined by the therapeutic treatment based on anti-TNF-α antibodies like infliximab or adalimumab as IBD treatments.

To quantify the severity of inflammation in the different intestinal segments of the $TNF^{+/\Delta ARE}$ mouse, a modified TJL-scoring system was used (Bleich et al. 2004). While the small intestine starting from the duodenum is only mildly affected, the Ileum is the most inflamed part followed by the colon from proximal to distal. This mimics Crohn's-like pathology in terms of locality. Additionally, the crypt and villus architecture as well as the epithelial lining seem to be intact judged from histological Haematoxylin and Eosin (H&E) staining. These facts make the $TNF^{\Delta ARE}$ mice an ideal animal model to study ion transport and epithelial barrier properties in the context of IBD.

1.4 Objective

Electrolyte transport dysregulation and barrier dysfunction have both been implicated in the complex pathophysiology of IBD. However, their contribution to epithelial pH homeostasis during intestinal inflammation has not been unraveled.

The aims of this thesis therefore were:

- to test the suitability of the TNF-α overexpressing mouse model $TNF^{\Delta ARE}$ in matters of intestinal ion-transport and epithelial barrier experiments for the first time

- to investigate if there are inflammation-associated disturbances in the HCO_3^- homeostasis in different segments of the intestine (Ileum, proximal colon and mid colon)

- to explore the underlying mechanisms for each examined part of the gut.

Since HCO_3^- output is modulated differently along the intestine, transepithelial (ion exchangers, transporters, and channels involved in HCO_3^- transport) as well as paracellular pathways (tight junction associated proteins) were examined. For this purpose, a multitude of methods was used including Ussing chamber experiments (HCO_3^- output, I_{sc}, ^{3}H-mannitol fluxes, dilution potentials, impedance spectroscopy), *in vivo* luminal perfusion experiments (HCO_3^- secretion and fluid absorption), classical histology, realtime PCR, western blot, and fluorescence immunohistochemistry.

2. Results

2.1 „Loss of Downregulated in Adenoma (DRA) Impairs Mucosal HCO3- Secretion in Murine Ileocolonic Inflammation"

Running head: **Ileocolonic HCO_3^- Transport in Inflammation**

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Author's contributions to the manuscript
provided the mouse model: GK
provided the DRA antibody: PD
designed experiments: FX, MJ, JL, BR, SY, AKS, SG, OB, US
performed experiments: FX, MJ, JL, BR, SY, AKS, LZ, SG
analyzed data: FX, MJ, US, OB
wrote the article: FX, MJ, US, OB
corrected and approved the manuscript: DT, GX, JZ

Abstract

Background: Ileocolonic luminal pH has been reported to be abnormally low in inflammatory bowel disease (IBD) patients, and one of the causative factors may be reduced epithelial HCO_3^- secretory rate (J_{HCO3}^-). Disturbances in J_{HCO3}^- may occur due to inflammation-induced changes in the crypt and villous architecture, or due to the effect of proinflammatory cytokines on epithelial ion transporters.

Methods: To discriminate between these possibilities, the tumor necrosis factor alpha (TNF-α) overexpressing ($TNF^{+/\Delta ARE}$) mouse model was chosen, which displays high proinflammatory cytokine levels in both ileum and colon, but develops only mild colonic histopathology and diarrhea. HCO_3^- secretion, mRNA expression, immunohistochemistry, and fluid absorptive capacity were measured in ileal and mid-colonic mucosa of $TNF^{+/\Delta ARE}$ and wildtype (WT) ($TNF^{+/+}$) mice in Ussing chambers, and in anesthetized mice in vivo.

Results: The high basal J_{HCO3}^- observed in WT ileal and mid-colonic mucosa were luminal Cl^--dependent and strongly decreased in $TNF^{+/\Delta ARE}$ mice. Downregulated in adenoma (DRA) mRNA and protein expression was strongly decreased in $TNF^{+/\Delta ARE}$ ileocolon, whereas cystic fibrosis transmembrane conductance regulator (CFTR), Na^+/H^+ exchanger 3 (NHE3), Na^+/HCO_3^- cotransporter (NBC), and epithelial sodium channel (ENaC) expression was not significantly altered. This indicates that the severe defect in ileocolonic J_{HCO3}^- was due to DRA downregulation. Fluid absorption was severely depressed in the ileum but only mildly affected in the mid-distal colon, preventing the development of overt diarrhea.

Conclusions: Even mild ileocolonic inflammation may result in a decrease of epithelial HCO_3^- secretion, which may contribute to alterations in surface pH, intestinal flora, and mucus barrier properties.

Keywords: inflammatory bowel disease; bicarbonate; anion exchanger; colon

2.2 „Increased epithelial permeability is the primary cause for bicarbonate loss in inflamed murine colon"

Running head: **HCO_3^- leakage in inflamed murine colon**

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Increased epithelial permeability is the primary cause for bicarbonate loss in inflamed murine colon

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Abstract

Background: Bicarbonate loss into the lumen occurs during intestinal inflammation in different species. However, candidate pathways like CFTR or DRA are inhibited in the inflamed gut. This study addressed the question whether and how inflammation-associated increased intestinal permeability may result in epithelial HCO_3^- loss.

Methods: Murine proximal colon was studied because it does not express functional DRA, but is inflamed in the TNF-α overexpressing mouse model ($TNF^{\Delta ARE}$). Luminal alkalization, ^{3}H-mannitol fluxes, impedance spectroscopy and dilution potentials were measured in Ussing chambers, while expression and localization of tight junction associated proteins were analyzed by Western blots and immunohistochemistry.

Results: Luminal alkalization rates and ^{3}H-mannitol fluxes were increased in $TNF^{+/\Delta ARE}$ proximal colon, while forskolin-stimulated I_{sc} was not altered. Epithelial resistance was reduced, but subepithelial resistance increased. The epithelial lining was intact, and enterocyte apoptosis rate was not increased despite massively increased Th1 cytokine levels and lymphoplasmacellular infiltration. Measurement of dilution potentials suggested a loss of cation selectivity with increased anion permeability. Western analysis revealed a downregulation of occludin expression, and an upregulation of both claudin-2 and claudin-5, with no change in ZO-1, E-cadherin, claudin-4 and claudin-8. Immunohistochemistry suggested correct occludin localization, but reduced tight junction density in $TNF^{+/\Delta ARE}$ surface epithelium.

Conclusions: Inflammation during TNF-α overexpression leads to increased epithelial permeability in murine proximal colon, decreased tight junctional cation selectivity, and increased HCO_3^- loss into the lumen. Inflammation-associated colonic HCO_3^- loss may occur via leaky tight junctions rather than via HCO_3^- secreting ion transporters.

Keywords: Tight junction; claudin; occludin; large intestine.

Introduction

The ability of the colonic epithelium to control surface and luminal pH is critical for many aspects of the epithelial interface barrier and transport function, including the absorption of short chain fatty acids (SCFAs), mucus production, as well as bacterial colonization and proliferation (1-3). Acid-base homeostasis is altered during intestinal inflammation, which is often the cause of acute and chronic diarrheal diseases, and has been associated with increased luminal HCO_3^- content in humans (4) and animal models (5). Particularly severe and chronic intestinal inflammation occurs in inflammatory bowel disease (IBD), and early studies have attempted to define the impact of chronic colonic inflammation on luminal pH. Due to the technical complexity, gastrointestinal pH profiles have only been measured in small numbers of patients suffering from IBD using a radiotelemetry capsule, and most of the more recent studies have demonstrated higher values in the colon of IBD patients than in the healthy controls [(6, 7); for review see (8)].

Intestinal inflammation and pro-inflammatory cytokines weaken the epithelial barrier by various mechanisms, including altered tight junctional anatomy (9). Tight junctions are formed by multiprotein complexes including e. g. occludin, tricellulin, claudins, and junctional adhesion molecules (JAMs), which restrict the free movement of ions across the epithelium. The continued exploration of barrier components has revealed that not all of them are “tightening” (10): While claudin-2 is selective for cations (11), several other claudins promote or regulate anion permeability (12-15). Intestinal inflammation influences the tight junction composition, reducing the expression of “tightening” claudins and occludin, leading to strand breaks (16), or up-regulating the pore-forming claudin-2 (17, 18).

