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Impact of large intestinal pancreatic protease activity on intestinal barrier and colitis susceptibility in experimental models for IBD

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Abstract

Frequent antibiotic therapy, especially metronidazole and fluoroquinolone treatment, is associated with increased risk for Crohn´s Disease (CD). However, the causal relevance of antibiotic therapy and the pathophysiological mechanisms underlying potential adverse effects are still unknown. An increased protease activity (PA) in the lumen of the large intestine is considered to be a consequence of antibiotic-mediated disturbance of the intestinal microbial ecosystem. It is known that exposure of the colonic mucosa to abnormally high levels of serine proteases resulted in detrimental effects on the intestinal barrier *in vitro* and *ex vivo*. In the present study, the aim was to unravel a role of antibiotic therapy on intestinal protease activity and associated adverse effects on the barrier function in the large intestine and the development of colitis in susceptible hosts.

Patient stools were analyzed before and after different antibiotic treatments in regard to the protease activity, the profile of proteases and protease inhibitors, and the impact on epithelial barrier function *in vitro*. Wildtype (WT) and IL10-/- mice were treated with an antibiotic mixture (vancomycin/metronidazole; V/M) and additional serine protease inhibitor (AEBSF) in order to investigate the functional relevance of enhanced protease activity. To identify anti-proteolytic bacteria, cecal protease activity was investigated in germ free (GF) mice colonized with single bacteria or simplified microbial consortia. The impact of antibiotic-mediated high protease activity on the intestinal barrier function was measured via FITC-dextran translocation and by *Ussing* chamber analyses *ex vivo*. The mRNA and protein levels of tight junctions and protease-activated receptor-2 (PAR-2) activation were measured by qPCR, western blot and immunofluorescence staining in order to reveal the mechanism of the defective intestinal epithelial barrier. The impact of repetitive V/M treatment on the intestinal microbiota (16S rDNA sequencing), the protease activity and the development of colitis were investigated in IL10-/- mice and WT mice subsequently treated with dextran sulfate sodium (DSS).

Approximately 20% of all patients showed a substantial increase (>5-fold) in protease activity upon antibiotic treatment, which is detrimental to the epithelial barrier function *in vitro*. Protease activity in the large intestine was reduced in SPF mice, however altered intestinal microbiota of V/M treated mice abrogated the anti-proteolytic capacity, supporting the role of enteric bacteria on protease activity reduction. None of the single bacteria and complex microbial consortia transferred to GF mice reduced protease activity *in vivo*. While V/M treatment transiently increased cecal permeability in WT mice, V/M treated IL10-/- mice showed a consistently impaired barrier function in the colon. The impaired barrier was associated with dysregulated tight junction proteins such as occludin and ZO-1 through PAR-2 activation, and a subsequent proinflammatory response. Repetitive V/M treatment resulted in

alterations in the intestinal microbial ecosystem and increased protease activity but did not affect the severity of subsequent DSS-induced colitis in WT mice. However, in genetically susceptible IL10-/- mice, the V/M-mediated rise in protease activity was associated with the acceleration of colitis development.

Therefore, the present study provides experimental evidence that increased protease activity is caused by the eradication of anti-proteolytic bacteria in the large intestine upon exposure to antibiotic, which is clinically relevant. The enhanced protease activity impairs intestinal barrier integrity and accelerates the development of colitis and colonic cancer in genetically susceptible individuals, suggesting that the antibiotic-mediated high protease activity may be an independent risk factor for the development of Inflammatory bowel diseases.

Zusammenfassung

Häufige Antibiotika Therapie, speziell Metronidazole und Fluorochinolone Behandlungen, ist mit erhöhtem Risiko für Morbus Crohn (CD) assoziert. Die kausale Relevanz von Antibiotika Therapie und die pathophysiologischen Mechanismen, welche potentiellen negativen Effekten zugrundeliegen, sind jedoch noch unbekannt. Eine erhöhte Protease Aktivität (PA) im Lumen des Dickdarms wird als Konsequenz von Antibiotika-vermittelten Störungen des intestinalen mikrobiellen Ökosystems in Betracht gezogen. Es ist bereits bekannt, dass eine Stimulation mit hoher Serinproteasen zu schädlichen Effekten der intestinalen Barriere *in vitro* und *ex vivo* führt. Ziel der gegenwärtigen Studie war es, herauszufinden ob spezifische Antibiotika Therapie die PA erhöht und ob die Antibiotika-vermittelten negativen Effekte eine prädisponierende Ursache für die Beeinträchtigung der Barriere Funktion im Dickdarm und der Entwicklung von Kolitis in empfänglichen Wirten haben.

Patienten Stuhlproben vor und nach verschiedenen Antibiotika Behandlungen wurden bezüglich der PA, dem Profil von Proteasen und Proteaseinhibitoren und dem Einfluss auf die epitheliale Barriere Funktion verglichen. Wildtyp (WT) und IL10-/- Mäuse wurden mit einer Antibiotika Mischung (Vancomycin/Metronidazole; V/M) und zusätzlichem Serine Protease Inhibitor (AEBSF) behandelt um die Relevanz von erhöhter PA in Antibiotika behandelten Patienten zu untersuchen. Um anti-proteolytische Bakterien zu identifizieren, wurde zökale PA in keimfreien (GF) Mäusen, welche mit einzelnen oder einem vereinfachten mikrobiellen Konsortium kolonisiert wurden, untersucht. Der Einfluss von Antibiotika-vermittelter hoher PA auf die intestinale Barriere Funktion wurde via FITC-Dextran Translokation und Ussing Chamber Analysen *ex vivo* gemessen. mRNA und Protein Level von Zelladhäsionsproteinen und PAR-2 Aktivierung wurde mit Hilfe von qPCR, Western Blot und Immunofluorescence Färbungen bestimmt um den Mechanismus der defekten intestinalen epithelialen Barriere aufzuzeigen. Der Einfluss von wiederholter V/M Behandlung auf das intestinale Mikrobiom (16S rDNA Sequenzierung), die PA und die Entwicklung von Kolitis wurde in IL10-/- und WT Mäusen, welche anschließend mit DSS behandelt wurden, untersucht.

Ungefähr 20% aller Patienten zeigte einen bedeutenden (>5-fach) Anstieg der PA auf Antibiotika Behandlung, was nachteilig für die epitheliale Barriere Funktion *in vitro* ist. Frische intestinale Mikrobiota von SPF Mäusen erniedrigte die PA, wohingegen die veränderte intestinale Mikrobiota von V/M behandelten Mäusen die anti-proleolytische Kapazität aufhob. Keine der einzelnen Bakterien und komplexen mikrobiellen Konsortien reduzierte die PA *in vivo*. In WT Mäusen erzeugte die Erhöhung der PA als Antwort auf V/M lediglich einen transienten Anstieg in zökaler Permeabilität, wohingegen IL10-/- Mäuse eine andauernde Beeinträchtigung der Barriere Funktionen im Dickdarm durch PAR-2 Aktivierung, Dysregulation von Zelladhäsionsproteinen, wie Occludin und ZO-1, sowie einer

anschließenenden proinflammatorischen Reaktion zeigten. Wiederholte V/M Behandlung resultierte in Veränderungen des intestinalen mikrobiellen Ökosystems und erhöhter PA, beeinflusste die Schwere der nachfolgenden DSS-induzierten Kolitis in WT Mäusen jedoch nicht. In genetisch empfänglichen IL10-/- Mäusen erzeugte der V/M-vermittelte Anstieg der PA eine Beschleinigung der Kolitis Entwicklung.

Die gegenwärtige Studie verschafft experimentellen Beweis, dass ein Anstieg in PA durch Ausrottung von antiproteolytischen Bakterien im Dickdarm aufgrund von Antibiotika Exposition hervorgerufen wird und somit klinische Relevanz besitzt. Die erhöhte PA beeinträchtigt die intestinale Barriere Integrität und beschleunigt die Entwicklung von Kolitis und Colonkrebs in empfänglichen Individuen, was Antibiotika-vermittelte hohe PA als unabhängigen Risikofaktor für die Entwicklung von IBD suggeriert.

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1 Introduction

1.1 IBD and microbiota

1.1.1 Etiology of IBD

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are complex chronic diseases in the gastrointestinal tract associated with dysregulation of intestinal immune response towards the intestinal microbiota and environmental factors. Whereas patients with UC exhibit inflammation and ulcers only from the cecum to the rectum, CD occurs in any part of the gastrointestinal tract. [1] Pathological symptoms of IBD include diarrhea, lower gastrointestinal bleeding, abdominal pain, weight loss and fever as is common for a chronic bowel-relapsing inflammatory disease. [2] Treatments for IBD target different inflammatory mechanisms in order to reduce or prevent acute flares of these chronic inflammatory diseases. State-of-the-art medical therapies comprise the use of aminosalicylates, anti-inflammatory steroids, non-steroidal anti-inflammatory drugs (NSAID) or biologics. Incidence of IBD is increasing all over the world. In North America, the incidence rate of IBD is 20-40 per 100,000 persons. [3] A steady rise in incidence and prevalence of IBD is seen in developing countries such as Asia and Latin America. [4]

The etiology of IBD involves a complex interplay of genetic susceptibility, [5, 6] environmental factors (e.g. diet, geography, smoking, stress and medications), [7–11] intestinal microbiota [12, 13] and immune response. [14, 15] Current genome-wide association studies (GWAS) identified more than 200 susceptibility loci for IBD in over 75,000 patients, [16, 17] indicating that genetic variants may affect the intestinal immune homeostasis through various mechanisms such as defects in immune regulatory mechanisms (e.g. interleukin 10) or defects in the innate immune defense (e.g. nucleotide-binding oligomerization domain containing protein 2 (NOD2) gene variants). [18, 19] A subsequent meta-analysis of GWAS found that around 30% of genetic loci in UC coincided with CD. [20] Additional genetic loci implicated in IBD were associated with intestinal immune homeostasis including microbial defense, immune regulation pathway, autophagy and cytokine receptor signaling. [21] However, it is important to realize that a single genetic locus of these IBD susceptibility genes cannot be used to predict the development of IBD in a given individual. There have been many advances in understanding the environmental risk factors related to the pathogenesis of IBD. Several studies consistently demonstrated that smoking is associated with a more severe disease course and more frequent relapse in CD patients, presumably via a nicotine-mediated increase in the plasmacytoid dendritic cells (DCs) and Th1 cells. [22, 23] Similarly, dietary fat consumption is a high risk factor for the development of IBD. [24, 25] Especially, the western diet consumption involving low fiber and increased intake of total

fat contributes to the development of IBD. Psychological stress also affects the immune functions, resulting in an increased risk of developing IBD. [26, 27] In the last decade, it became more and more clear that the intestinal microbial ecosystem plays an important role in the development and maintenance of intestinal immune homeostasis. [28] Currently, the focus of scientific and medical research is to unravel the impact of the intestinal microbiota on the development and progression of IBD.

1.1.2 Microbe-host interaction in IBD

The gastrointestinal tract contains about 10^{11} (bacteria/g wet stool) diverse microorganisms including bacteria, viruses, archaea and eukaryotes (yeasts and protozoa). [29, 30] The human intestinal microbiota is estimated to comprise approximately 1,500 different bacterial species, and is composed of the phyla *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* which account for about 99% of all bacteria in the intestine. [31, 32] The intestinal microbiota plays an essential role in different aspects of host physiology. [33, 34] The intestinal microbiota affects host metabolism, intestinal homeostasis, development and regulation of the immune system. [35–37] Alterations in the intestinal microbial ecosystem have been found to be associated with metabolic diseases such as diabetes, atherosclerosis and obesity. [38–41] Some bacterial species contribute to nutrient digestion e.g. via indirect regulation of host enzymes and metabolic pathways or through secreted bacterial enzymes. [42, 43] Other bacterial species metabolize dietary fiber into short chain fatty acids (SCFA), which have been shown to affect the metabolism and immune functions of the host. [44, 45] In addition, the microbiota degrades complex substrates such as plant-derived polysaccharides, [46] synthesis of vitamins and absorption of calcium and magnesium. [47–49] The intestinal microbiota is a highly diverse ecosystem which plays a role in the bile acid metabolism and degradation of pancreatic proteases in the large intestine. [50, 51] Furthermore, the presence of a diverse microbial ecosystem in the intestine is of major importance to prevent the proliferation and colonization of pathogenic microorganisms, not only via direct competition for pivotal substrates but also by the stimulation of the host to produce antimicrobial peptides and IgA.[52–54] Importantly, the development and functionality of the immune system in the intestine is strongly dependent on the presence of intestinal microorganisms. GF mice have deficits in the number of immune cells in the gut associated lymphoid tissue. [55] Specific bacterial species promote the maturation and expansion of T lymphocytes, the regulation of DCs and Th17 cells. [56–58] In addition, intestinal microorganisms were found to promote immune tolerance mechanisms, e.g. via the prevention of NF-kB activation, the detoxification of LPS or the downregulation of endotoxin signaling. [59–61]

The intestinal microbiota plays a pivotal role for the onset and progression of IBD. Several studies revealed that alteration of microbiota composition, repeatedly reduced abundance of *Bacteroidetes* and *Lachnospiraceae*, [62] and increase in *Gammaproteobacteria* and *Enterobacteriaceae* was shown in UC and CD patients. [63, 64] This implicates the triggering of inflammation in disease susceptible hosts. In other studies, depletion of *Faecalibacterium prausnitzii* was observed in CD patients compared to healthy people, suggesting that anti-inflammatory properties of *F. prausnitzii* prevents ileitis development. [65] Thus, compositional changes in the intestinal microbiota induce chronic inflammation and IBD development. **IBD are characterized by a vicious circle of inflammation, impaired barrier function and microbial dysbiosis in the intestine** (Figure 1). **However, the initial triggers leading to chronic inflammation is unknown and may differ between patients**.