HCO_3^- movement into the intestinal lumen occurs in a highly segment-specific and tightly regulated fashion (19-21), and represents a major mechanism for luminal pH control (22). During chronic colitis, HCO_3^- could potentially reach the lumen via the paracellular pathway, which plays a minor role in the healthy epithelium (23). However, the significance of tight junction modification for acid-base balance in the inflamed intestine has not been studied, and it is not known whether specific changes to components of the epithelial barrier by the inflammatory process can lead to HCO_3^- losses. One major obstacle to such studies is the influence of inflammation on active HCO_3^- transporters, predominantly luminal Cl^-/HCO_3^- transporters from the Slc26 family. The proximal colon of rats was shown to lack DRA (down-regulated in adenoma, Slc26a6), which is the primary pathway for basal HCO_3^- output into the

colonic lumen in the absence of nutrients (24, 25), and murine proximal colon was found to be similar [(26), and this study]. The expected rates of HCO_3^- transport across the apical membrane of the proximal colonic epithelium under resting conditions should be negligible, which makes this segment ideal to test the hypothesis that changes to the epithelial barrier during intestinal inflammation cause HCO_3^- leakage.

Materials and Methods

Animals: $TNF^{\Delta ARE}$ mice (TNF^{tm2Gkl}) (27) were bred on a C57BL/6J background at the animal care facility at Hannover Medical School under standardized conditions. Sex- and age-matched heterozygous mice ($TNF^{+/\Delta ARE}$) and their healthy littermates ($TNF^{+/+}$) at the age of 6-8 months were used for the experiments. Mice were sacrificed by CO_2 narcosis and subsequent cervical dislocation. Animal care and experimentation were approved by and carried out in accordance with the Medical School of Hannover and the local authorities for the regulation of animal welfare (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit).

pH-Stat titration of HCO_3^- secretory rates and I_{sc}: Experiments were carried out exactly as described previously (26). Excised proximal colon was stripped of external muscle layers, mounted into Ussing chambers and bathed with equiosmolar solutions, pH 7.4 (serosally (in mM): 108 NaCl, 25 $NaHCO_3$, 3 KCl, 1.3 $MgSO_4$, 2 $CaCl_2$, 2.25 $KH_2PO_4^-$, 8.9 glucose, 10 sodium pyruvate, gassed with 95% O_2/5% CO_2, and luminally with 154 mM NaCl, gassed with 100% O_2). Neural activity and prostaglandin generation were blocked with 1 µM tetrodotoxin and 3 µM indomethacin (serosal). HCO_3^- secretory rates were determined by pH-stat titration and electrical parameters recorded under open circuit conditions. I_{sc} was calculated from recording tissue resistance (R_t) and potential difference (PD) in 2-min intervals.

Fluxes of [^{3}H] mannitol: To assess tissue permeability, bilateral [^{3}H] mannitol fluxes were measured as previously described (28) in isolated proximal colonic $TNF^{+/\Delta ARE}$ and $TNF^{+/+}$ mucosa, with bilaterally identical, CO_2/O_2 gassed solutions (as described for the serosal solution above, except for 9 mM lactose in the mucosal bath and 1 mM mannitol in both), under short circuit conditions. After 30 min equilibration, 74 KBq/ml ^{3}H-mannitol (Perkin Elmer, Waltham, MA, USA) were added either to the serosal or the mucosal bath, and samples were taken as quadruple in 15 min intervals for 90 min.

Dilution potentials: The permeability of sodium and chloride ions was measured with a modified dilution potential technique (29). In brief, pieces of stripped proximal colon were mounted into Ussing chambers and equilibrated in our basal solution with mannitol instead of glucose on the luminal side (see pH-Stat titration). The mucosal solution was then substituted with a 54 mM NaCl containing solution while osmolarity was maintained with 108 mM of mannitol. Electrical parameters were recorded in 30 sec intervals for 30 min before and after the substitution. Ion permeability ratio (P_{Na}/P_{Cl}) was calculated from the dilution potential by using the Goldman-Hodgkin-Katz equation. Using Ohm´s law, the total conductance *G* was calculated. The absolute permeabilities of sodium (P_{Na}) and chloride (P_{Cl}) were obtained by using a simplified Kimizuka-Koketsu equation.

Impedance spectroscopy: Tissues were mounted in Ussing chambers and one-path impedance spectroscopy was performed in order to discriminate between epithelial (R_{epi}) and subepithelial (R_{sub}) resistance exactly as described previously (30). To adjust mucosal surface area from $TNF^{+/\Delta ARE}$ and $TNF^{+/+}$, images of hematoxylin/ eosin stained paraffin sections were analysed with the processing software Image J (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). Villus height and crypt depth of the epithelium as well as the thickness of the subepithelial tissue layers were measured. The number of villi and crypts were counted. On average, 10 adjacent sections were analyzed. The ratio of mucosal to serosal surface area was determined, representing apical epithelial area and subepithelial area corresponding to the opening area of the Ussing chamber.

See Supplementary Materials for quantitative real-time polymerase chain reaction (PCR), measurements of apoptosis, western blot analysis, immunofluorescence, and statistical analysis.

Results

Characterization of proximal colonic inflammation in the $TNF^{\Delta ARE}$ colitis model

As a model of chronic intestinal inflammation, $TNF^{+/\Delta ARE}$ mice and their wild-type littermates were utilized. These mice develop a Crohn's disease-like phenotype with ileocolitis and pronounced arthritis (26, 27) (Figure 1A). A significant separation of the weight curves after 16 weeks for both male and female mice was observed (Figure 1B). In analogy to human inflammatory bowel disease (31-33), it was previously established that heterozygeous animals suffer from a pronounced intestinal absorptive defect, but also display a reduced net secretory response (26).

Histologically, mild to moderate colonic inflammation with a proximal-to-distal gradient was observed (Figure 2A,B). The inflammatory infiltrate (consisting mostly of B- and T-lymphocytes, supplementary fig. 1B,C) partially extended into the submucosa, but no gross ulceration was present. There was no indication of disrupted continuity of the epithelium, which could have represented a bias for transport- and permeability studies. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining did not reveal obvious differences in the frequency of apoptotic cells (Figure 2C), which was further substantiated by caspase 3/7 activity assay (Figure 2D). In line with the histological signs of inflammation, the content of the inflammatory cytokines TNF (tumor necrosis factor)-α and IL (interleukin)-1β was markedly elevated on an mRNA level (Figure 2E,F). The results of Figure 1 and 2 therefore substantiate the presence of a mild, non-erosive colitis with chronically strongly elevated Th1 cytokines in the proximal colon.

Basal luminal alkalization rates in the proximal colon of $TNF^{\Delta ARE}$ mice and analysis of active transport involvement

Luminal HCO_3^- output in the isolated proximal colonic mucosa was low, consistent with previous results (26), and due to virtual absence of DRA (Figure 3A,C and 4A). However, basal alkalization rates were significantly higher in $TNF^{+/\Delta ARE}$ than in wild-type proximal colon (Figure 3A,C) without a difference between male and female animals. The I_{sc} was not significantly different (Figure 3B,D), suggesting that electrogenic Cl^- secretion was not disturbed. To exclude that chronic inflammation leads to an up-regulation of Cl^-/HCO_3^- exchangers, DRA (Slc26a3), and PAT1 (Slc26a6), mRNA abundance was measured by semiquantitative RT-PCR. The expression of both was very low in murine proximal colon (Figure 4A,B). No differences in anion exchanger expression between wild-type and heterozygous mice were detected with all

reference genes used (Figure 4C,D). This suggested that neither alterations in electrogenic HCO_3^- secretion nor luminal Cl^-/HCO_3^- exchangers were involved.

Epithelial permeability and barrier selectivity in the inflamed proximal colon

To assess permeability changes in the proximal colon of $TNF^{+/\Delta ARE}$ mice and their normal littermates as a potential cause of HCO_3^- leakage, ^3H-labelled mannitol flux rates were measured in isolated proximal colonic epithelium. Indeed, higher fluxes were observed in $TNF^{+/\Delta ARE}$ tissue (Figure 5A), which indicates increased paracellular permeability for small uncharged solutes. Impedance spectroscopy, which allows the differentiation of epithelial (R_{epi}) and subepithelial (R_{sub}) resistances, demonstrated a markedly lower R_{epi} (Figure 5B). In contrast, R_{sub} was elevated, probably due to subepithelial thickening (Figure 2B, 5B, and supplementary fig. 1A).

Next, we measured dilution potentials in heterozygous compared to healthy colonic epithelia during isoosmolar dilution of the apical NaCl concentration by 50% (see Materials and Methods). Interestingly, the dilution potential was significantly altered in heterozygous animals, reflecting a calculated shift in the permeability ratio towards Cl^- (Table 1). Using the measured conductance and the modified Kimitsuka-Koketsu equation, the Na^+ permeability, which was overall higher, was found to be unchanged (Figure 5C). This suggested a decrease in cation selectivity of the inflamed proximal colonic mucosa.

Expression and localization of tight junction associated proteins

The influence of proinflammatory cytokines on the differential expression of tight junction components has been documented in several studies (34, 35) and could explain the observed permeability changes. To investigate whether an altered abundance of the different proteins can be observed in inflamed vs. healthy mice, the expression of occludin as well as representative members of pore-forming (claudin-2) and tightening (claudin-5, -4, and -8) claudins as well as adherens junction components was assessed. Occludin, which was suggested as a general indicator for tight junction integrity, and which was shown to be strongly regulated by TNF-α, was downregulated in $TNF^{+/\Delta ARE}$ proximal colonic tissue. Localization of occludin, however, was unchanged in heterozygous vs. wild type animals, but disturbed strand morphology was present, with an apparent reduction in tight junction density than in WT mucosa. (Figure 6C,D). While claudin-2 was upregulated on a protein level, analysis of the

tightening claudins gave a mixed image, with an upregulation of claudin-5 and no change of claudin-4 or claudin-8. The adherens junction associated proteins E-Cadherin (E-Cad) and Zona Occludens Protein 1 (ZO-1) were not changed (Figure 6A,B).

Discussion

Intestinal inflammation results in disturbed barrier function and a dysregulation of electrolyte transport (31, 36). However, changes in epithelial pH homeostasis due to altered permeability and paracellular leakage have not been studied. The experiments presented here demonstrate the existence of a higher paracellular permeability and loss of cation selectivity in otherwise mildly and chronically inflamed proximal colonic mucosa, leading to enhanced HCO_3^- leakage into the proximal colonic lumen.

We and others have previously investigated the impact of intestinal inflammation on epithelial HCO_3^- transport (26, 37). Zhang et al. injected mice with an anti-CD3 monoclonal antibody and measured ileal I_{sc} and HCO_3^- secretion in the Ussing chamber (37). In this acute inflammation model, the authors found a shift from electroneutral Cl^-/HCO_3^- exchange to a predominantly electrogenic anion secretion with an increased intracellular cAMP content, but no expressional changes for CFTR. Similarly, the chronically inflamed ileum and mid-distal colon of $TNF^{\Delta ARE}$ heterozygotes displays reduced Cl^- dependent HCO_3^- secretion, which is due to the loss of DRA (26). A down-regulation of this exchange process was also found in the colon of these mice (26), the HLA-B27/β2m transgenic rat, the interleukin-10 (IL-10) knockout mouse with spontaneous colitis, and in patients with ulcerative colitis (38). While these studies had established a dysregulation of pH regulating ion transporters during intestinal inflammation, the importance of barrier changes for intestinal pH homeostasis remained unresolved, since the experimental design did not permit to separate active transport from paracellular leakage.

Recent studies have reported a pronounced segmental heterogeneity for ion transporter expression (24), leading to significant variability within even the same organ such as the colon, and different species (20). In particular, the proximal murine colonic mucosa displays very low rates of luminal alkalization (39), and DRA expression in this segment is almost negligible (24). We therefore chose the murine proximal colon of TNF-α overexpressing mice (which develop an ileocolitis, and in which the proximal colon is more affected than the distal colon) for the present study, assuming that it would permit us to measure the passive leakage of HCO_3^- without the influence of an inflammation-associated decrease in DRA-mediated Cl^-/HCO_3^-

exchange. Indeed, luminal alkalization occurred at almost negligible rates in WT mucosa, compared to the tenfold higher rates in the ileal and mid-distal murine colonic mucosa (26), paralleled by a very low DRA expression, which was unchanged with respect to mRNA abundance in $TNF^{+/\Delta ARE}$ mice (Figure 4). Luminal alkalization rate was significantly increased in proximal colonic mucosa of $TNF^{\Delta ARE}$ heterozygotes, which was in contrast to the decreased HCO_3^- output rates in the mid-distal colon (26). I_{sc}, on the other hand, was not significantly altered by the inflammatory state in the proximal colon, neither the basal nor the forskolin-stimulated value. The crypt-expressed CFTR anion channel (or any other channel) is therefore not responsible for the higher luminal alkalization rates. In addition, CFTR expression levels were unchanged. We speculated that another potential reason may be less luminal acidification by the apical Na^+/H^+ exchanger NHE3, which has been shown to be dysfunctional despite normal expression and localization in moderately severe murine and human intestinal inflammation (31, 33, 40). However, NHE3 mRNA expression was also equal in $TNF^{+/\Delta ARE}$ and $TNF^{+/+}$ mice, and luminal application of fully NHE3 inhibitory concentrations of the specific NHE3 inhibitor S1611 did not affect luminal alkalization rates in the chambered proximal colon (supplementary fig. 2A,B), indicating that this transporter is quiescent under the conditions of our experiments. The likely reason for this is the complete absence of CO_2 in the lumen (which would diffuse into the enterocytes, acidify them, and stimulate NHE3), the absence of any other acidifying substances in the luminal bath (such as SCFAs), and therefore an inactivation of NHE3 exchange by the high pH_i in colonic surface cells under the given experimental conditions. Thus, transporter-mediated events do not explain the observed higher alkalization rates in $TNF^{\Delta ARE}$ heterozygote mucosa.

In addition to disturbed electrolyte transport, changes in intestinal epithelial barrier function have been described in many experimental models of bowel inflammation, sometimes preceding inflammation (32, 35, 41). Cytokines can directly influence barrier components (42). As a major inflammatory messenger, TNF-α has been shown to down-regulate claudin-1, up-regulate claudin-2 (43), and to degrade occludin mRNA in a micro-RNA-dependent manner (44). Occludin was shown to regulate macromolecule flux across the intestinal epithelium (45), which may explain our observation of increased ^{3}H-mannitol permeability during TNF-α overexpression, but also that of increased HCO_3^- leakage.

Due to the cation selectivity of small and large intestinal mucosa, paracellular permeation of anions occurs at a much lower rate than cations, i.e. Na^+, in healthy mucosa. A paracellular

passage of HCO_3^- ions may occur when the charge-selectivity for cations is decreased, which is what we found. We therefore searched for the reason for the decrease in cation selectivity. Expression of WT and mutated claudins in Madin-Darby Canine Kidney (MDCK) cells under an inducible promoter first clearly demonstrated that claudins can mediate charge selectivity of the paracellular pathway for ions. When the charge of selected extracellular amino acids was reversed, ion selectivity increased Na^+- (claudin-4) or switched the preference from Na^+ to Cl^- permeability (claudin-15) (10). When claudin-2 is overexpressed in MDCK monolayers, paracellular permeability increased 5.6-fold compared to the vector control, with relative cation selectivity (11). Several studies have linked claudins to increased Cl^- permeability, but whether this occurs via an increase in paracellular permeability or via upregulation of transcellular transport was not clearly determined in most of them (12-15). Recently, claudin-10a and -17 have been characterized as being anion selective (46), but claudin-10a is expressed at extremely low levels in the proximal colon (data not shown), and claudin-17 mRNA is absent in the colon (47) and is also not up-regulated in the inflamed intestine (J.-D. Schulzke, personal communication). In our study, the observed changes in claudin expression pattern included increased expression of “tightening” as well as “pore-forming” components, and no obvious explanation for the decrease in cation selectivity. However, it is known that claudins are expressed in rodent colon which have not been studied for their ion permeation properties in detail, but do pose additional pathways for cations and/ or anion (48, 49). We did find a strong down-regulation in occludin expression (as mentioned above), and the immunohistochemistry revealed a normal localization of the tight junction components in the zones of cell-cell contacts, but a coarser architecture in the $TNF^{\Delta ARE}$ heterozygote mucosa. Given the complexity of the tight junction, it may be that in the present case, not a single protein directly confers increased HCO_3^- permeability, but that expressional changes of other, even “tightening” components alter TJ regulation and increase permeability for HCO_3^-, as it has been suggested for renal cells (50).