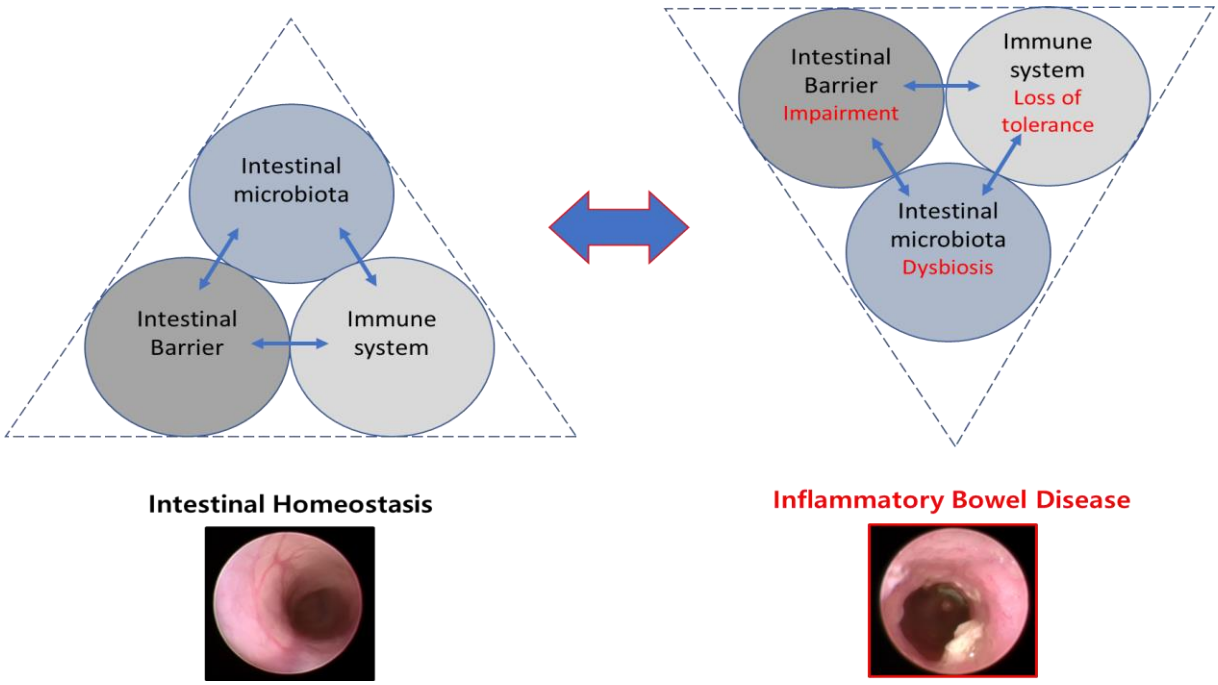

Figure 1: Characterization of inflammatory bowel diseases
Maintenance of intestinal homeostasis depends on a complex interplay between the intestinal microbiota, the intestinal barrier and the immune system. Dysfunction in one or more of these pivotal factors may lead to loss of immune tolerance towards the intestinal microbiota, resulting in chronic intestinal inflammation.

1.2 Antibiotics-mediated alteration of microbiota and IBD

1.2.1 Antibiotics and microbial dysbiosis

The short-term administration of antibiotics transiently alters the composition of the intestinal microbiota, but after ceasing antibiotic treatment, the disturbed intestinal microbiota is commonly normalized. [66, 67] Infrequent use of antibiotics does not induce immediate health problems. [68] Controversially, the long-term antibiotic treatment is well known for disrupting the ecological balance

in the bacterial community, [69] potentially leading to the selection of resistant opportunistic pathogens which cause acute or chronic disease . [70, 71] For example, clindamycin treatment caused long-term alteration in the microbial community, resulting in a subsequent susceptibility to *C. difficile* infection. [72] Vancomycin treatment had a long-lasting detrimental impact on the intestinal microbial ecology and susceptibility to any secondary infections. [73, 74] Several studies showed a loss of resilience in the gut microbiota after administrating antibiotics. [75, 76] Jernberg et al. showed that the long-term compositional alteration in the microbiota persisted for more than one year after antibiotic therapy. [77] Another study showed that antibiotics resulted in a substantial loss of microbial diversity and specific taxa, while the resistant opportunistic pathogens and resistance genes were increased. [76] A rapid perturbation of intestinal microbial ecosystem by antibiotic in early-life affects the development of inflammatory disorders [78] and accelerates metabolic diseases. [79] Recently, development of multi-omics analysis such as transcriptomics, proteomics and metabolomics for microbial community profiling demonstrated that antibiotics affect the microbial community structure and function such as gene expression, functional activity and overall metabolism. [80, 81] Ampicillin treatment decreased microbial diversity in the intestine and greatly increased *Enterobacter spp*. [82] Ciprofloxacin treatment resulted in a reduction in *Enterobacteria*, bacterial diversity and SCFA production. [66, 83] Especially, treatment of patients with antibiotics in early-life has a major impact on health during later years. For example, treatment of infants with antibiotics leads to a reduction in the microbiota diversity. [84] Therefore, antibiotic treatment may induce compositional and functional disturbances, potentially promoting development of various diseases.

1.2.2 Clinical relevance of antibiotic in IBD

The use of antibiotics in IBD patients appears to lead to conflicting effects. There is only little evidence showed that a clinical benefit in the treatment of IBD especially when the patient has a clinical symptom such as septic complications of IBD, pouchitis, abscesses and toxic megacolon. [85, 86] Even though the reduction of intestinal inflammation was observed in CD and pouchtis patients treated with antibiotics, several clinical studies showed that not only antibiotics have shown success in a limited number of CD patients, but also this was even less conclusive in UC. [87–89] Moreover, there was limited evidence that antibiotic therapies such as ciprofloxacin and metronidazole showed a remission of active CD or even prevention of CD recurrence. [87] Another study showed that the treatment of sulfasalazine was effective in patients with mild to moderate UC. [90] Considering the limited evidence and unclear effectiveness, antibiotic use is only recommended in the case of infection or ileal pouch surgery. [91]

Contradictory to the clinical benefits, negative effects of antibiotics in the context of IBD have been reported as well. [92] Except for the effective prevention of pouchitis after ileal pouch anal anastomosis, there are conflicting data on the therapeutic relevance of antibiotic treatments in CD and UC patients. [93, 94] A frequent side effect of antibiotic treatment in IBD patients was an increase in antibiotic resistance to ciprofloxacin, vancomycin and rifaximin. [95–97] Especially, after frequent metronidazole treatment, adverse effects on gastrointestinal and peripheral neuropathy have been reported. [98] Recently, several studies showed that antibiotic treatment of IBD patients was associated with an increase in risk for *C.difficile* infection and recurrence of IBD. [99, 100] Another adverse effect associated with the exposure of antibiotics, especially in childhood, is the risk of developing IBD later in life. [101] For instance, antibiotic exposure in the first year of life significantly increased pediatric IBD, diagnosed at the age of 8 years. [10] A population based cohort study showed that antibiotic therapies in early-life increased the ratio of pathogenesis of CD in adults. [102] In particular, either metronidazole or fluoroquinolone treatment is suspected of contributing to disease pathogenesis of new-onset CD. [103] A recent study revealed that the treatment age, repetition time and specific class of antibiotic were associated with early-onset of IBD. [104] Shaw et al. showed that the use of metronidazole was strongly connected with the development of IBD rather than that of other antibiotics. [105]

Considering these adverse effects, the use of antibiotics might be rather detrimental than protective for IBD patients in the long term. However, the pathophysiological mechanisms of both the development and the recurrence of intestinal inflammation upon discontinuation of antibiotics treatment have not yet been investigated.

1.3 Physiological aspect of proteases in gastrointestinal tract

1.3.1 Type and role of proteases in GI tract

The gastrointestinal (GI) tract contains the highest amount of proteolytic enzymes from host and enteric bacteria classified as serine, threonine, cysteine, aspartic or metalloproteases (Table 1 and Figure 2). [106, 107] The proteases can be classified into two subtypes according to their degradation sites. While exopeptidases cleave their substrates at the end of C-terminus, endopeptidases degrade in the middle of proteins. The proteases in the GI tract have different origins and are either produced by the host tissue or the microbiota (Table 1). First, host proteases consist of digestive proteases, circulation proteases and brush-border proteases. Digestive proteases, such as trypsin, chymotrypsin and elastase, are secreted from the pancreas and released in the lumen of the GI tract. [108] Circulation proteases e.g. plasminogen are mostly produced by the liver and endothelial cells in blood

vessels, and reach the GI tract. The major role of plasminogen is plasmin cleavage. Brush-border proteases e.g. enteropeptidase play an important role in digesting the dietary proteins in the lumen of intestine, especially through conversion of inactive trypsinogen into active trypsin. Second, bacterial proteases are also present in the lumen of the GI tract. Most bacterial proteases in feces originate from *Bacteroides, Streptococcus*, and *Clostridium* species. [109] *Enterobacteriaceae* family such as *Neisseria, Shigella, Citrobacter rodentium* and pathogenic *Escherichia coli* produce serine protease autotransporters associated with cytoskeleton stability, autophagy or innate and adaptive immunity. [110] *E. faecalis* secretes a gelatinase being implicated in the degradation of collagen, fibrinogen, fibrin and complement components C3. [111, 112] *P. gingivalis* produces gingipains K and R (Kgp and Rgp). [113] *S. pyogenes* secretes SlyCEP associated with necrotizing fasciitis lesions. [114] Third, the cellular proteases, which are secreted from resident immune cells and intestinal epithelial cells (IEC), play a role in modulating the bioactivity of inflammatory mediators connected with the infiltration and activation of inflammatory cells. [115] The major proteases from mast cells are tryptase and chymase. The macrophage produces different subtypes of proteases such as matrix metalloproteases sub-family, caspase, and cathepsins. Elastase and cathepsin G5 are specifically produced by neutrophils. [116] The IECs produce matriptase and matrix metalloproteases. IECs are affected by the different sources of endogenous and exogenous proteases.

Dysregulation of proteolytic homeostasis in the GI tract induces physiological and intracellular effects relevant to matrix remodeling, mucus degradation, impairment of epithelial barrier, cellular homeostasis, receptor activation and inflammatory mediator processing (Figure 2). [117] Active proteases cleave the extracellular N-terminal domain of protease-activated receptors (PAR) on the apical and basolateral sides of IECs, [118] resulting in the regulation of physiological functions such as barrier integrity and gastrointestinal motility. [119, 120] Active proteases regulate inflammatory cytokines such as IL1β, IL8, IL18 and TNFα, [121, 122] and chemokines such as the epithelial-derived neutrophil-activating peptide 78. [123] Active proteases directly and indirectly influence cell to cell integration through modulating tight junction proteins, [124] and cleave the adherent junctional protein. [119, 125] Proteases, derived from digestive enzymes such as trypsin, and from bacteria such as *Akkermansia muciniphila* and *Porphyromonas gingivalis,* degrade mucins composing the mucus. [126–128] **The different proteases contribute to cell proliferation, tissue homeostasis and immune response in the intestine.**

Table 1: Classification and origin of host and bacterial proteases

	Host protease		Microbial protease	
Protease category	**Origin**	**Protease**	**Origin**	**Protease**
Aspartic	Intracellular	Cathepsin D	*C.albicans*	Secreted aspartic
	Matrix, Plasma, Intracellular	Renin	*P.aeruginosa, E. coli*	Type 4 prepilin
			Archaeal bacteria, *M.voltae*	Preflagellin
Cysteine	Intracellular	Caspases	Gram positive bacteria, E.*faecalis*	Sortases
	Matrix, Plasma	Cathepsins (B, L)	*P.gingivalis*	Gingipain
	Intracellular	Autophagins	*S.aureus*	Staphopain
	Intracellular	Calpains		
	Intracellular	Deubiquitinases		
Metalloprotease	Matrix, Plasma, Intracellular	MMPs	*B.fragilis*	Fragilysin
	Matrix, Plasma, Intracellular	ADAMTS	*E.faecalis*	Gelatinase
	Intracellular	Deubiquitinases	*S.epidermidis, H.pylori, P. aeruginosa*	Elastase
			C.perfringens, S.typhimurium	Collagenase
Serine	Luminal, Matrix, Plasma, Intracellular	Elastases	*C.difficile*	Subtilisin
	Luminal, Matrix, Plasma, Intracellular	Proteinase-3	*P.aeruginosa*	Elastase
	Luminal, Matrix, Plasma	Chymase	*H.pylori*	High temperature requirement A
	Luminal, Matrix, Plasma	Kallikreins	*B.subtilis*	Subtilisin
	Luminal, Matrix, Intracellular	Granzymes		
	Luminal, Matrix, Plasma	Tryptase		
	Matrix, Plasma	Plasminogen activator		
	Luminal, Matrix, Plasma	Trypsins		
	Luminal, Matrix, Plasma, Intracellular	Cathepsin		
	Luminal, Matrix, Plasma	Thrombin		
	Luminal, Matrix, Plasma	Factors V and VIII		
	Matrix, Intracellular	Matriptase		

1.3.2 Characteristics of pancreatic proteases in GI tract

The most abundant proteases in the GI tract are digestive enzymes which are synthesized in the pancreas, secreted as inactive zymogens into the lumen of the duodenum, and activated by enteropeptidases. Afterwards, digestive proteases are inactivated by autocatalysis passing through the GI tract. In the small intestine, dietary proteins are digested into small peptides and amino acids by digestive proteases. Several studies provided further evidence that digestive proteases are continuously self-degraded passing through the GI tract. [129, 130] Proteases activity in the ileum is 20-60-fold greater than in feces, [109, 131, 132] indicating that pancreatic proteases are disrupted more in the large intestine than in the small intestine. While high amounts of digestive proteases (1-3g trypsin analogues and 0.5g elastase) are secreted from the pancreas in humans, [133] large amounts of these proteases are already inactivated in the feces. Analogous to the observation in humans, cecal protease activity in different animals were higher than ileal protease activity. [134, 135] One study showed that a substantial proportion of fecal protease activity was still observed in patients who failed

to produce digestive enzymes due to exocrine pancreatic insufficiency, [136] suggesting that bacterial proteases (e.g. cell-bound proteases of *Bacteroides fragilis*) are also part of luminal proteases. Inactivation of digestive proteases rapidly and widely occurs at the transition between the ileum and cecum, indicating that **the small intestine is exposed to high amounts of active pancreatic proteases, while the large intestine has a low pancreatic protease activity**.