What may be the clinical implications of our findings? On a local level, the increased HCO_3^- leakage will change mucosal surface pH and likely affect the mucosa-associated hydrogenotrophic gut flora (51) as well as luminal flora (3), alter the microbial fermentation, and influence colonic health. Alterations of gut flora composition have indeed been observed during intestinal inflammation (52), and are believed to be involved in IBD pathogenesis (53). The functional down-regulation of both Na^+/H^+ as well as Cl^-/HCO_3^- exchangers of the surface

epithelium, in combination with increased HCO_3^- leakage, observed even in this mild non-erosive inflammatory state as observed in the $TNF^{+/\Delta ARE}$ proximal colon, will completely alter the segmental surface cell pH profiles, as well as luminal bulk pH in the different colonic segments and thus be one of the reasons for alteration of gut microbiome during intestinal inflammation, with possible detrimental consequences. The second consequence of paracellular HCO_3^- leakage in the inflamed intestine may be a very considerable loss of base equivalents from the systemic circulation. It is likely that this leakage occurs wherever intestinal epithelium is inflamed, although, as discussed above, this will be difficult or eve impossible to prove in intestinal segments with significant active HCO_3^- transport masking additional HCO_3^- leakage. Since inflammation also down-regulates DRA-mediated Cl^-/HCO_3^- exchange, this loss of HCO_3^- is in part compensated, but for the price of losing Cl^-. A third consequence of paracellular HCO_3^- leakage will be the loss of acidic microclimate in those segments of the gut where it is present and necessary to drive proton-coupled nutrient uptake, such as in the jejunum and ileum, or nonionic diffusion of weak acids, such as SCFAs in the proximal colon.

In summary, we describe an enhanced luminal alkalization associated with a tight junction defect in chronic intestinal inflammation, which is likely to have a significant effect on epithelial pH homeostasis, making it an important pathophysiological factor in inflammatory bowel disease.

Acknowledgements

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Figures, Tables and Legends

Figure 1:

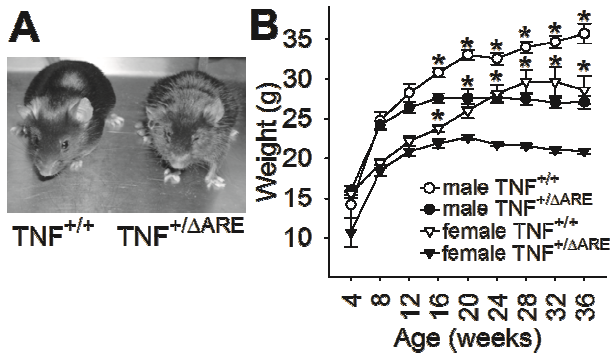

Figure 1: *Phenotype and weight development of TNF$^{\Delta ARE}$ mice.* Heterozygous TNF$^{+/\Delta ARE}$ mice display a fuzzy fur and signs of arthritis (A). From the 16th week of life, the body weight of TNF$^{+/\Delta ARE}$ males and females (● and ▼) is significantly lower compared to TNF$^{+/+}$ males and females (○ and ▽), respectively. n= 4-17 per data point.

Figure 2:

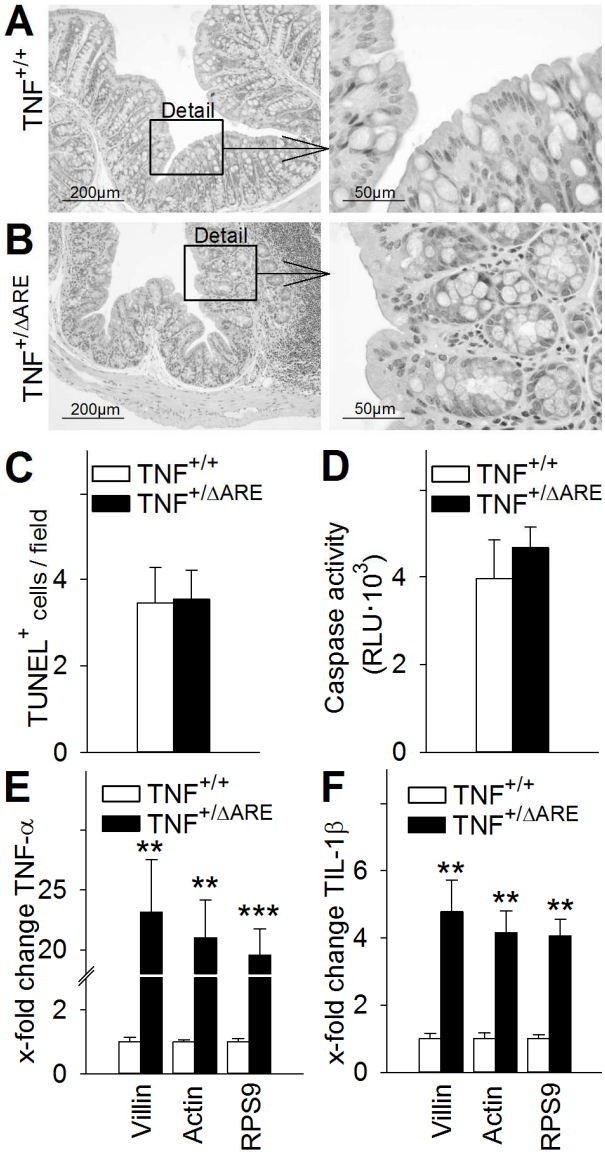

Figure 2: *Histology and cytokine RNA expression in the proximal colon of TNF$^{\Delta ARE}$ mice.* Compared to TNF$^{+/+}$ (A), mild to moderate inflammation can be observed in the proximal colon of TNF$^{+/\Delta ARE}$ mice (B), leaving the epithelial lining intact. There is no sign of a higher apoptotic rate as assessed by TUNEL staining (C, n=4) and a Caspase 3/7 assay (D, n=4). TNF-α mRNA expression of TNF$^{+/\Delta ARE}$ animals (■) is 23.1-, 21- and 19.6-fold increased compared to villin, actin, and RSP9 as a control gene, respectively (E). IL-1β mRNA is 4.8 (villin)- and 4.1 (actin and RSP9)-fold higher expressed compared to TNF$^{+/+}$ (F)(□, mRNA expression value taken as 1, n=6).

Figure 3:

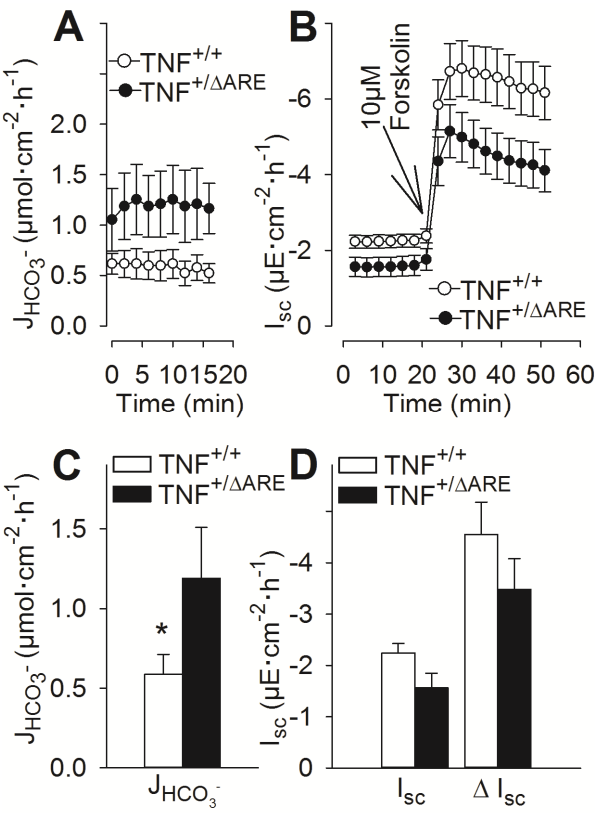

Figure 3: *Time course of basal HCO_3^--output (A) and I_{sc} (B) in the proximal colon of $TNF^{\Delta ARE}$ mice.* Basal HCO_3^--output of $TNF^{+/\Delta ARE}$ mice (■) is significantly higher compared to $TNF^{+/+}$ animals (□, A,C). No significant differences are found in basal and forskolin-stimulated I_{sc} (ΔI_{sc}) among the two groups (B,D, n=6/5).

Figure 4:

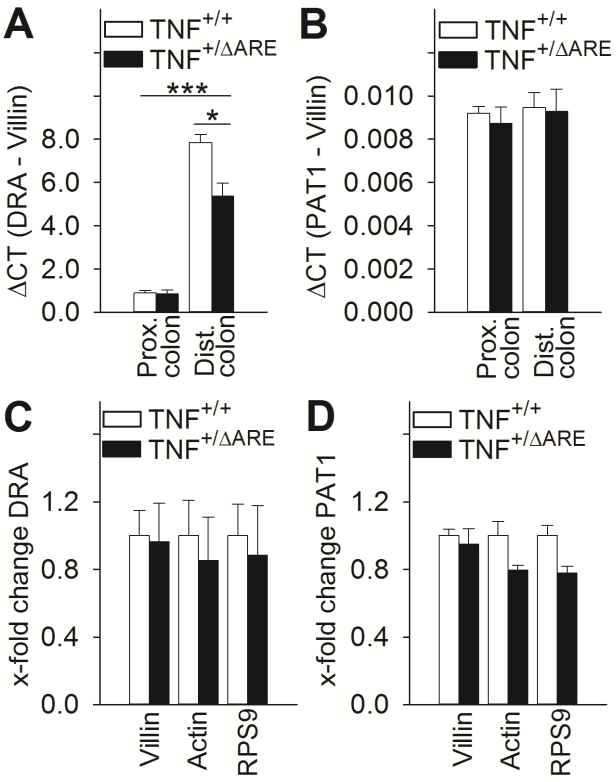

Figure 4: *RNA expression of the apical Cl^-/ HCO_3^--exchanger DRA and PAT1 in the proximal and distal colon of $TNF^{\Delta ARE}$ mice.* To illustrate the heterogeneity of DRA, ΔCT values of proximal and distal colonic mRNA are shown. DRA mRNA is significantly higher expressed in the distal colon, where it additionally differs significantly between $TNF^{+/+}$(□) and $TNF^{+/\Delta ARE}$ (■) mice (A). PAT1 at a very low expression level does neither differ between the two colonic segments nor the two groups (B). Independently of the control gene, the expression level of both exchangers does not differ between $TNF^{+/\Delta ARE}$ and $TNF^{+/+}$ mice in the proximal colon (C,D) (mRNA expression value of the control group taken as 1, n=6).

Figure 5:

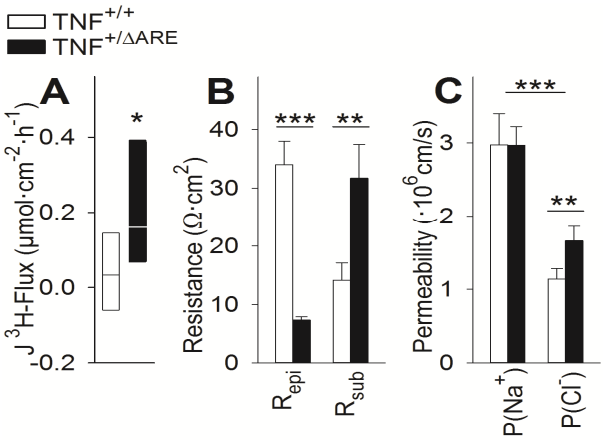

Figure 5: *Permeability studies in the proximal colon of TNF$^{\Delta ARE}$ mice.* As an indication for paracellular permeability, TNF$^{+/\Delta ARE}$ (■) proximal colon displays a significantly higher ^{3}H-mannitol flux rate compared to TNF$^{+/+}$ (□, A, n=4) colon. Impedance spectroscopy measurements (B, n=8) reveal furthermore a significantly lower epithelial resistance (R_{epi}), and coevally significantly elevated subepithelial resistance (R_{sub}) in TNF$^{+/\Delta ARE}$ tissue. Additionally, dilution potentials (C, n=7/8) show an overall higher permeability of Na^+ vs. Cl^-, but also a significantly higher permeability for Cl^- ions in TNF$^{+/\Delta ARE}$ proximal colon compared to the control group.

Figure 6:

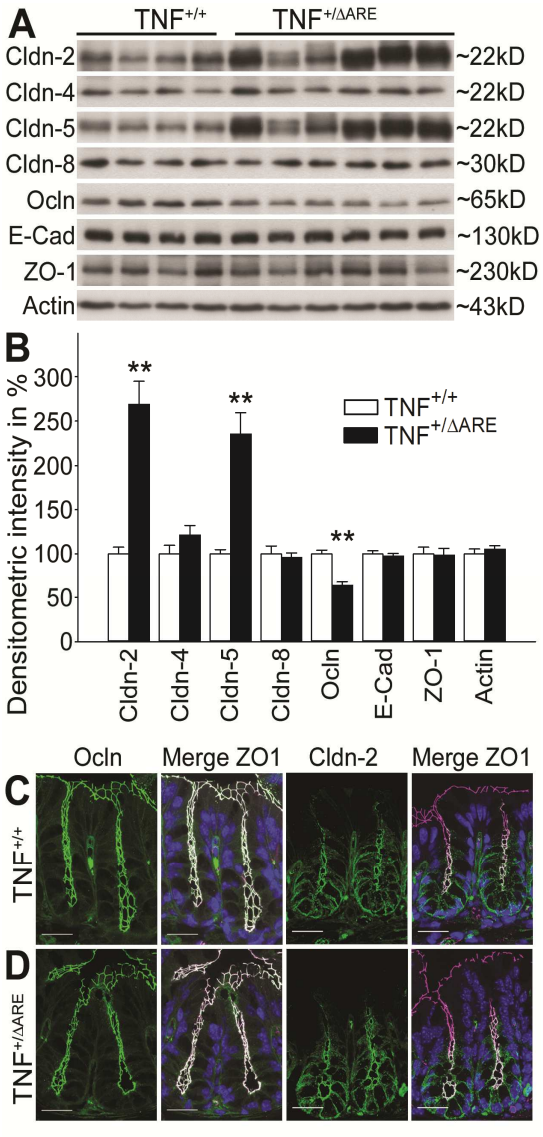

Figure 6: *Expression of tight junction associated proteins and localization of zona occludens protein 1 (ZO-1, magenta), occludin (Ocln, green) and claudin-2 (Cldn-2, green) in the proximal colon of TNF$^{\Delta ARE}$ mice.* Densitometric analysis of the original western blots (A, n=4/6) shows a significant upregulation of claudin-2 (Cldn-2) and claudin-5 (Cldn-5) in TNF$^{+/\Delta ARE}$ tissue. While claudin-4 (Cldn-4), claudin-8 (Cldn-8), e-cadherin (E-Cad) and zona occludens protein 1 (ZO-1) remain unchanged, occludin (Ocln) expression is found to be reduced in the inflamed colon (B).

In TNF$^{+/+}$ (C) and to TNF$^{+/\Delta ARE}$ animals (D), ZO-1 and Ocln are expressed in the same apical region of every enterocyte (merge). Occludin seems not to be differentially localized but tight junction density appeared to be lower. Cldn-2 localization is restricted to the lower part of the crypt in both groups. Bar=25µm.

Table 1:

	DP [mV]	$P(Na^+)/P(Cl^-)$ [$\cdot 10^{-6}$ cm/s]	$J(Na^+)$ [$\mu Eq \cdot cm^{-2} \cdot h^{-1}$]	$J(Cl^-)$ [$\mu Eq \cdot cm^{-2} \cdot h^{-1}$]
$TNF^{+/+}$	-8.00 ± 0.24	2.62 ± 0.08	6.43 ± 0.37	2.46 ± 0.21
$TNF^{+/\Delta ARE}$	-5.06 ± 0.01	1.80 ± 0.07	6.41 ± 0.13	3.59 ± 0.16
p-value	< .001	< .001	n.s.	< .001

Table 1: *Results of dilution potentials in the proximal colon of $TNF^{\Delta ARE}$ mice.* Data represent mean ± standard error of the mean (SEM). Abbreviations: DP= dilution potential, P= permeability, J= flux, n.s.= not significant.