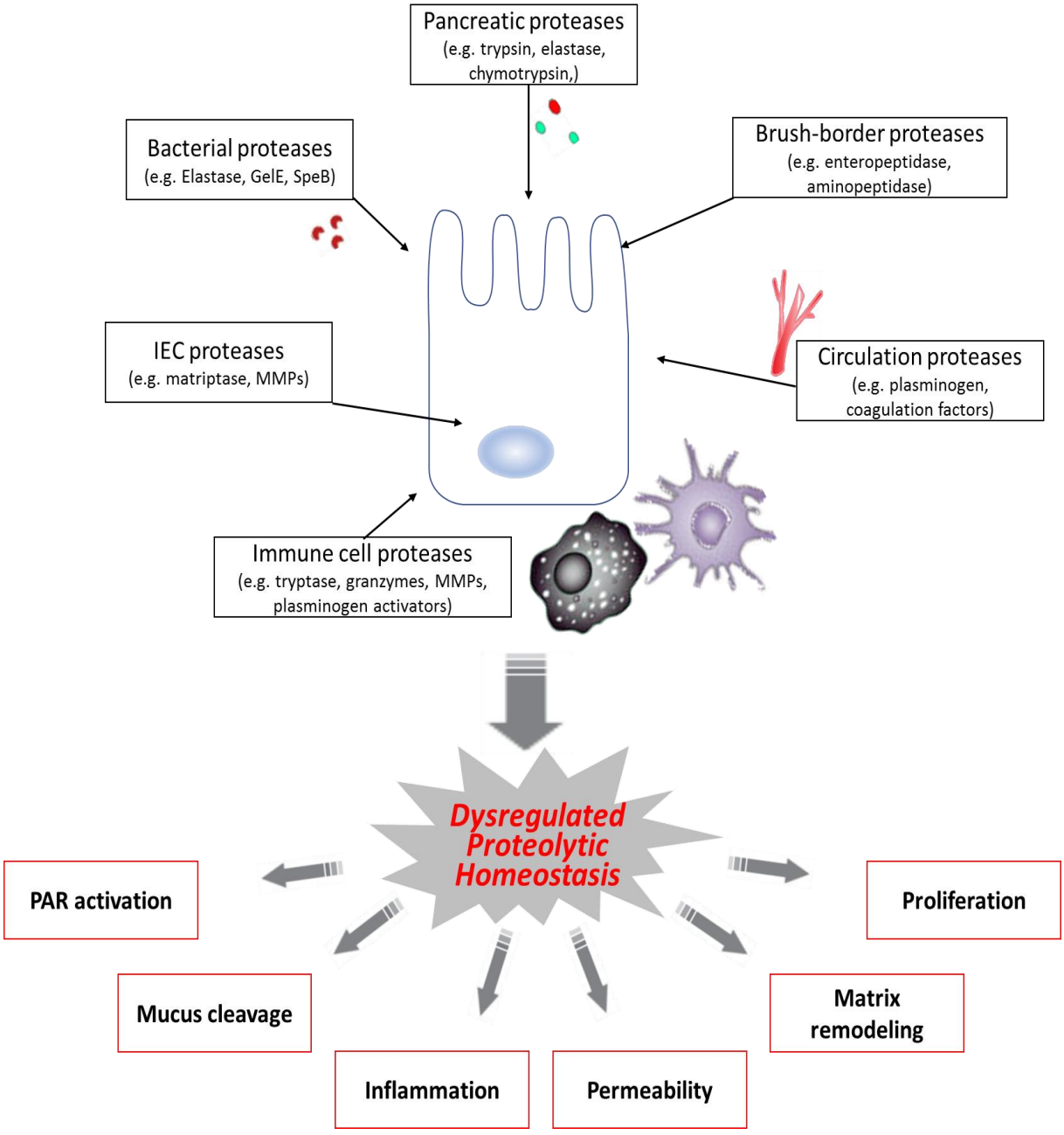

Figure 2: Major identified proteases and mechanism of action in the gastrointestinal tract
Major proteases are identified from luminal, bacterial, intracellular and extracellular milieus in the gastrointestinal tract. The figure illustrates role of proteases in the intestinal homeostasis. IEC, intestinal epithelial cell

1.3.3 Microbial regulation of pancreatic proteases in GI tract

In the large intestine, intestinal microbiota is pivotal for the regulation of proteolytic homeostasis. [136] It is well known that colonic protease activity in germfree (GF) mice was higher than colonic protease activity in conventional mice, suggesting that enteric bacteria modulate host pancreatic proteases. [137] Other studies provided similar evidence that chickens, rats and rabbits in the gnotobiotic facility enhanced protease activity in the cecum compared to the animals in conventional housing. [134, 135, 138]

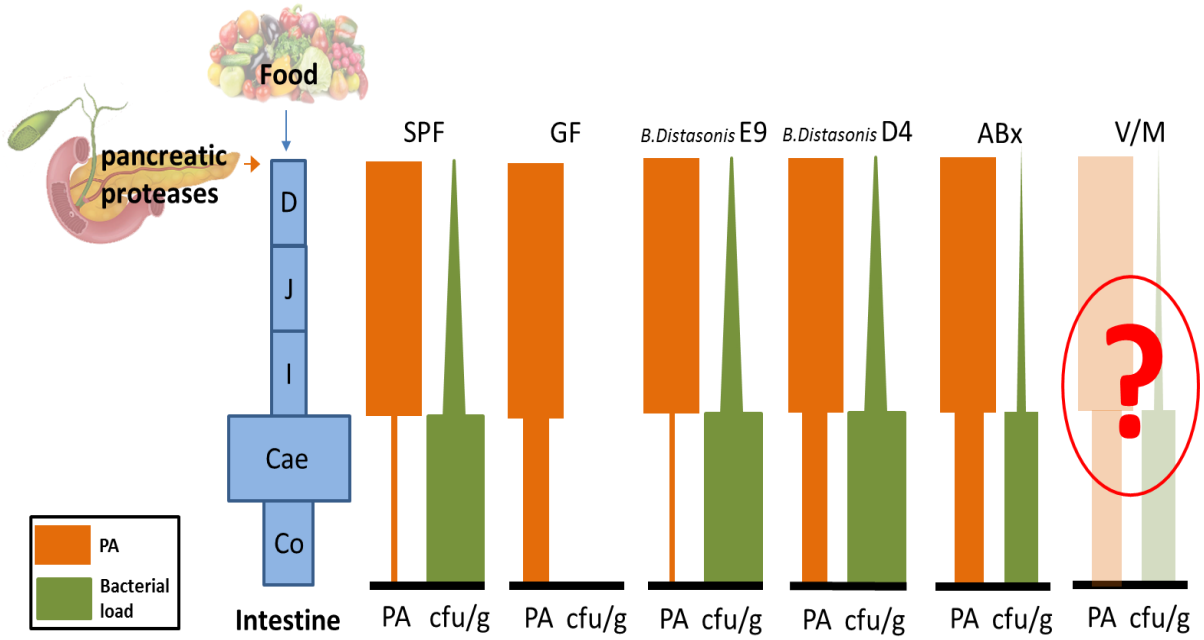

Figure 3: Specific bacteria regulates the pancreatic proteases in the large intestine
GF mice are characterized by a high pancreatic PA in the large intestine. A complex microbiota (e.g. SPF microbiota) or *P. distasonis* strain E9 mediate the inactivation of pancreatic proteases in the large intestine. Antibiotic therapy that mediates the eradication of these anti-proteolytic commensals results in a major rise of the pancreatic protease activity in the large intestine. PA in V/M treatment still undiscovered. PA: proteolytic activity, D: duodenum, J: Jejunum, I: ileum, Cae: caecum, Co: colon, SPF: specific pathogen free, GF: germ free, ABx: antibiotics, V/M: vancomycin/metronidazole

From this point of view, however, not every microbiota contributes to physiological regulation of pancreatic proteases in the large intestine. [139] A disturbance of intestinal microbiota in rats by benzylpenicillin, ampicillin, doxycycline, or clindamycin treatment increased cecal protease activity to the level observed in GF rats compared to conventional rats. [140] A similar result has been reported concerning clindamycin treatment influencing a long-lasting increase in protease activity in rats. [141] Analogous to the animal studies, a clinical study revealed that antibiotic treatments elevated trypsin

and elastase in the feces, resulting in altered microbiota-dependent inactivation of pancreatic proteases. [142] Additionally, a quantitative analysis of pancreatic proteases in feces showed that antibiotic treatment in patients strongly increased trypsin (100-fold), chymotrypsin and elastase 2 (2-3-fold) concentration compared to untreated persons. [143] **Specific antibiotic treatment has been reported to result in a substantial increase of pancreatic proteases, mostly trypsin in the large intestine**. This major increase in pancreatic protease activity in the large intestine is thought to be due to the eradication of specific microbes which are essential for inactivation of the high load of digestive proteases in the large intestine under physiological conditions (Figure. 3). **The commensals which have the ability to the commensals that modulate pancreatic proteases in the large intestine are so far mostly unknown except for *Parabacteroides distasonis* E9**. [139, 144, 145] A single human-derived *P.distasonis* strain E9 has been proven to normalize the high protease activity in GF rats in a monoassociation experiment, while colonization of *E.coli* in GF minipigs and rats did not decrease protease activity in the large intestine. [146] Although *P.distasonis* E9 had been proven to normalize the protease activity *in vivo*, the underlying anti-proteolytic mechanism of microorganisms is unknown. It is unclear whether the reduction of the high load of trypsin in the large intestine is due to direct degradation or secretion of protease inhibitors. Thus, the question **which kind of antibiotic disturbs the proteolytic balance still has to be addressed.**

1.4 Regulation of physiological barrier and protease activity in the large intestine

1.4.1 Intestinal barrier and junctional proteins

The intestinal epithelium consists of a single layer of epithelial cells that separate the intestinal lumen from the underlying lamina propria. The single layer of epithelial cells is renewed every 4-5 days and represents a physical barrier. [147] Pluripotent intestinal epithelial stem cells permanently self-renew, and thus, regenerate all lineages of differentiated intestinal epithelial cells. [148] All differentiated IECs migrate from crypt bottoms up along the villus structures and are released into the luminal space at the villus tips. More than 80% of IECs are absorptive enterocytes, adapted for metabolic and digestive functions, and the remaining 20% are either enteroendocrine cells, goblet cells, microfold cells (M cells) or Paneth cells. [149] The role of the intestinal barrier involves biochemical, immunological, and physical barrier functions which maintain the mechanical integrity of the barrier through the formation of the proteins complex between epithelial cells. The intestinal epithelium selectively absorbs dietary nutrients and water, and prevents the invasion of pathogenic antigens and microbiota. The intestinal barrier selectively regulates transcellular permeability, which is associated with transporting the amino acids, ions and SCFAs, microorganisms, and their molecules in the area between adjacent epithelial

cells. [150, 151] The transcellular pathway requires active transport mechanisms through selective transporters, pumps and channels localized on the apical and basolateral plasma membrane. In contrast, the paracellular pathway is dynamically regulated by the intracellular apical junctional complex. [152] Desmosomes, adherence junctions, gap junctions and tight junctions constitute the protein complex in para-cellular space between epithelial cells (Figure 4). [153, 154] **These junctional proteins allowing exchange of various molecules and organisms are important for the epithelial permeability,** and are linked to the perijunctional actomyosin ring which is a regulatory factor for paracellular permeability. [155, 156] Functional and structural regulation of these junctional proteins is mediated by the contraction of actin cytoskeleton through the phosphorylation of the myosin light chain in the epithelial cells. [157] The paracellular permeability is influenced by the intestinal microbiota, their molecules and cellular specificity, hence the intestinal barrier adapts to physiological and pathological circumstances. [158–160]

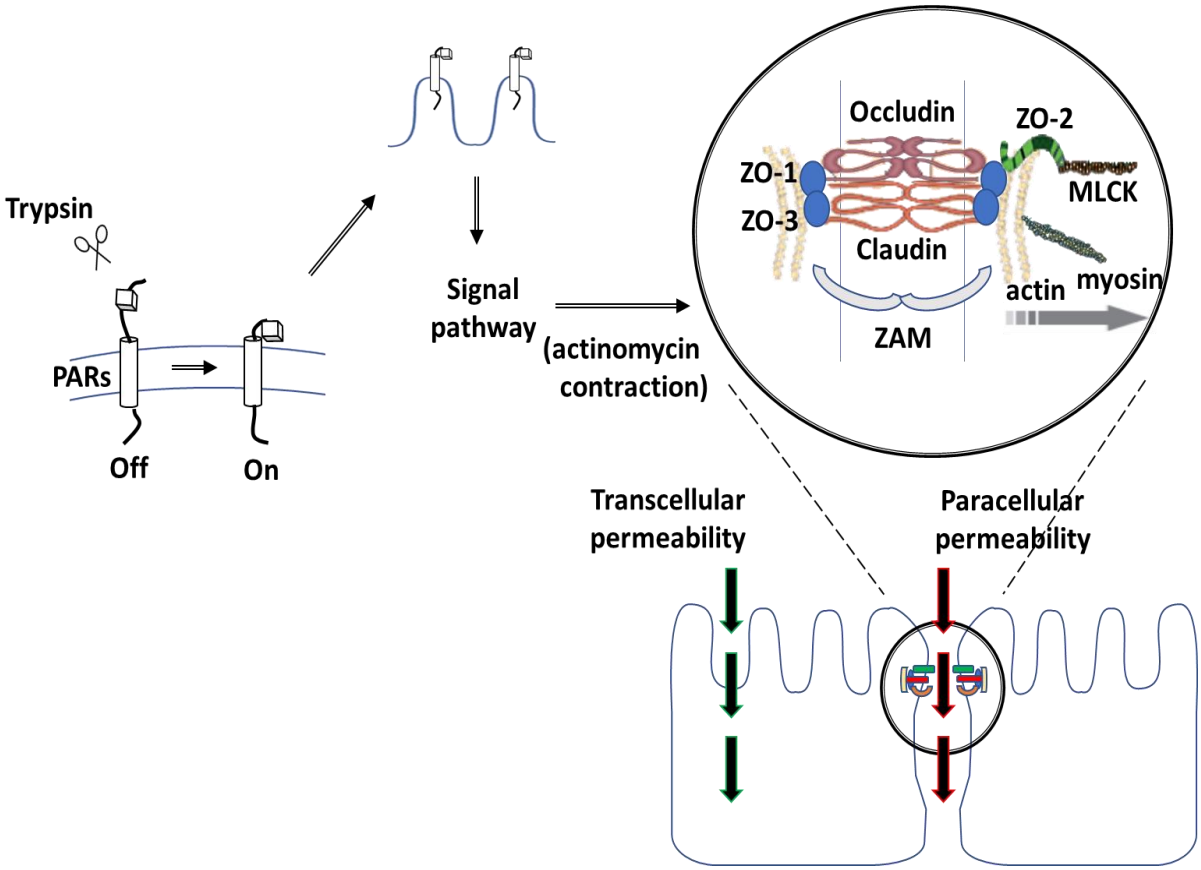

Figure 4: Overview of tight junction and regulation mechanism of paracellular permeability
Tight junctional proteins such as occludin, ZO, claudin and ZAM play a role in regulating paracellular permeability in the intestinal epithelial cells. PARs activated by proteases induce signal pathways which trigger the opening of tight junctional proteins through the phosphorylation of myosin light chain (pMLC). The pMLC is regulated by myosin light chain kinase (MLCK) which is associated with actin/myosin contraction and junctional protein regulation. PARs: protease-activated receptor, ZO: zonula occludens, ZAM: junctional adhesion molecule