Supplementary Materials

Increased epithelial permeability is the primary cause for bicarbonate loss in inflamed murine colon

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Supplementary Materials and Methods

Materials: All the reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) unless mentioned otherwise. S1611 was kindly provided by Sanofi-Aventis (Frankfurt, Germany). The following antibodies were used for Western blotting and/ or Immunhistofluorescence: monoclonal mouse anti-occludin, monoclonal mouse anti-claudin 2 (Invitrogen, Camarillo, CA, USA), polyclonal rabbit anti-claudin 4, monoclonal mouse anti-claudin 5, polyclonal rabbit anti-claudin 8, polyclonal rabbit anti-E-cadherin (Abcam, Cambridge, UK), polyclonal rabbit anti-ZO-1 (Proteintech, Chicago, IL, USA), rabbit anti-actin (santa cruz, Santa Cruz, CA, USA), polyclonal rabbit anti-CD3, polyclonal chicken anti-SLC26A3 (DRA) (Sigma-Aldrich, Deisenhofen, Germany), monoclonal rat anti-CD45R (B220) (AbD serotec, Oxford, UK).

Quantitative real-time PCR: Total RNA isolation and real time quantitative PCR was performed exactly as described previously (Xiao et al. 2012). Data were standardized to three different housekeeper genes [villin, actin and ribosomal protein S9 (RPS9)] for each sample and then normalized to control tissue samples ($TNF^{+/+}$). The sequences of the primers used are:

Villin: 5`-TCATACTCAAGACTCCGTCC-3´ and 5´-TACCACTTGTTTCTCCGTCC-3´

Actin: 5´-AGAGGGAAATCGTGCGTGAC-3´ and 5´-CAATAGTGATGACCTGGCCGT-3´

RPS9: 5´-AAGCACATCGACTTCTCCC-3´ and 5´-ACAATCCTCCAGTTCAGCC-3´

TNF-α: 5´-CATCTTCTCAAAATTCGAGTGACAA-3´ and 5´-TGGGAGTAGACAAGGTACAACCC-3´

IL-1β: 5´-CAACCAACAAGTGATATTCTCCATG-3´ and 5´-GATCCACACTCTCCAGCTGCA-3´

DRA: 5´-TTCCCCTCAACATCACCATCC-3´ and 5´-GTAAAATCGTTCTGAGGCCCC-3´

PAT1: 5´-GGCTCCTGGGTGATCTGTTA-3´ and 5´-CCAAACATAGGAGGCAATCC-3´

TUNEL staining: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (In Situ Cell Death Detection Kit, Fluorescein, from Roche, Mannheim, Germany) was used to quantify apoptotic cells following the producer's directions. For permeabilization, 20µg/ml proteinase K solution was used for 15 min at room temperature. Six random, non-overlapping pictures of 20x magnified optical fields of two different colon layers were taken (n=4), and the number of TUNEL-positive cells per field was counted by a person blinded to the genotype of the sample.

Caspase 3/7 assay: To access caspase activity, 2 pieces of proximal colon of 4 healthy and 4 inflamed mice were homogenized in lysis buffer (in mM) 10 Tris-HCl pH 7.4, 10 MgCl, 150 NaCl, 10 dithiothreitol, 0.5% octylphenoxypolyethoxyethanol (Nonidet P-40), 1% Protease-Inhibitor (Sigma Aldrich), and the total protein concentration was assessed by a Bradford assay (BioRad, Munich, Germany). Activity of caspase 3 and caspase 7 was measured by a luminescent substrate assay (Caspase-Glo; Promega, Mannheim, Germany) according to the manufactures' instruction. Luminescence of each sample was measured twice and the results are given in relative light units (RLU).

Western blot: To determine tight junction protein expression, western blot analysis was performed with protein lysates of proximal colonic tissue of $TNF^{+/+}$ and $TNF^{+/\Delta ARE}$ mice. Scratched mucosa was put into Lysate buffer containing 1mM EGTA, 1% Triton X100, 0.1% SDS and 10% Protease Inhibitor Cocktail (ProteoBlock, Fermentas, St. Leon-Rot, Germany) in PBS (pH7.4), homogenized and sucked through a 24Gx1" needle. The samples were centrifuged at 12.000g and 4°C, and protein concentration of the collected supernatant was determined by a Bradford assay (Quick Start BSA Standard Set and Bradford Dye Reagent, Biorad, München, Germany). 20µg protein were separated on 12-15% SDS-polyacrylgels and blotted on a PVDF Transfer Membrane (Hybond-P, GE Healthcare, Buckinghamshire, UK). The Blots were blocked in RotiBlock-Solution (Carl Roth, Karlsruhe, Germany) for 2 hours before being incubated with the corresponding primary antibody overnight at 4°C. Between two washing steps with TBS-Tween, the Membranes were incubated with the corresponding horseradish peroxidase conjugated secondary antibodies (Cell Signaling, Danvers, MA, USA), and developed for visualisation of protein with ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Densitometric analysis was performed using Image J Software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/).

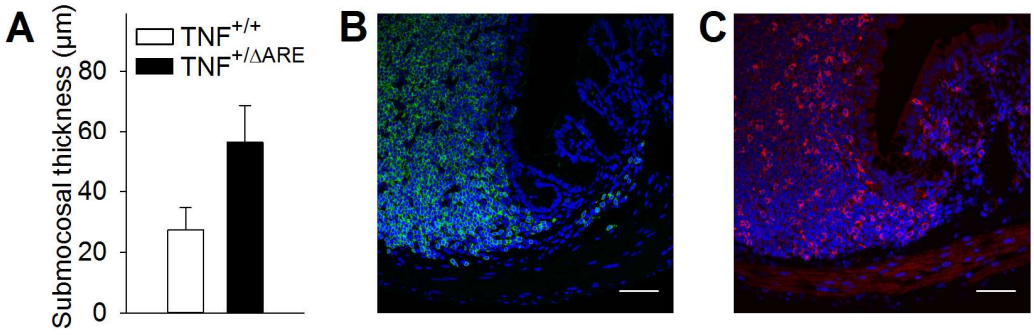
Immunofluorescence: 5µm paraffin sections from proximal colonic tissue were deparaffinized and except for the anti-B220 staining heat induced epitope retrieval was performed with Dako Target Retrieval Solution pH9 (Dako, Glostrup, Denmark) for 20 min at 96°C. Nonspecific binding sites were blocked by an incubation in 10% natural horse serum (NHS) diluted in PBS-Tween for at least 2 hours. The first antibody (-mix) diluted in 5% NHS-PBS-Tween was applied over night at 4 °C. The secondary antibody (-mix) consisted of corresponding fluorescent dye conjugated antisera (Cy3, Alexa Flour 488 (Sigma Aldrich St. Louis, MO, USA, Invitrogen, Camarillo, CA, USA) as well as 0.5µg/ml DAPI in 5% NHS-PBS-Tween and was incubated for 1 hour at room temperature. Sections were mounted, and images were taken on a Leica DM IRB confocal microscope with a TCS SP2 AOBS scan head. 10 consecutive optical sections were taken and subsequently put together to a 4µm thick maximum projection using Image J Software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/).

Submucosal thickness: Haematoxylin and Eosin stained slides of four $TNF^{+/+}$ and four $TNF^{+/\Delta ARE}$ mice were pictured in a 10x magnification. One image of six different proximal colonic sections of each mouse were taken and the thickness of the submucosa was measured with the "line" tool of Image J Software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/) at 6 random positions per image (144 data points per group).