Adherence junctional proteins are located under the tight junctions and formed on the lateral membrane between the epithelial cells and by interactions between cadherin and catenin superfamilies. [161, 162] Additionally, adherence junctions also interact with cytoskeleton proteins through intracellular adaptor proteins. [163, 164] The role of adherence junctional proteins such as E-cadherin together with desmosomes is the mechanical regulation of adjacent cells strength. [165] Interaction between cadherin and catenin is associated with the maintenance of cell polarity, [166] migration and homeostasis. [167, 168] Dysregulated E-cadherin leads to the leaky barrier through light cellular adhesion and the disturbance of cellular proliferation. [169, 170]

The paracellular pathway for barrier functions is maintained by apical tight junctional proteins such as claudin, occludin, zonula occludens-1 (ZO-1) and junctional adhesion molecule (JAM). [171] These proteins are located between the apical and lateral membrane regions and have a highly dynamic structure, capable of being constantly remodeled. [160] The transmembrane proteins such as claudin and occludin are connected to the zonula occludens family, which is linked with the actin cytoskeleton, in order to regulate interepithelial permeability of the intestinal barrier. [154, 172] Similar to adherence junctions, tight junctional proteins regulate cellular polarity, signaling and vesicle trafficking. [173] Regarding the function of tight junctional proteins, cleaved occludin increased paracellular permeability when the allergen DerP1 disrupted occludin through proteolytic cleavage. [174] Interestingly, phosphorylated occludin led to disrupted ZO-1, resulting in the impairment of barrier functions. [175] JAM-A deficient mice demonstrate that JAM-A plays a role in formation and assembly of tight junctional proteins, and maintenance of barrier integrity. [176] Therefore, **the complex regulation of tight junctional proteins is highly related to barrier functions in the intestine.**

1.4.2 Protease-activated receptors and barrier functions

The GI tract contains the highest amount of proteases that activate PARs in the lumen of the intestine. In addition to direct activation of PARs, the intestinal luminal proteases indirectly engage in proteolytic cleavage of junctional proteins. [177] PARs consist of 7 transmembrane domain G-protein coupled receptors with 4 identified family (PAR1~4), [178] and activated by proteolytic cleavage of N-terminal termini, thereby binding the second loops on the amino terminus and activating signaling cascades (Figure 5). [179, 180] PARs are present on epithelial, neuronal and inflammatory cells, [181, 182] and play a role for intestinal barrier integrity, neuronal activation and immune regulation. [178, 183, 184] Activation of PAR-1 by thrombin and PAR-2 by trypsin analogues are associated with barrier functions in the intestine. [180, 185] While PAR-1 activation regulates epithelial and smooth muscle functions in

the intestine, PAR-3 and 4 are associated with neutrophil functions rather than epithelial barrier regulation. [181, 186]

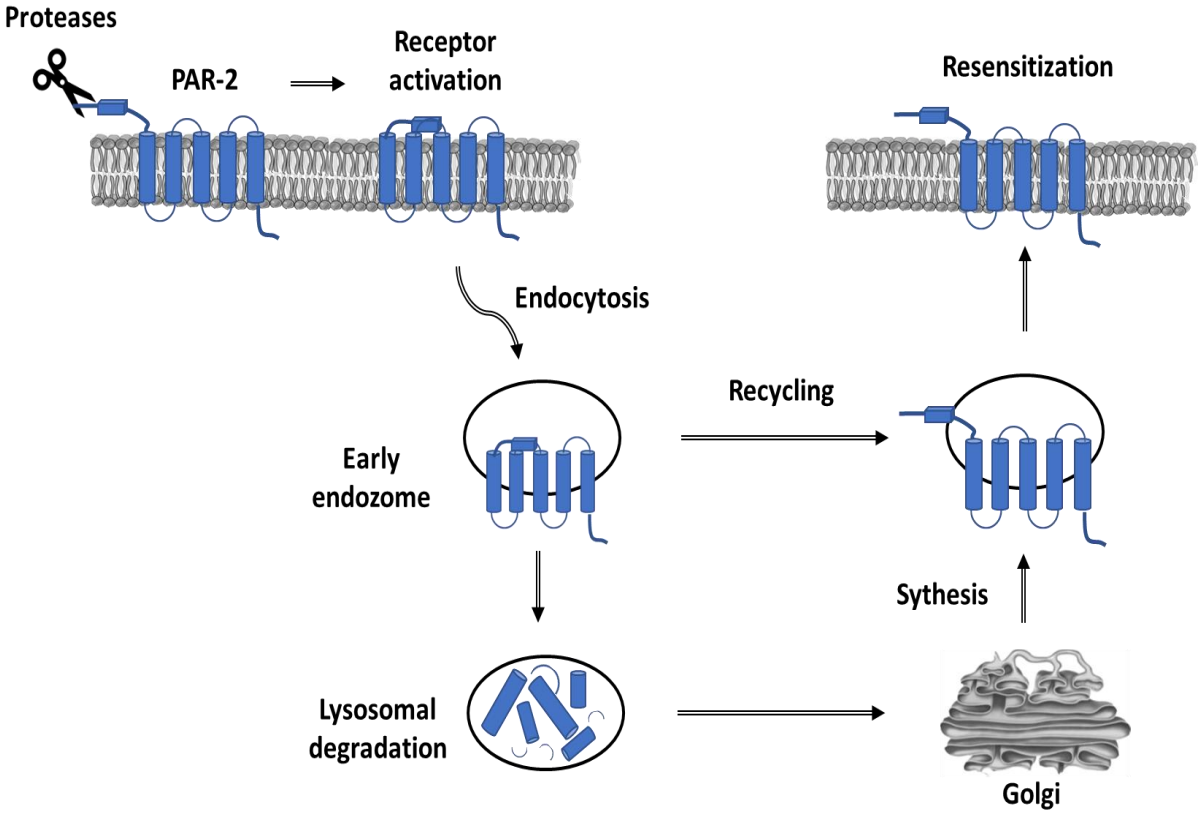

Figure 5: Mechanism of PAR-2 activation
PAR-2 is activated by agonist degradation of N-terminal extracellular domain and then subjected to endocytosis. The internalized receptor is recycled or subjected to lysosomal degradation and then synthesized in Golgi pools. Finally, the recycled or re-synthesized receptor is relocated in extracellular domain.

PAR-2 activation affects epithelial permeability, motility and immune regulation. [187] Several studies showed that apical and basolateral activation of PAR-2 mediate different signaling cascades; apical PAR-2 is activated by trypsin, matriptase and bacterial serine proteases, [188, 189] while basolateral activation is induced by cellular tryptase. [190] The stimulation of PAR-2 agonist SLIGRL revealed that PAR-2 activation increases the intestinal permeability in the apical and basolateral sides. [191] Phosphorylation of myosin light chain induced by SLIGRL-mediated PAR-2 activation is also linked to promoting paracellular permeability. [192] PAR-2 promotes ERK1/2 activation belonging to the mitogen-activated protein kinases, resulting in the relocation of tight junctional proteins. [193] PAR-2 activation contributes to the physiological barrier function as well as disease initiation such as irritable

bowel syndrome (IBS), IBD and colorectal cancer. [194] PAR-2 deficient mice demonstrated that PAR-2 activation mediates the intestinal inflammation associated with colitis development. [195, 196] It is well known that **PAR-2 activation is influenced by serine proteases, and subsequently dysregulates tight junctional proteins**, [196] **suggesting that PAR-2 activation breaks down barrier tightness, and consequently affects intestinal inflammation**.

1.4.3 Effect of impaired barrier functions in IBD

A major task of the intestinal barrier is to protect the intestinal mucosa from translocation of commensal bacteria and the passage of foreign antigens. [153] A breakdown in intestinal barrier function is associated with the pathogenesis of IBD, and caused by the disruption of tight junctional proteins such as occludin and ZO-1. [194, 197] JAM-A deficient mice showed increased intestinal permeability and susceptibility to DSS colitis, suggesting that barrier impairment is closely associated with disease development in the intestine. [198] The dysregulation of tight junctions affects barrier tightness, which allows microbial components to infiltrate into the epithelium and drives gastrointestinal inflammation. [199] An increase in paracellular permeability and dysregulation of tight junctional proteins are implicated in immune-mediated diseases such as allergy, celiac disease and IBD. [195, 200] Several studies demonstrated that the activation of PAR-2 by trypsin or PAR-2 agonist increased epithelial permeability and interrupted tight junctions, [191, 201] suggesting that **alteration of proteases in the intestine may impair barrier functions through dysregulated junctional proteins, and subsequently predispose the initiation of IBD**.

Extensive animal studies showed that a barrier defect in the intestine is implicated in microbiota-mediated intestinal inflammation, [202] resulting in the onset and severity of immune-mediated experimental ileitis and colitis. [203–205] Colitis development in IL10-/- mice is associated with an increase in intestinal permeability caused by dysregulation of zonulin. [206] IL10-/- mice induce a defective barrier and the dysregulated immune response. [203, 207] In addition to the dysregulated tight junctions, an overexpression of MLCK increased numbers of CD4 lymphocytes in the lamina propria, resulting in acceleration of development and severity of colitis. [205] In clinical studies, a defected intestinal barrier is associated with active disease and relapse in CD patients. [208, 209] Mutation of caspase recruitment domain family member 15 in CD patients was associated with increased intestinal permeability. [210] Furthermore, several studies revealed that an increase in intestinal permeability is related to disease initiation in IBD patients. [211, 212] Notably, downregulated tight junctions such as occludin and claudin were observed in IBD patients, [213, 214] indicating that the enhanced paracellular permeability leads to the triggering of intestinal

inflammation. Proinflammatory cytokines such as IFNγ and TNFα in the intestinal mucosa were elevated in IBD patients, which is connected with impairment of barrier through dysregulated tight junctions. [215, 216] **These studies demonstrate that impairment of barrier functions in the intestine can be a predisposing cause of IBD onset**.

2 Hypothesis and Aim

Frequent antibiotic therapy is associated with increased risk for the onset of IBD, however, the cause and effect as well as pathophysiological mechanisms are still unknown. In spite of the high clinical relevance, potential long term adverse effects of transient antibiotic therapy on the development of inflammation in the IBD susceptible host have not yet been addressed in experimental studies.

Previous studies revealed that the use of antibiotics disrupts the composition of intestinal microbiota and enhances protease activity via eradication of unknown microorganisms in the large intestine. With regard to the physiological barrier in the small and large intestine, the small intestinal mucosa is faced with exposure to very high levels of luminal protease activity. In contrast to the small intestine, the large intestine constantly adapts to low protease activity under normal circumstance. Therefore, the large intestine might not be accustomed to a rapid increase in protease activity. Hence, it is highly probable that the rise in protease activity in response to antibiotics exerts similar detrimental effects on the large intestinal barrier and immune homeostasis. The present study is the first to provide a causal link between ABx therapies and increased risk for CD, especially in IBD susceptible individuals.

In the present work, the first aim of this thesis was to assess the detrimental impact of antibiotics on fecal protease activity in patients. The level of protease activity in stool samples of patients before and after antibiotic therapy was examined in order to reveal the clinical relevance of antibiotic-increased protease activity.

The second aim of this thesis was to assess the impact of antibiotic treatment on the protease activity as well as on the intestinal barrier in mice. The change in protease activity and barrier function in the large intestine as well as the mechanisms underlying affected barrier function were investigated referring to major readouts including intestinal protease activity, epithelial permeability, tight junctions, and PAR-2 activation in WT and IL10-/- mice treated with short-term V/M.

The third aim of this thesis was to elucidate the relevance of the acute adverse effect for the accelerated colitis development after antibiotic in IBD susceptible hosts. The correlation between protease activity and colitis development was evaluated in repetitive V/M treated WT mice upon subsequent treatment of DSS and IL10-/- mice.

The last aim of this thesis was to Identify and isolate anti-proteolytic bacteria from murine gut content and human stool. Since the commensals that mediate the physiologically low protease activity in different hosts are mostly unknown, the isolation of anti-proteolytic bacterial strains is essential for

the anti-proteolytic mechanism of these bacteria as well as for their protective relevance in the context of high protease activity-mediated barrier dysfunctions.

3 Material and Methods

3.1 Ethics statement

The breeding and experimental use of mice in the facilities at the Technische Universität München (School of Life Sciences Weihenstephan) were approved by the regulatory authority (Regierung von Oberbayern; approval number 55.2-1-54-2531-99-13 and 55.2-1-54-2532-17-2015).