Statistical analysis: All results are given as means ± SEM. A Students t-test (group mean comparison test) was used to compare groups of generally 6, but at least 4 and at most 17 samples/ animals. $P < 0.05$ was considered statistically significant. Significance levels are denoted n.s. = not significantly different, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

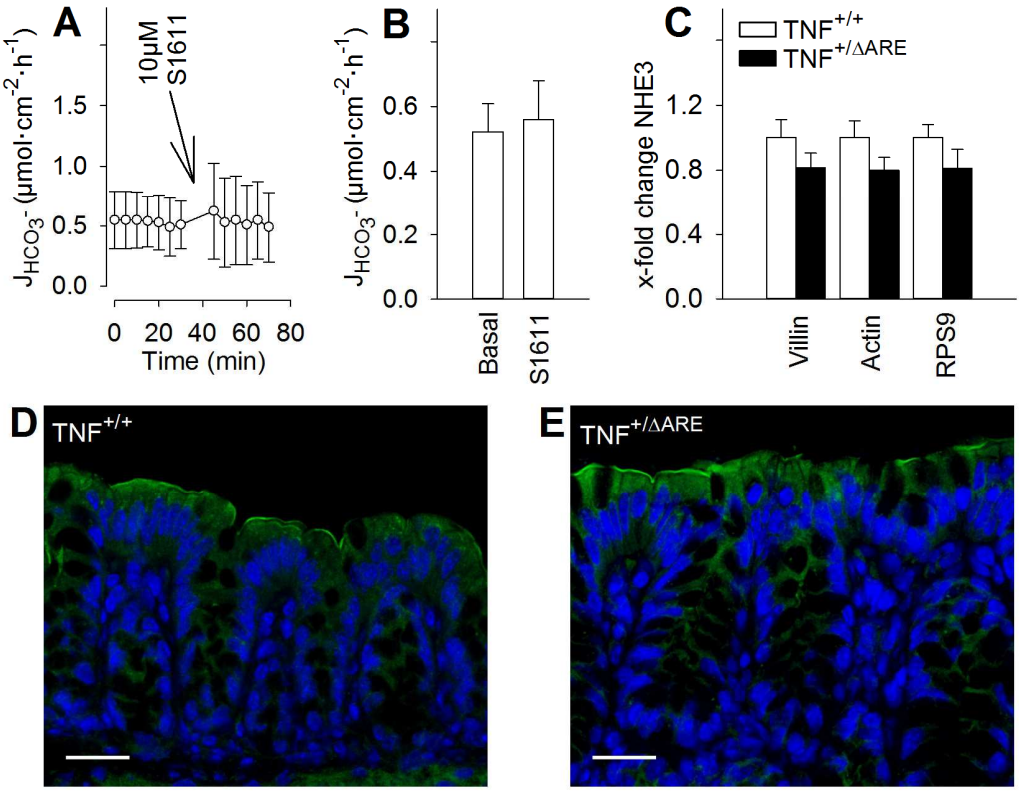
Supplementary Figures

Supplementary Figure 1:



Supplementary Figure 1: *Thickness of proximal colonic submucosa and Lymphocyte infiltration of TNF$^{+/+}$ and TNF$^{+/\Delta ARE}$ mice.* TNF$^{+/\Delta ARE}$ animals show a significantly thicker proximal colonic submucosa in comparison to their healthy littermates (A, n=4). The inflammatory infiltrate of TNF$^{+/\Delta ARE}$ proximal colon consists mainly of Lymphocytes. (B) shows a B220 staining (B-lymphocytes, lytically active subsets of Natural Killer cells and non-major histocompatibility complex (MHC) restricted cytotoxic T Lymphocytes (CTL´s),(C) shows a CD3 staining (mature T-lymphocytes). Bar=50µm.

Supplementary Figure 2:



Supplementary Figure 2: *Luminal alkalization of murine wildtype proximal colon during NHE3 inhibition, RNA expression and localization of NHE3 in TNF$^{+/+}$ and TNF$^{+/\Delta ARE}$ mice.* Addition of a fully NHE3 inhibitory concentration of S1611 to the luminal side did not change the basal alkalization rate. A: time course of luminal alkalization, where the pH deviation occurring in response to addition of the acidic S1611 solution is omitted. B: Mean basal luminal alkalization rates, t-test: not significant. n= 6. Independently of the control gene, the RNA expression level of NHE3 does not differ between TNF$^{+/\Delta ARE}$ and TNF$^{+/+}$ mice in the proximal colon (C) (mRNA expression value of the control group taken as 1, n=5). There is no indication of a changed DRA protein localization (D, E), although it has to be taken into consideration that reduced specificity has been reported for the available DRA antibodies, which may lead to staining of other related transport proteins. Bar=25µm.

3. General Discussion

Luminal HCO_3^- has been found essential for the release and proper function of intestinal mucus (Garcia et al. 2009), intestinal wound healing (Feil et al. 1989) and intestinal electrolyte reabsorption (Binder et al. 2005). Intraluminal pH has been also found to be changed in the colon of UC patients, but the importance of this has so far been discussed predominantly in the context of anti-inflammatory drug release (Nugent et al. 2001). Additionally, changes in epithelial pH homeostasis due to altered permeability and paracellular leakage have rarely been studied in the context of intestinal inflammation.

Therefore, the presented studies were designed to get further insight into the mechanisms that are responsible for an alteration in HCO_3^- output, if any, in intestinal inflammation, with special emphasis on the segmental heterogeneity. Recent studies have reported a highly segment specific expression of ion transporters which leads to a large variability even within the same organ such as the colon (Talbot and Lytle, 2010). The considered parts of the intestine were the ileum, the proximal colon and the mid colon, which represent the most affected intestinal parts in IBD patients. The used TNF-α overexpressing mouse model $TNF^{\Delta ARE}$ mimics this disease pattern in matters of locality and develops mild to moderate inflammation in the respective segments without gross destruction of the crypt-villlus architecture.

The key findings of this project were:

- basal HCO_3^- secretion was found to be Cl^- dependent and significantly reduced in the Ileum and the mid colon of inflamed $TNF^{+/\Delta ARE}$ mice. DRA protein and mRNA were strongly decreased and proved to cause this circumstance.

- in the inflamed proximal colon of $TNF^{+/\Delta ARE}$ animals, increased HCO_3^- output and epithelial permeability together with a loss of cation selectivity was observed. This was likely due to disturbances in tight junctional protein expression or formation.

HCO_3^- ions can pass the luminal membrane in various ways: either in exchange against another anion, through anion conductance or anion-selective pores in the tight junction complexes, or they can be generated from CO_2 that perfuses to the lumen. In most segments of the intestine,

the discrimination of paracellular in contrast to transcellular pathways is difficult or even impossible, since passive leakage would be masked by active HCO_3^- transport.

In Ussing chamber experiments, it is possible to discriminate between HCO_3^-/anion exchange and conductance by removing the dependent anion from the luminal solution. In the ileum and mid colon, we found a significantly lower HCO_3^- secretion in inflamed versus non-inflamed tissue. Further experiments revealed that this secretion was Cl^- dependent in the basal state, which pointed to an alteration of HCO_3^-/Cl^- exchange. Both probable apical proteins, DRA and PAT-1 (which is only weakly expressed in the colon, Wang et al. 2002) were found to be significantly downregulated on an mRNA level. However, PAT-1 knockout mice had no defect in ileal HCO_3^- secretion (Xiao F. and Singh A., unpublished observations), and since Yang et al. (1998) as well as Farkas et al. (2011) alike found a drastic decrease in the expression of DRA in UC patients and rodent colitis models, this exchanger can be considered to be the responsible one.

Concerning the reduced ΔI_{sc} in the ileum (which is an index of active Cl^- secretion, Clarke and Harline (1996)), CFTR mRNA expression was examined, but no changes were found. Other potential defects in HCO_3^- uptake or supply pathways in the Ileum were further explored, but did not give a distinct explanation. While Carbonic anhydrase IV (CA IV) mRNA was found to be downregulated in the inflamed mucosa, CA IV-deficient mice did not show any disturbances in ileal HCO_3^- -secretion. Thus, we assumed that disturbances in the signaling pathway for regulating the CFTR HCO_3^- conductivity during inflammation, or the overall villus shortening with reduction of epithelial surface area, may be explanations for the observed defect.