3.2 Housing conditions

WT (C57BL/6) or IL10-/- mice (129/SvEv) (N>=5/group) were bred under SPF conditions (12h light/dark cycles at 24-26°C) until a maximum age of 16 weeks.

3.3 Antibiotic treatment and experimental schedule

Antibiotics were freshly prepared by mixing vancomycin (0.25 g/L, Fluka) and metronidazole (1.0 g/L, Sigma) twice a week. Prepared antibiotics were mixed with mashed Chow powder (ratio 1:1) every day. In the short-term antibiotic treatment, mice at the age of 8 weeks were fed with control mash or V/M-containing chow mash for 2 days or 7 days respectively. Except for the period of V/M treatment, all mice were fed chow diet ad libitum. For protease inhibitor treatment, mice were gavaged with 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma) or water one day before V/M treatment as indicated in the experimental design in Figure 13 and 24. Feces were collected every day for the measurement of protease activity. To evaluate intestinal barrier integrity, FITC-dextran (4kDa, Sigma) was administered orally via gavage 4h before sacrificing the mice.

In the long-term treatment, mice were left untreated or were repeatedly treated with V/M for 7 days at the age of 4 and 8 weeks. WT mice were given 1.5% DSS (Sigma) in the drinking water for 7 days at the age of 12 weeks and sacrificed 2 days after DSS treatment. In the case of IL10-/- mice, from 2 weeks after the second V/M treatment, facial blood was repeatedly collected every second week. Mice were sacrificed at 16 weeks. To determine the protease activity, the feces were collected every week from WT and IL10-/- mice.

3.4 Colonization of GF mice

Cecal microbiota from untreated or V/M treated mice were isolated and orally administered to GF mice at10 weeks of age. Mice were colonized for 2 weeks and sacrificed. For the mono-association of GF mice, mice were colonized by gavage with different numbers of bacteria as described in Table2. The mono-colonization study was performed by Jelena Calasan. For the colonization with the complex consortia, GF mice were colonized with each of the indicated consortia of intestinal microbiota as

described in Table 3 and sacrificed. To colonize GF mice with 5 different simplified microbial consortia, our colleagues and collaborators provided cecal intestinal contents of the associated mice as indicated in Table 3.

Table 2. Colonization condition in mono-association of GF mice

Bacterial Strain	**Cell Num.**	**Duration**	**Mice age**
Bacteroides sartorii	$10^{3\sim4}$	14.1 weeks	18 weeks
Alistipes sp.	10^{10}	4 weeks	12 weeks
Lactobacillus murinus	$10^{6\sim7}$	4 weeks	12 weeks

Table 3. The candidate consortia from mice and human intestinal microbiota for mice associations

Consortia Name	**Bacterial Strain**	**Strain Num.**
OligoMM [(1)]	*Akkermansia muciniphila*	YL44
	Bacteroides sp. nov.	I48
	Bifidobacterium animalis	YL2
	Blautia sp.	YL58
	Clostridiales gen. nov.	KB18
	Clostridium innocuum	I46
	Enterococcus sp.	KB1
	Flavonifractor plautii	YL31
	Lactobacillus reuteri	I49
	Porphyromonadaceae fam. nov.	YL27
	Sutterellaceae gen. nov.	YL45
	Clostridium clostridioforme	YL32
Altered Schaedler Flora (B6.ASF) [(2)]	*Clostridium* sp.	ASF356
	Lactobacillus sp.	ASF360
	Lactobacillus murinus	ASF361
	Mucispirillum schaedleri	ASF467
	Eubacterium plexicaudatum	ASF492
	Firmicutes bacterium	ASF500
	Clostridium sp.	ASF502
	Parabacteroides sp.	ASF519
MiBC [(3)]	*Akkermansia muciniphila*	YL45
	Bacteroides acidifaciens	JJM0207_2
	Blautia sp. nov.	SJ18
	Intestimonas butyriciproducens	BLS 21
	Clostridium ramosum	SRB509-5-F-B
	Clostridium sp. *(C. bifermentans)*	G7K1R3-PYG-90
	Enterococcus sp. *(E. hirae)*	SB
	Enterohabdus mucosicola	DSM19490(T)_(AM747811)
	Enterobacteriaceae sp. *(E.coli)*	Mt1B1_(AM944637)
	Lactobacillus sp. *(L. murinus)*	M-6244-3B
	Parabacteroides goldsteinii	BS-C3-2_(GQ456205)

	Porphyromonadaceae fam. nov. *(Barnesiella)*	YL27
Coriobacteriacea [4]	*Atopobium parvulum*	DSM20469
	Eggerthella lenta	DSM2243
	Collinsella aerofaciens	DSM3979
	Enterorhabdus mucosicola	DSM19490
Anti-PA 1 [5]	*Acetatifactor muris*	CT-m2
	Anaerotruncus colihominis	JM4-15
	Blautia sp.	A-C6-2
	Clostridium innocuum	A-C3-1
	Lactobacillus reuteri	MJJ0609-4-1
	Clostridiaceae gen. nov.	BARN-424-CC-10
	Clostridium symbiosum	SRB539-5-G-R
	Paenibacillus sp.	pT2-260P
	Alistipes sp.	CC-5826-WT-bac
	Bacteroides sartorii	A-C2-0
	Bacteroides vulgatus	39a-cc-B-5824-ARE
	Parabacteroides distasonis	SAB-131-CoC-3
	Parabacteroides goldsteinii	BS-CS-2
Anti-PA 2 [6]	*Bacillus subtilis* group	Amp-T18
	Lactobacillus reuteri	MJJ0609-4-1
	Clostridium ramosum	SRB509-5-F-B
	Parabacteroides goldsteinii	BS-CS-2
	Enterohabdus mucosicola	Mt1BB

[1, 2] Colonization of GF mice with respective consortia was performed and fecal samples were provided by Prof. Dr. Stecher Barbara.

[3] This was performed by Prof. Thomas Clavel.

[4] This was performed by Dr. Sarah Just and Prof. Thomas Clavel.

[5, 6] This was performed by master student Carina Deli, Tabea Moll and Dr. Gabriele Hörmannsperger

3.5 Stool collection from patients treated with antibiotics

Stool samples were collected from untreated subjects and patients receiving antibiotics by Dr. Patrizia Kump and Andrease Blesl (Medizinische Universität Graz, Austria, ethical approval number 17-199 ex 05/06) and by Suchita Panda and Dr. Chaysavanh Manichanh (Digestive Unit, VHIR, Barcelona, Spain). [217] The samples were stored at -20 °C and delivered on dry ice. The characteristics of patients receiving antibiotics in the study are summarized in Table 4.

Table 4. Patient characteristics and antibiotics therapy

Classification	**Antibiotic**	**Antibiotic therapy duration**	**fold change in PA**	**Diarrhea (after antibiotic)**	**Disease**
Fluoroquinolone	Levofloxacin	7d	**7.57**	No	Bronchitis
(+Imidazole)	Levofloxacin	7d	0.30	No	Bronchitis

	Levofloxacin	7d	3.04	No	Bronchitis
	Levofloxacin	7d	2.35	No	Bronchitis
	Levofloxacin	7d	**5.50**	No	Bronchitis
	Levofloxacin	7d	1.83	No	Bronchitis
	Ciprofloxacin	7d	**13.59**	No	UC
	Levofloxacin	7d	0.32	No	Bronchitis
	Levofloxacin +Metronidazole	7d	**8.27**	No	*C.difficile* infection
β-Lactam	Amoxicillin+Clavulanate	7d	**12.74**	No	Bacteremia
	Amoxicillin+Clavulanate	7d	0.96	No	Bronchitis
	Amoxicillin+Clavulanate	7d	1.43	No	Bacteremia
	Amoxicillin+Clavulanate	7d	1.02	No	Bronchitis
	Amoxicillin+Clavulanate	7d	2.23	No	Urinary infection
	Amoxicillin+Clavulanate	7d	0.26	No	Bronchitis
	Amoxicillin+Clavulanate	7d	0.37	No	Bronchitis
Cephalosporin (+Macrolide)	Azithromycin	2d	**6.10**	No	CD
	Ceftriaxone +Azithromycin	7d	0.41	No	Pneumonia
	Ceftriaxone +Azithromycin	7d	1.29	No	Bronchitis
	Ceftriaxone	7d	0.51	No	Urinary infection
Rifamycin	Rifaximin	3d	**10.49**	No	IBS
	Rifaximin	3d	0.34	No	IBS
	Rifaximin	3d	0.45	No	IBS
	Rifaximin	3d	2.49	No	IBS
	Rifaximin	3d	2.17	No	IBS
	Rifaximin	3d	0.82	Yes	IBS
	Rifaximin	3d	0.77	No	IBS
	Rifaximin	3d	1.85	No	IBS
	Rifaximin	3d	0.92	Yes	IBS
	Rifaximin	3d	0.73	Yes	IBS
	Rifaximin	3d	0.64	Yes	IBS
	Rifaximin	3d	**5.36**	No	IBS

3.6 Preparation of supernatant from gut contents of mice and stool of patients

The stools of patients and gut contents (100mg) including ileal, cecal, colonic contents and feces from V/M-treated WT and IL10-/- mice were homogenized using sterile glass beads and dissolved in 10 % w/v of sterile phosphate buffered saline. Supernatants were acquired by two step centrifugations (5 min, 1000 rpm, 4 °C and 10 min, 6000 rpm, 4 °C). The collected supernatants were stored at -20 °C for

measuring protease activity and pattern, proteomic analysis, and *in vitro/ex vivo* permeability assay. To inactivate protease activity, supernatants were incubated with phenylmethane-sulfonylfluoride (PMSF, 5 mM, Sigma) for 1 h at 37°C.

3.7 Measurement of protease activity and serine protease pattern

Protease Assay Kit (Calbiochem®, EMD Biosciences) was used for the quantification of protease activity. 10 µL of prepared supernatant was incubated together with FTC-casein and incubation buffer for 1 h at 37 °C. The protease activity in the reaction was determined by emission at 538 nm after excitation at 485 nm using Infinite® 200 PRO plate reader (Tecan).

For the serine protease pattern, ActivX® TAMRA-FP Serine Hydrolase Probes (TAMRA, Thermo Scientific) were used to detect active serine proteases. 10 µL of prepared supernatant was incubated with 0.5 µL of a 0.1 mM TAMRA stock solution. Next, 10 µL of the solution was analyzed by a SDS-PAGE (15% SDS gel, 15 mA per gel, 75 V). Serine protease pattern was detected by fluorescent gel scanner (Typhoon™ Trio+, Amersham Biosciences) with excitation at 552 nm and emission at 575 nm.

3.8 Colonic epithelial cell culture

The murine colonic epithelial cell line PTK6 was grown in the RPMI-1640 cell culture medium containing 5% of fetal calf serum, 1% of Insulin-Transferrin-Sodium Pyruvate (Gibco) and 1% of antibiotic mixture (Sigma Aldrich) in the incubator (5 % CO2, 37°C). Upon confluency, the cells were subcultured by addition of trypsin EDTA.

3.9 TEER measurement and permeability assay

PTK6 cells (1.5 x 10^5 cells/well) were seeded on a 12-transwell cell culture plate (polyester membrane with a pore size of 0.4 µm, Corning). The cells were cultured until the transepithelial electrical resistance (TEER) reached 1,0 kΩ. Cells on the apical side were stimulated with either cecal or stool supernatant (10% w/v) or supplement-free medium for the control group, and TEER was measured on a heating block (Thermo) in order to maintain a constant temperature at 37 °C. After the TEER measurements, the medium on both sides was replaced by fresh Krebs buffer on the basolateral side and by a fresh Krebs buffer containing fluorescein on the apical side for permeability measurements. PTK 6 cells were incubated for 30 min at 37 °C. Krebs buffer on the basolateral side was determined by the emission at 538 nm after excitation at 485 nm.

3.10 TEER measurement in *Ussing* chamber

For TEER measurement *ex vivo*, 1 cm of cecal and colonic tissue of WT and IL10-/- mice was used. Tissue was excised and basolaterally mounted in the *Ussing* chamber. Samples were pre-incubated in Krebs buffer containing 5% CO_2 at 37°C and stimulated with prepared supernatant from gut contents on the apical side. In the case of PAR-2 antagonist pre-stimulation, apical side of tissues were exposed to 2 µM of PAR-2 antagonist for 20 min before stimulation with the supernatant. Meanwhile, the tissues were connected to a voltage clamp apparatus and electrical resistance measured every hour using Acquire & Analyze 2.3 software. For *in vivo* measurement of TEER, the cecal and colonic tissue of untreated or antibiotic treated WT and IL10-/- mice were prepared in the same procedure as described above, without any stimulation. After measurement of TEER, 50mg/ml of fluorescein was apically loaded and incubated for an additional 30 min. The penetrated fluorescein on the basolateral side was analyzed by fluorescence measurements using the Infinite® 200 PRO plate reader (Tecan).

3.11 Tissue processing, H&E, IF and IHC staining

The cecal and colonic tissues of untreated or V/M-treated WT and IL10-/- mice were fixed in formaldehyde (4%) and embedded in paraffin. Afterwards, the embedded tissues were cut into 4 µm or 5 µm sections using a microtome. For H&E staining, the sections were stained with hematoxylin and eosin (0.2%) before mounting.

Junctional proteins such as E-cadherin, ZO-1 and Occludin were visualized by the immunofluorescence (IF) staining. After deparaffinization of the tissue sections, antigen unmasking was performed by boiling the sections with 10mM citrate buffer (pH 6, 900 W, 23 min) in a pressure cooker. For the immunohistochemistry (IHC) staining of Ki76 and PAR-2, sections were incubated in 3 % hydrogen peroxide for another 5 min after washing with PBS. Additionally, sections were incubated in blocking buffer with 5% serum for 1h at room temperature after rinsing with PBS. The sections were then incubated overnight with primary antibodies for junctional proteins (Occludin, 1:100, Cell Signalling; Ecadherin, 1:200, Abcam; ZO-1, 1:200, Life technologies), Ki67 (1:200, Abcam) and PAR-2 (1:100, Alomone) at 4°C and rinsed with PBS. Fluorescence-conjugated secondary antibodies for IF staining of junctional proteins (AlexaFlour546 or AlexaFlour488 coupled for junctional proteins, Life Technologies) were incubated for 1h at room temperature. All sections were counterstained with DAPI (1:2000, Sigma-Aldrich) to visualize nuclei and were detected by Fluoview FV10i microscope (Olympus). In the case of IHC staining, the sections were treated with DAB after secondary antibody incubation (HRP-coupled, 1:200, Life technologies) and counterstained with hematoxylin. The stained sections were visualized on a Zeiss Axioskop 40 microscope (Zeiss).

3.12 Disease severity

Disease severity of DSS-induced mice was assessed by determining daily weight loss, stool consistency and fecal occult blood. The score of each metric was within a range of 0 to 4 and an overall mean was calculated to determine the disease activity index (DAI) as described in Table 5.

Table 5. Score of disease activity index (DAI)

Score	weight loss (%)	stool consistency	blood in stool
0	0	Normal	no blood
1	lower than 5	/	/
2	lower than 10	slightly changed	positive
3	lower than 15	/	positive for 2 days
4	lower than 20	Diarrhea	positive for more than 2 days

3.13 Histopathology

Histopathology of H&E stained cecal and colonic tissue sections were blindly analysed by determining infiltration of mononuclear cells in lamina propria, architecture imbalance and crypt hyperplasia using an established scoring system (scored from 0–12). [218, 219] Scanned images were acquired by Touch microscope V. precipoint (PreciPoint GmbH).

3.14 Quantitative real-time PCR and Western Blotting

RNA and protein were extracted with RA1 buffer (Macherey-Nagel, Düren) using NucleoSpin RNAII kit (Macherey-Nagel GmbH) from the cecum and colon of untreated or V/M treated WT and IL10-/- mice. cDNA was synthetized using 500 ng total RNA, random hexamers and MMLV reverse transcriptase Point Mutant Synthesis System (Promega). Quantification of target gene was performed using the Universal Probe Library system with 1 µl of cDNA and Brilliant III Ultra-Fast 2x master mix (Agilent) in a Light Cycler® 480 system (Roche Diagnostics). The primers and probes were described in Table 6. Relative mRNA expression was normalized to the expression of GAPDH using the Light Cycler® 480 software (Roche Diagnostics).

For western blotting, the isolated protein was diluted with 2X SDS buffer. The same amount of protein was electrophoresed on 10% and 15% SDS-PAGE gels. Junctional protein (Occludin, Cell Signalling; ZO-1, Thermo Fisher Scientific; Claudin-2, Cell Signalling) and PAR-2 (Abcam) antibodies were used to identify the respective proteins, and the membrane was developed with an enhanced chemiluminescence light-detecting kit (Amersham). The target proteins were normalized to actin.

Table 6. Information of primer sequences and UPL probe ID

Gene	Forward primer	Reverse primer	Probe Num.
ALPI	5’-catctccaacatggacattga	5’-ggttccagactggttactgtca	109
CD3	5’-cttgtacctgaaagctcgagtg	5’-gatgattatggctactgctgtca	10
Foxp3	5’-cccacacctcttcttccttg	5’-catgactaggggcactgtagg	33
C3	5’-accttacctcggcaagtttct	5’-ttgtagagctgctggtcagg	76
IFABP	5’-ggtttctggtaatgaactaatccag	5’-aaatctgacatcagcttagctcttc	1
IFNγ	5’-ggaggaactggcaaaaggat	5’-ttcaagacttcaaagagtctgagg	21
IL1ß	5’-tgtaatgaaagacggcacacc	5’-tcttctttgggtattgcttgg	78
IL6	5’-tgatggatgctaccaaactgg	5’-ttcatgtactccaggtagctatgg	6
IP10	5’-aatgaaagcgtttagccaaaaa	5’-aggggagtgatggagagagg	56
Ki67	5’-gctgtcctcaagacaatcatca	5’-ggcgttatcccaggagact	80
LgR5	5’-cttcactcggtgcagtgct	5’-cagccagctaccaaataggtg	60
Occludin	5’-cacgacaggtggggagtc	5’-ttgatctgaagtgataggtggatatt	17
PAR2	5’-ggaccgagaaccttgcac	5’-ggaacccctttcccagtg	75
TNFα	5’-tgcctatgtctcagcctcttc	5’-gaggccatttgggaacttct	49
ZO1	5’-aggcagctcacgtaggtctc	5’-ggttttgtctcatcatttcttcag	12
GAPDH	5’-tccactcatggcaaattcaa	5’-tttgatgttagtggggtctcg	9

3.15 Serum amyloid A (SAA) and complement C3 ELISA

SAA levels in plasma and complement C3 levels in fecal supernatants were measured by ELISA kit (Immunology Consultants Laboratory) and determined by absorbance at 492 nm using Multiskan® spectrophotometer (Thermo Scientific).

3.16 Isolation of metagenomic DNA

For genomic analysis of the isolated bacteria, metagenomic DNA was isolated from cecal content of repetitive V/M treated WT and IL10-/- mice using a protocol with modification according to Godon et al.. [220] Thawed samples were mixed with 600 µl stool DNA stabilizer (Stratec biomedical). Samples were then transferred into 2 ml screw-cap tubes containing sterile 500 mg silica/zirconia beads (0.1 mm-diameter). Next, samples were mixed with 250 µl 4 M guanidine thiocyanate in 0.1 M Tris (pH 7.5) and 500 µl 5 % N-lauroyl sarcosine in 0.1 M PBS (pH 8.0) and were incubated at 70 °C in the shaking incubator (700 rpm for 60 min). For cell disruption, a FastPrep® instrument (MP Biomedicals) was run with 24 × 2 ml cooling adaptor 3 times for 40 s at 6.5 M/s. Dry ice was added to the cooling adapter in

between runs. The samples were mixed with 15 mg Polyvinylpyrrolidone (PVPP) followed by 3 min centrifugation at 15.000 x g and 4 °C. The supernatant (650 µl) was transferred into a new 2 ml tube, and was centrifuged again (3 min, at 15.000 x g, 4 °C). Transferred supernatant (500 µl) in a 2 ml tube was mixed with 50 µg of RNase. After spin down (20 min, at 37 °C, 700 rpm), the NucleoSpin® gDNA Clean-up Kit (Macherey-Nagel) was used for the isolation of genomic DNA. Isolation was performed following the NucleoSpin® gDNA Clean-up Kit protocol. DNA was eluted twice using columns with elution buffer (40 µl). Concentration of eluted gDNA was measured using a NanoDrop® (Thermo Scientific). The gDNA was then stored at -20 °C.

3.17 16S ribosomal RNA gene sequence analysis

Sequence analysis was performed by Dr. Gabriele Hörmannsperger and Dr. Illias Lagkouvardos. Library preparation and sequencing, and data analysis were performed as described in detail previously. [221] The 16S rRNA genes containing V3-V4 region was amplified for 25 cycles using primers 341F-785R following a two-step protocol. Sequencing was performed using paired-end reads (PE275) on a MiSeq system (Illumina) following the manufacturer's protocol and the PhiX standard library (25% (v/v)). Each sample was processed using a developed pipeline (www.imngs.org) based on UPARSE. All sequences were trimmed to the first base with a quality score <3 and then paired. The filtered sequences excluded the nucleotides less than 380 and more than 480. All paired reads with an expected error >3 were filtered out. Each end of remaining reads was trimmed by ten nucleotides in order to avoid GC bias and non-random bases. Chimeras in the paired reads were removed by UCHIME. Operational taxonomic units (OTUs) were selected at 97% sequence similarity. Those OTUs with a relative abundance >0.5% in at least one sample were further analysed. The classified taxonomies were assigned using the RDP classifier and Silva.

Downstream analysis for diversity and bacterial composition was performed using Rhea pipelines (https://lagkouvardos.github.io/Rhea/) written in the R programming language. OTUs were normalized to the differences of abundance in each group. Alpha-diversity was analysed on the basis of species richness and the calculated Shannon index. Beta-diversity was assessed based on the calculation of generalized UniFrac distances. For the statistical analysis, only prevalent taxa in one given group were defined as detected in at least 30 % of the samples. The ANOVA and Benjamini-Hochberg method were used for P-values calculation and corrected multiple comparisons.

3.18 Protein extraction

In-gel digestion of protein extracts: Protein from the stool supernatants from patient and the cecal supernatants of untreated, V/M treated and GF WT mice were analyzed by LC-MS/MS analysis. The cecal proteins were mixed with 2 × NuPAGE® LDS Sample Buffer (Invitrogen) and were diluted by 10 mM dithiothreitol and alkylated by 55 mM iodoacetamide. Before trypsin digestion, cecal proteins were electrophoresed on 4–12% NuPAGE gel (Invitrogen) to approximate 1 cm. The concentrated cecal proteins were digested by trypsin digestion buffer in the NuPAGE gel according to standard protocol [222].

3.19 LC-MS/MS analysis

Proteomic analysis was performed by Dr. Hannes Hahne and Stephanie Heinzlmeir. Trypsinized cecal peptides were dried down in a speed-vacuum concentrator and were resuspended in 20 µL of 0.1% formic acid buffer. For LC–MS/MS analysis, accurate quantitative proteomics measurements were conducted by LC–nanoESI-MS/MS using a nanoLC-Ultra (Eksigent) and a LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific). The cecal peptides were automatically loaded onto a commercial trap column (ReproSil-pur C18-AQ, 5 µm, Dr. Maisch, Ammerbuch, Germany, 20 mm × 75 µm, self-packed) with 100% solvent A, consisting of 0.1% formic acid in HPLC grade water, for 10 min at a flow rate of 5 µL/min. After washing the column, the prepared peptides were loaded onto an analytical column (ReproSil-gold C18-AQ, 3 µm, Dr. Maisch, Ammerbuch, Germany, 400 mm × 75 µm, self-packed) with 2% to 32% of gradient solvent B, consisting of 0.1% formic acid in acetonitrile, for 210 min at 300 nL/min flow rate. 5% of DMSO was added to both solvent A and B in order to boost the nanoESI response of peptides. We performed the LTQ Orbitrap Velos during the data dependent acquisition mode to evaluate the automatic gain between MS and MS/MS. Full MS scans were acquired in the Orbitrap at m/z 400 and 30,000 resolution. Accumulation precursor ions reached a target value of 1,000,000 for a maximum of 100 ms. The calibration of the internal lock was detected in solvents using an ion signal (m/z 401.922720). Up to ten of the most intense peptide precursors were fragmented in HCD (target value of 40,000, isolation width 2.0 Th) for a maximum of 100 ms accumulation time with normalized collision energy of 30%. Tandem mass spectra detected fragment ions in the Orbitrap mass analyser with resolution 7500. Maximized numbers of target precursors for dynamic exclusion were allowed one repeat count at 10 s and 20 s exclusion time (mass tolerance +/- 10 ppm).

For qualitative experiments, deisotoped and deconvoluted peak lists were generated from raw tandem mass spectra using the Mascot Distiller v2.3.0 (Matrix Science, London). All MS/MS samples were

analysed using the Mascot (Matrix Science, London, UK; version 2.4.1). The Mascot was set up to search for SwissProt (download date 07/08/2014; 515,319 entries) including common contaminants (Ref: http://www.thegpm.org/crap/index.html) using the built-in target decoy option. Further search parameters included trypsin as a digestion enzyme, a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 ppm. For fixed modification and oxidation of methionine, carbamidomethylation of cysteine was specified. The Mascot result files were imported into Scaffold (version Scaffold 4.1.1, Proteome Software Inc., Portland, OR) and peptide and protein identifications were accepted at 1% and 2% target decoy FDR, respectively. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

3.20 Statistics

Statistical significance was determined by unpaired t-test or analysis of variance with One-way ANOVA was followed by Tukey's post-test. All data in charts are presented as mean±SD. Statistically significant differences were considered with p values below 0.05. Graph-Pad Prism (Graph Pad, version 6.01) was used for statistical analyses and creation of graphics.

4 Results

4.1 Antibiotic therapy in patients is linked to change in protease activity

4.1.1 Protease activity is dependent on individual and antibiotic class

It is a well-known fact that antibiotic treatments lead to changes in the microbial ecosystem of the intestine that are highly associated with various intestinal diseases. [103, 223–225] Many experimental and clinical studies evaluated the impact of antibiotics on the microbial community and its related functional disturbances in the intestine. In this context, a few studies show that specific antibiotic treatment resulted in a major increase in protease activity in the large intestines of rodents and humans. [140–143] However, the clinical relevance is unclear due to limited evidence on the impact of specific antibiotic therapies on the large intestinal protease activity.

Thus, to investigate the role of different antibiotics on the alteration of protease activity, protease activity was analyzed in stools of healthy individuals as well as patients with different antibiotic treatments. There was no significant difference in stool protease activity between healthy individuals and patients with gastrointestinal diseases (Figure 6A). However, it was observed that protease activity varied greatly among individuals in the group of healthy persons and patients. To examine whether antibiotics lead to change in protease activity, stool samples from patients undergoing different antibiotic treatments were analyzed. Stool protease activity did not change in antibiotics-treated patients compared to untreated patients (Figure 6B). A high variation of protease activity among each individual was observed. Reasons for the observed variations might be previously prescribed antibiotics, eating behavior and different diseases. Interestingly, when the group of patients receiving antibiotics was classified according to the presence of diarrhea, the patients with diarrhea showed a significant increase in protease activity (~3.5-fold) compared to those without diarrhea (Figure 6C). However, different routes of administration of the antibiotics (eg. intravenous and oral administration) did not affect protease activity in stool samples of patients (Figure 6C). The treatment with specific antibiotics such as fluoroquinolone (+imidazole) showed a significant increase in protease activity compared to untreated and other antibiotic treated patients (Figure 6D). The ratio of rise in protease activity was 31.4% in the fluoroquinolone (+imidazole) treatment. Whereas the β-lactam treatment was 13.3%, the cephalosporin (+macrolide) treatment increased the protease activity in 20% of the patients, more than the highest level of protease activity in untreated patients. Overall, the fluoroquinolone (+imidazole) treatment strongly induced an increase in protease activity compared to the other antibiotic treatments.