Cytokines like TNF-α and INF-γ are known to downregulate Na^+ absorptive mechanisms in cells (Amin et al. 2006 and 2011), and reduced Na^+ or fluid absorptive rates were also found in biopsies from IBD patients (Yeruva et al. 2011). When we measured the ileal and mid-colonic fluid absorptive capacity in anesthetized mice, a reduced capacity was found in the ileal mucosa of $TNF^{+/\Delta ARE}$. In contrast to that, the mid-colonic fluid absorptive capacity was unexpectedly well maintained even though the mRNA expression of TNF-α, INF-γ and IL-1β were higher in our inflamed animal model than those in the previously mentioned biopsies. Therefore we studied the epithelial ion channel ENaC, which is expressed in the mid to distal colon and reabsorbs Na^+ actively. In the distal colon, no change of ENaC activity could be detected between inflamed and healthy mice, and its transport rate could be further induced by a three week given low sodium diet. All three subunits of ENaC mRNA remained unchanged between the groups,

indicating a still intact response to aldosterone despite the inflammation. The studies of Bergann et al. (2009) which showed that that TNF-α may enhance glucocorticoid-induced ENaC expression via stabilization of glucocorticoid receptors, may explain this finding.
Neither ENaC nor NHE3 or CFTR were affected in mRNA expression which demonstrates a discriminating sensitivity of DRA to TNF-α induced inflammation. This sensitivity may possibly be related to the observed downregulation of DRA promoter activity by INF-γ (Saksena et al. 2010). An additional defect in ileal barrier function cannot be excluded, but is impossible to distinguish from the high amount of actively transported HCO_3^-.

However, in the rodent proximal colon the expression of the Cl^-/HCO_3^- exchanger DRA and PAT-1 was found to be almost negligible and very low, respectively (Talbot and Lytle 2010; Wang et al. 2002), and the rates of luminal alkalization are very small (Yu et al. 2009). The second part of the study was therefore addressed to the murine proximal colon which would permit measurements of possible passive leakage of HCO_3^- without the influence of an inflammation-associated alteration in active Cl^-/HCO_3^- exchange.
In this study we indeed found very low luminal alkalization rates in wildtype mice, but these were significantly higher in the inflamed mucosa of $TNF^{+/\Delta ARE}$ animals. Also, the expression of DRA mRNA was found to be unchanged between the groups and at a very low level. There was no change in the basal or forskolin stimulated I_{sc} and CFTR mRNA levels, which would have pointed to active transport. To rule out other possible transporters involved, we studied the role of NHE3, the apical Na^+/H^+ exchanger, which potentially could lead to a lesser luminal acidification if downregulated or impaired in function. Such dysfunction was shown in murine and human inflamed mucosa, despite normal mRNA and protein expression as well as localization (Seidler et al. 2006; Yeruva et al. 2010; Farkas et al. 2011). Here, NHE3 mRNA expression was unchanged. Additionally, the application of the NHE3 specific inhibitor S1611 did not lead to altered luminal alkalization of murine proximal colonic mucosa in Ussing chamber experiments, excluding a participation of NHE3.
TNF-α as well as other cytokines are known to cause disturbances in epithelial barrier function by various mechanisms, e.g. direct influence the expression or the intracellular localization of one or several claudins (Amasheh et al. 2010) or other tight junctional proteins. Occludin expression was shown to be downregulated in a micro-RNA-dependent manner when exposed to TNF-α (Ye et al. 2011). In Caco2 monolayers and the intact murine intestine, Al-Sadi et al.

2011 revealed that occludin regulates epithelial macromolecule flux, suggesting that it plays a crucial role in the maintenance of tight junction barrier. Since we found a significant occludin protein downregulation in the proximal colon of $TNF^{+/\Delta ARE}$ mice, this could explain not only our observed increased ^{3}H flux but also the increased HCO_3^- leakage.

We further found increased anion permeability with a decrease in cation selectivity in the inflamed mucosa. Adjusted ion selectivity was also reported for claudin-4 and -15, which, when expressed in Madin-Darby Canine Kidney (MDCK) cells and challenged with extracellular amino acids with reversed charge, increased their Na^+ selectivity or switched the preference from Na^+ to Cl^- permeability (Colegio et al. 2002). Several claudins have been linked to increased Cl^- permeability, like e.g. claudin-7, which was shown to promote paracellular Cl^- permeability when phosphorylated at the Ser^{206} phosphorylation site by WNK4 (Tatum et al. 2007). Several claudins are known to have splice variants, some with fundamental differences between these isoforms. Only recently, the splice variant claudin-10a, which is barely detectable in the proximal colon but evident amongst others in the nephron, was described as anion specific, while one of the other variants, claudin-10b was identified as cation selective (Krug et al. 2012).

In the $TNF^{+/\Delta ARE}$ mouse, we saw an upregulated expression of tightening (claudin-5) as well as pore-forming (claudin-2) claudins, but no apparent explanation for the decrease in cation selectivity. However, we did see a downregulation of occludin. Given the fact that the properties of the epithelial barrier function are massively influenced by the combination of the expressed claudins (Krug et al. 2012), it is possible that not a single protein causes higher HCO_3^- permeability in intestinal inflammation, but the combination of altered tight junction components.

In our two studies we showed that $TNF^{\Delta ARE}$ mice are a suitable model to study ion transport and epithelial barrier properties under mild inflammatory conditions. With its high expression of Th1 cytokines and its segmental inflammation pattern it resembles Crohn´s disease in humans, although there are limitations like in all established inflammatory mouse models (e.g. IL-10 knockout, transfer colitis or DSS colitis). However, similar to many CD patients, these mice do not have overt diarrhea. Therefore, our studies underline the fact that the absence of diarrhea does not necessarily indicate the absence of ion transport defects in inflamed colon, and that other pathophysiological aspects of disturbed transport function need to be considered.

The segment-specific expression pattern of transporters involved in HCO_3^- secretion (especially DRA) allows us to gain insights in defective transcellular pathways in the ileum and mid colon, as well as paracellular pathways in the proximal colon, where active transport occurs at extremely low levels. Although a defective barrier function is not unlikely to occur in other segments, it would be masked by active transport.

The relevance of defective HCO_3^- secretion or leakage in the lower gut may be manifold. In the upper digestive tract, the importance of HCO_3^- for mucosal protection against low luminal pH has been broadly studied (Shorrock and Rees 1988; Flemström and Sjöblom 2002). In addition, epithelial wound healing requires an alkaline surface pH both in the upper GI tract and in the colon (Feil et al. 1989, Semble and Wu 1989). Furthermore, intact HCO_3^- secretion has been shown to be essential for mucus secretion in intestine and airways (Garcia et al. 2009, Chen et al. 2010). In this context, an intact mucus layer has recently been shown to be crucial for the prevention of bacteria to attach to the colonic mucosa and elicit an inflammatory response (Johansson et al. 2010). A disturbed HCO_3^- secretion or leakage is most likely to change mucosal surface and luminal pH and thereby influence several circumstances, for example the acidic microclimate which is needed to drive the nonionic diffusion of week acids like SCFAs. Furthermore, a changed pH presumably affects the mucosa-associated hydrogenotrophic gut flora (Nava et al. 2012) as well as luminal flora (Duncan et al. 2009). A shift of this flora composition has indeed been observed during intestinal inflammation (van Nuenen et al. 2004), and are believed to be involved in IBD pathogenesis (Swidsinski et al.2005).

Taken together, our studies show that TNF-α-induced inflammation has differential effects on HCO_3^- output in the mildely inflamed ileum, proximal colon and mid colon of $TNF^{\Delta ARE}$ mice. The exquisite sesitivity of DRA expression and the resultant reduced ileal HCO_3^- secretion in the ileum and mid colon as well as the enhanced colonic luminal alkalization associated with a changed tight junctional protein expression cause a changed surface and luminal pH and thus influence epithelial pH homeostasis in chronic intestinal inflammation.

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Submitted Manuscript:

Singh AK, Xia W, Riederer B, **Juric M**, Li J, Zheng W, Cinar A, Xiao F, Bachmann O, Song P, Praetorius J, Aalkjaer C, Seidler U (2013). Essential role of the electroneutral Na^+/HCO_3^- cotransporter NBCn1 in murine duodenal acid/base balance and colonic mucus layer build-up *in vivo. J Physiol*

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Juric M*, Xiao F*, Amasheh S, May O, Wahl K, Bantel H, Manns MP, Seidler U, Bachmann O (2012/13). Increased epithelial permeability is the primary cause for bicarbonate loss in inflamed murine colon. *Inflamm Bowel Dis*
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Declaration

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

Segment-specific impact of TNF-α-induced inflammation on HCO_3^- homeostasis and epithelial barrier function in the murine intestine

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Marina Juric