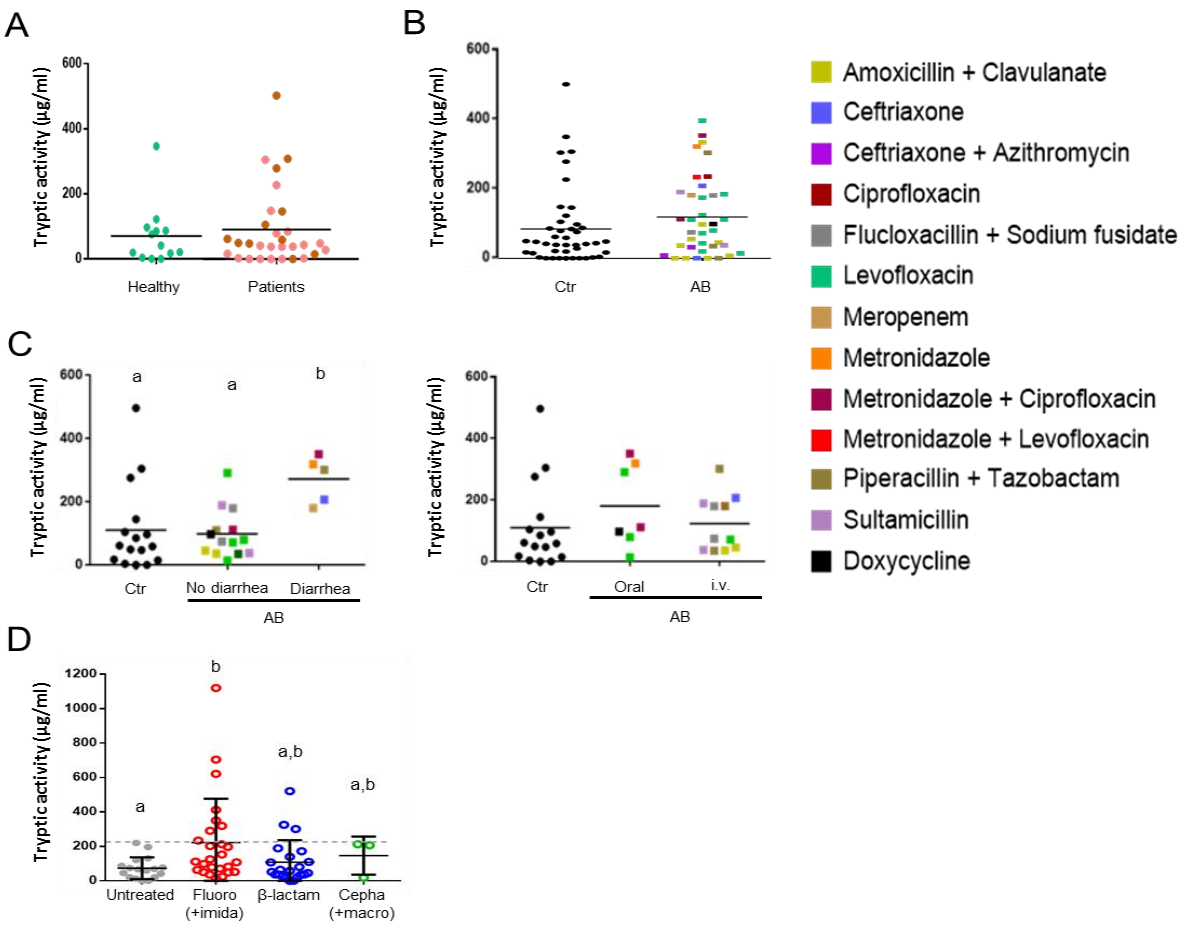

Figure 6: Patients show individual- and antibiotic-specific increase in PA.
(A) Protease activity (PA) in stool supernatant (stool_sup) of healthy persons and patients. (B) The PA in stool_sup of healthy persons and different antibiotics (AB) treated patient. (C) Effect of diarrhea in the change of PA in stool_sup of AB treated patients (left panel) and comparison of the PA in stool_sup from patients treated with antibiotic using different way of administration (right panel). (D) The PA in stool_sup of healthy people versus patients treated with different antibiotic. Fluoro (+imida): fluoroquinolone (+imidazole) antibiotic, Cepha (+macro): cephalosporin (+macrolide) antibiotic. One-way ANOVA was followed by Tukey's post-test. Different superscripts (a and b) were significantly different between groups ($P < 0.05$).

Since specific antibiotics resulted in increased protease activity in stool of patients, stool protease activity was compared before and after antibiotic treatment in same patients. Stool protease activity was not significantly changed after antibiotics treatment (Figure 7A). Interestingly, 9 out of 19 patients showed a more than 2-fold increase in protease activity after start of antibiotic treatment (Figure 7B). More precisely, however, 66% of the patients treated with fluoroquinolone (+imidazole) showed a more than 2-fold increase of the stool protease activity, in contrast to only 20% of the patients treated with β-lactam or Cephalosporin (+macrolide). None of the patients had diarrhea, neither before nor

after the antibiotic treatment. Levofloxacin and levofloxacin+metronidazole treatment showed a strong increase in stool protease activity of each patient compared to before treatment.

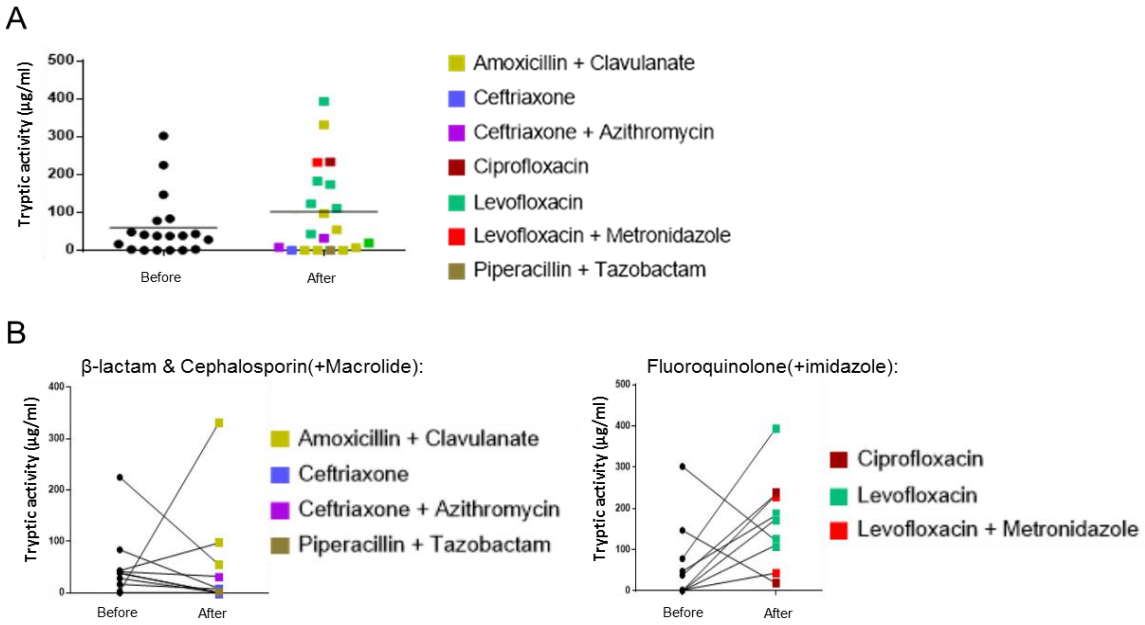

Figure 7: Fluoroquinolone (+imidazole) treatments increase PA in patients.
(A) Stool PA in patients after antibiotic treatment versus before treatment. Stool samples were collected before antibiotic treatment and over 7 days of antibiotic treatment. (B) PA in stool_sup of patient before and after β-lactam/Cephalosporin+macrolide or fluoroquinolone+imidazole treatment. Only 20% of the patients treated with β-lactam/Cephalosporin+macrolide showed an increase in PA, more than 2-fold. After 7 days of fluoroquinolone+imidazole treatment, 67% of the stool PA in patients showed a more than 2-fold increase.

Limited evidence was provided for intestinal bacteria playing a pivotal role in the regulation of protease activity in the large intestine. [134, 137, 138] Antibiotic treatments result in changes in microbial composition, total bacterial numbers, richness in microbiota and capacity of microbiota-mediated proteolytic inactivation. [70, 129] To demonstrate the correlation between changes in the intestinal microbiota and an increase in protease activity upon antibiotic treatments, protease activity and microbial composition in stool samples of patients before and after antibiotic treatment were analyzed. We randomly selected 3 patients who showed changes in protease activity after antibiotic treatment. While ceftriaxon treatment decreased stool protease activity, leovofloxacin+metronidazole and ciprofloxacin classified as the fluoroquinolone (+imidazole) strongly increased stool protease activity (Figure 8A). The patient treated with leovofloxacin+metronidazole showed an increase in protease activity, more than 25-fold. The ciprofloxacin treatment enhanced protease activity more than 8-fold. At the phylum level, no changes were observed between before and after ceftriaxone treatment

(Figure 8B). Compositional dynamics of intestinal microbiota at phylum level from a leovofloxacin+metronidazole treated patient was close to the level attained by a ceftriaxone treated patient, rather than the level achieved by ciprofloxacin treatment. *Proteobacteria* was strongly increased in the leovofloxacin+metronidazole treatment. Moreover, the ciprofloxacin treatment strongly increased *Bacteroidetes* and decreased *Firmicutes*.

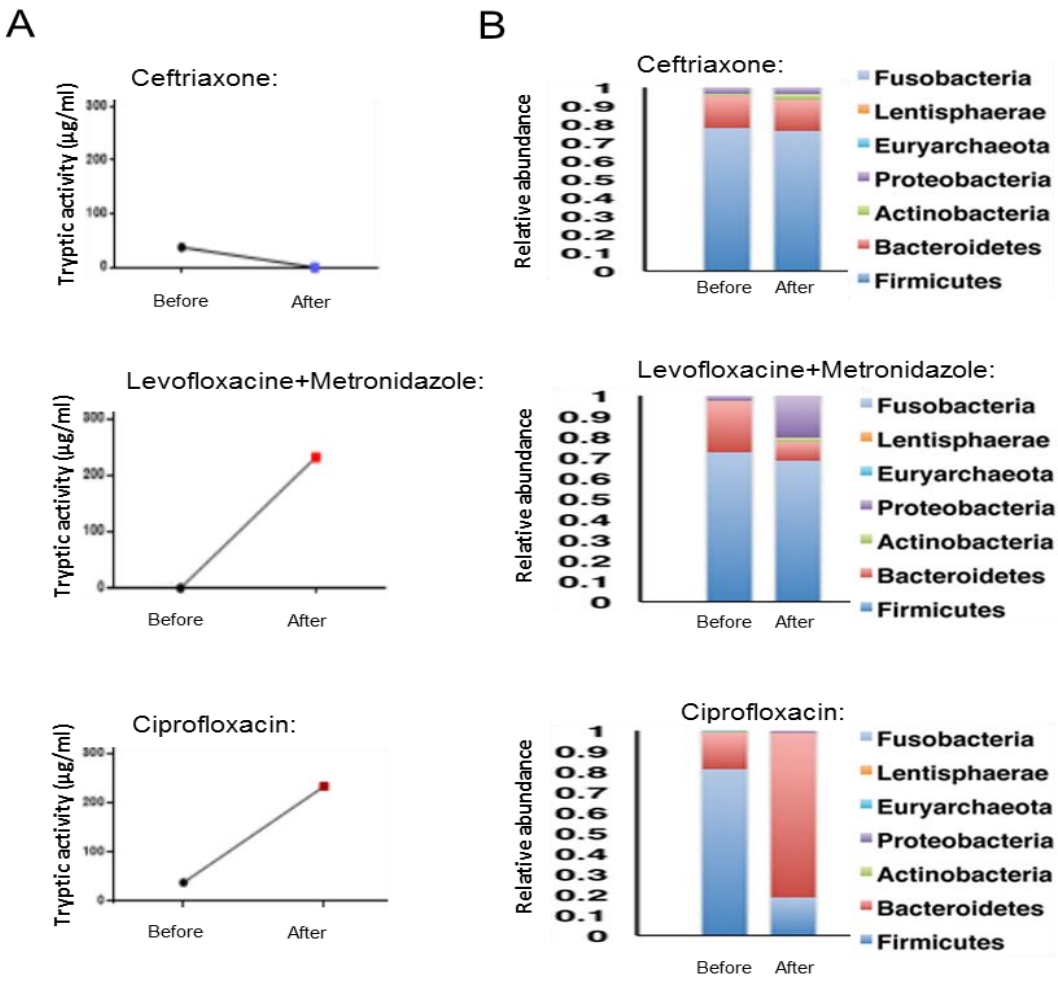

Figure 8: Increase in PA depends on specific antibiotics, but is not correlated with gut microbiota community. (A) The PA in stool_sup of patient before and after different antibiotic treatment. Ceftriaxon was classified as the treatment of Cephalosporin antibiotic. The ceftriaxone treatment did not show an increase in PA (upper panel). The treatment with fluoroquinolone+imidazole resulted in an increase in PA (middle panel: Leovofloxacin/Metronidazole, Lower panel: Ciprofloxacin). (B) The gut microbiota community in stool_sup of patient before and after respective antibiotic treatments. The antibiotic treatments revealed the alteration in gut microbiota composition.

4.1.2 Antibiotic treatment increases pancreatic serine protease-dependent activity

Our previous data (Figure 6 to 8) and several published studies provide evidence that antibiotic treatment in rodents and humans results in increased protease activity in luminal contents of the large

intestine. [140, 142, 143] To investigate the causal link between antibiotics and protease activity, and origin of proteases, we investigated protease activity and profiles in stools of patients before and after antibiotic treatments. Stool protease activity was analyzed in 32 different patients receiving 11 different antibiotics classified into four different types (Table 2). Patients treated with different antibiotics showed that the ratio of increase in protease activity in fluoroquinolone (+imidazole) treatments was higher than the ratio in the other antibiotic treatments (Figure 9A). 8 out of 32 patients after antibiotic therapy had a over 5-fold increased protease activity. Especially, fluoroquinolone (+imidazole) treatment such as levofloxacin, ciprofloxacin and leveofloxacin/metronidazole mixture showed an increase in protease activity more than 5 times in 4 out of 9 patients (44.4% of the patients). In contrast, treatment with β-lactam antibiotics increased protease activity more than 5-fold in 14.3% of the patients. Treatment with cephalosporin (+macrolide) antibiotics enhanced protease activity in 25% of patients, and treatment with ansamycin antibiotics increased protease activity in 16.7% of patients. We confirmed that the major rise in protease activity in fluoroquinolone (+imidazole) treated patients had the same tendency as the result indicated in Figure 6D.

To identify the relevant proteases, stool supernatants from patients treated with different antibiotics, which showed a high protease activity, were randomly selected, and were incubated with the serine proteases probe TAMRA. In the analysis of active serine protease patterns, different antibiotics treatment showed a strong increase of low size proteases (<25 kDa) compared to the pattern before antibiotic treatment, suggesting that serine proteases such as trypsin (~23.5 kDa) and chymotrypsin (~24.8 kDa) were causal for the observed rise in protease activity (Figure 9B). Host and bacterial proteases as well as protease inhibitors were determined in stool supernatants of patients before and after different antibiotic treatments by LC-MS/MS. The fluoroquinolone (+imidazole) treatment showed that the most abundant and strongly elevated proteases were host pancreatic proteases such as trypsin, chymotrypsin and chymotrypsin-like elastase family members, whereas some protease inhibitors (e.g. Serine protease inhibitor A3K and Alpha-1-antitrypsin 1-2) declined (Figure 9C). These data indicate that the increased protease activity in response to fluoroquinolone (+imidazole) was caused by an elevation of pancreatic proteases and a decrease in protease inhibitors. In contrast, treatment with β-lactam antibiotic and the cephalosporin (+macrolide) antibiotic increased non-pancreatic serine proteases and other proteases rather than pancreatic proteases. Additionally, protease inhibitors were not reduced in the treatment with β-lactam and cephalosporin (+macrolide) antibiotics. Taken together, these findings suggest that **fluoroquinolone (+imidazole) treatment increased protease activity through elevated pancreatic proteases and decreased protease inhibitors**.

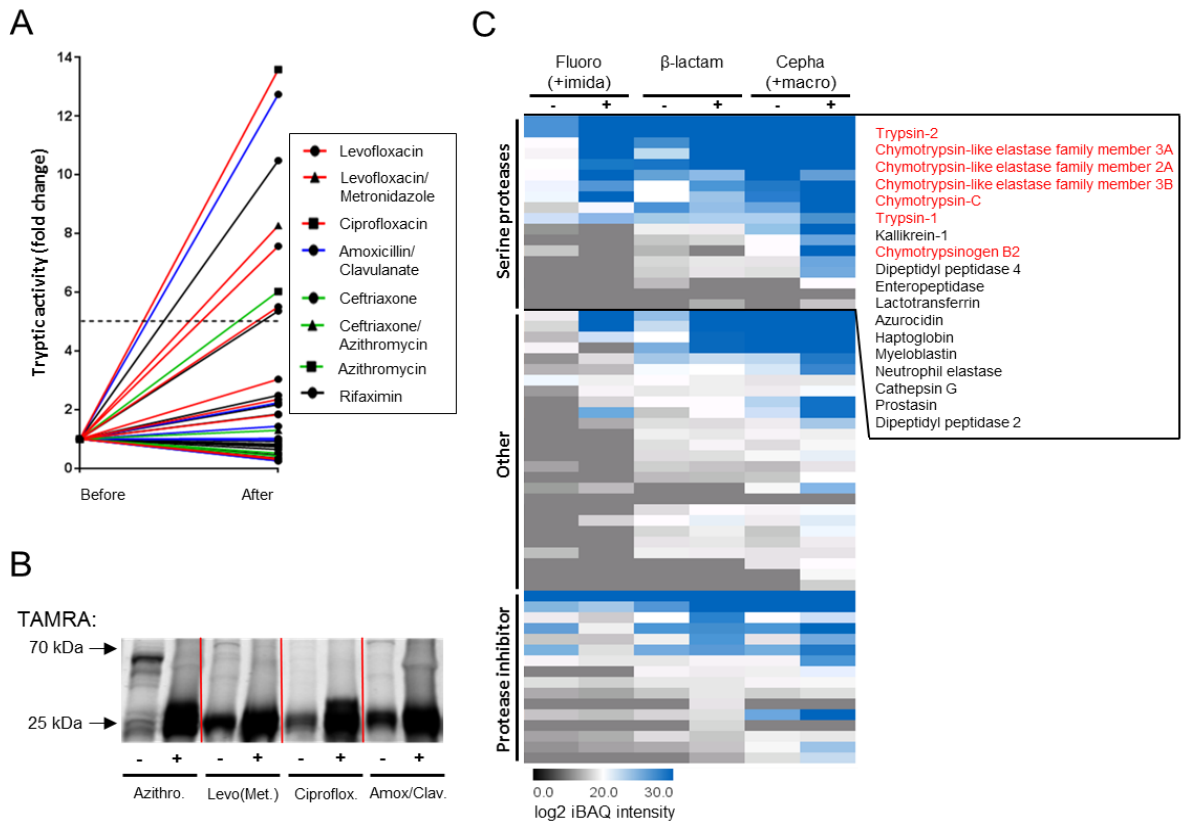

Figure 9: Specific antibiotic therapies increase the number and activity of pancreatic proteases in patients. (A) Change in PA in stool supernatants (stool_sup) of each patient before and after antibiotic treatment. Either levofloxacin, levofloxacin/metronidazole or ciprofloxacin treatment was classified as fluoroquinolone(+imidazole) antibiotic (red line, n=9). Amoxicillin/clavulanate were classified as β-lactam antibiotic (blue line, n=7). Either ceftriaxone, azithromycin or ceftriaxone/azithromycin were classified as cephalosporin(+macrolide) antibiotic (green line, n=4). Rifaximin was classified as rifamycin (black line, n=12). (B) A major increase in host proteases, including pancreatic trypsin (active trypsin ~23.5 kDa), in stool_sup of patient before or after Azithromycin (Azithro.), Ciprofloxacin (Ciproflox.), Amoxicillin/Clavulanate (Amox/Clav.) or Levofloxacin/Metronidazole (Levo(Met.)) detected by TAMRA-FP staining. (C) The heatmap shows all human and bacterial proteases detected via the LC-MS/MS analysis in stool_sup of patient before/after treatment with Ciproflox., Amox/Clav., Levo(Met.) or Azithro. Proteases highlighted in red were significantly abundant in patients after antibiotic treatment compared to before treatment. iBAQ: intensity Based Absolute Quantitation.

4.1.3 Increased protease activity is detrimental to barrier function in colonic epithelial cells

In several *ex vivo* and *in vivo* studies in which the apical membrane of colonic tissue was exposed to high levels of active proteases, a highly active trypsin has been found to result in acute impairment of the epithelial barrier in the large intestine. [196, 201, 226] The specific antibiotic treatment such as fluoroquinolone (+imidazole) elevated stool protease activity of patients. In consequence, it is highly probable that the rise in protease activity exerts similar detrimental effects on the large intestinal

barrier. So, the effects of this increased protease activity on the large intestinal barrier were further analyzed.

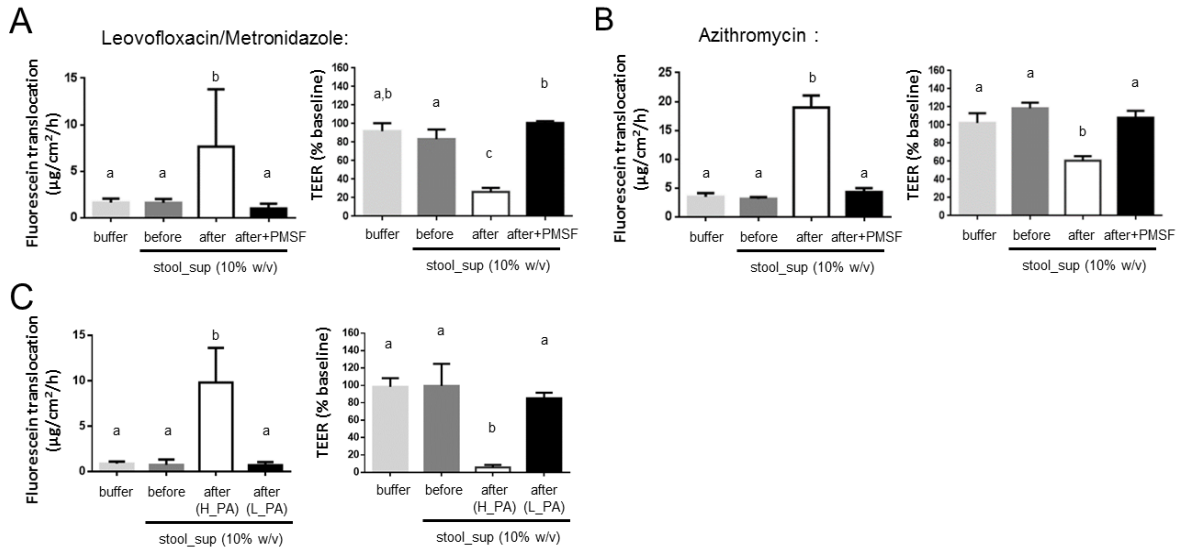

Figure 10: Increased PA in response to antibiotics impairs barrier functions in trans-well PTK6 cell cultures.
(A & B) The PTK6 cells were apically stimulated with either the stool_sup (10% w/v) of the respective antibiotic treated patient or the stool_sup pre-incubated with PMSF. (A: levofloxacin+metronidazole, B: azithromycin). The transepithelial electrical resistance (TEER) and the translocation of fluorescein on basolateral side in transwell were measured. (C) The rise in PA induced barrier dysfunction in the PTK6 cells stimulated with the stool_sup (10% w/v) from patients having the high PA before and after different antibiotic treatment (H_PA: high PA, L: low PA). One-way ANOVA; Tukey's post-test. Different superscripts indicated significant difference ($P < 0.05$).

To investigate the detrimental effects of enhanced protease activity on the intestinal barrier, we used PTK6 cells which are non-carcinogenic epithelial cells from the murine large intestine. [227] This cell line is suitable to measure barrier integrity and transepithelial electric resistance on transwell permeable supports using double electrodes across the epithelial cell layer. PTK6 cells stimulated with the stool supernatant of the patient treated with Levofloxacin/Metronidazole showed highly increased fluorescein translocation to the basolateral side of transwell, and decreased TEER compared to the stimulation with the stool supernatant of the patient before treatment (Figure 10A). However, pre-incubation of the protease inhibitor PMSF with the stool supernatant negated this detrimental effect. In the stimulation with the stool supernatant of the patient treated with azithromycin, fluorescein translocation was strongly increased, and TEER was significantly decreased (Figure 10B). These results suggest that a major rise in protease activity impaired barrier functions in colonic epithelial cells. To confirm the observed detrimental effect of high protease activity, PTK6 cells were apically stimulated with stool supernatant from patients before and after different antibiotic treatments that showed

either high or low protease activity. High PA stool supernatant induced extreme barrier dysfunction, while the stimulation with low PA stool supernatant showed normal barrier function as observed in the buffer stimulation (Figure 10C). Since the pre-incubation of PMSF effectively prevented the barrier dysfunction, it can be assumed that **the barrier impairment was indeed due to the high load of active serine proteases in response to antibiotic treatment.**

4.2 Increased protease activity relevant to microbial dysbiosis and functional disturbance in the large intestine

4.2.1 Presence of intestinal bacteria is a prerequisite for inactivation of protease activity

In the intestine, digestive enzymes such as pancreatic trypsin and chymotrypsin are secreted into the lumen of the duodenum, and are passed through the gastrointestinal tract. [108] These digestive proteases are inactivated by several mechanisms including auto-inactivation and reactions with host/bacterial protease inhibitors. [228] Thus, the large intestine shows physiologically low protease activity compared to the small intestine. It is well known that GF mice show an increase in the large intestinal protease activity, since pancreatic proteases are not inactivated in the absence of the intestinal microbiota. The observed results indicate that specific antibiotics treatment is linked to dysregulation of pancreatic proteases in patient (Figure 9).

To confirm the impact of antibiotic treatment on the increase in protease activity and intestinal microbiota to be an indispensable factor for inactivation of pancreatic proteases in large intestine, V/M-treated mice and GF mice colonized with different bacteria were used. Abrupt proteolytic inactivation was observed at the transition between the ileum and the cecum (Figure 11A). The V/M-treated mice showed a significant increase in protease activity in the large intestine (>10-fold) to the level observed in GF mice compared to untreated mice, suggesting that certain bacteria which colonized in the cecum are essential for the inactivation of proteases. Analysis of active serine protease patterns revealed an increase in presence of low molecular serine proteases in GF and V/M-treated mice (Figure 11B), whereas ampicillin treatment did not result in any change in the protease pattern (data not shown). V/M-treated mice showed a major increase in low molecular weight of serine proteases similar to the patterns in stool supernatant of the patient receiving different antibiotics (Figure 9B). Changes in proteases and protease inhibitors of cecal supernatant from V/M-treated mice and GF mice were analyzed using a proteomic approach in order to find a cause for the rise in protease activity (Supplementary Table 1). The proteomic profile observed by LC-MS/MS analysis revealed that the most increased and abundant protease was pancreatic trypsin in V/M-treated and GF mice (Figure

11C). Only a few bacterial proteases were detected in V/M-treated mice. These results suggest that V/M treatment eradicates the bacterial commensals associated with the degradation of pancreatic proteases in the large intestine.

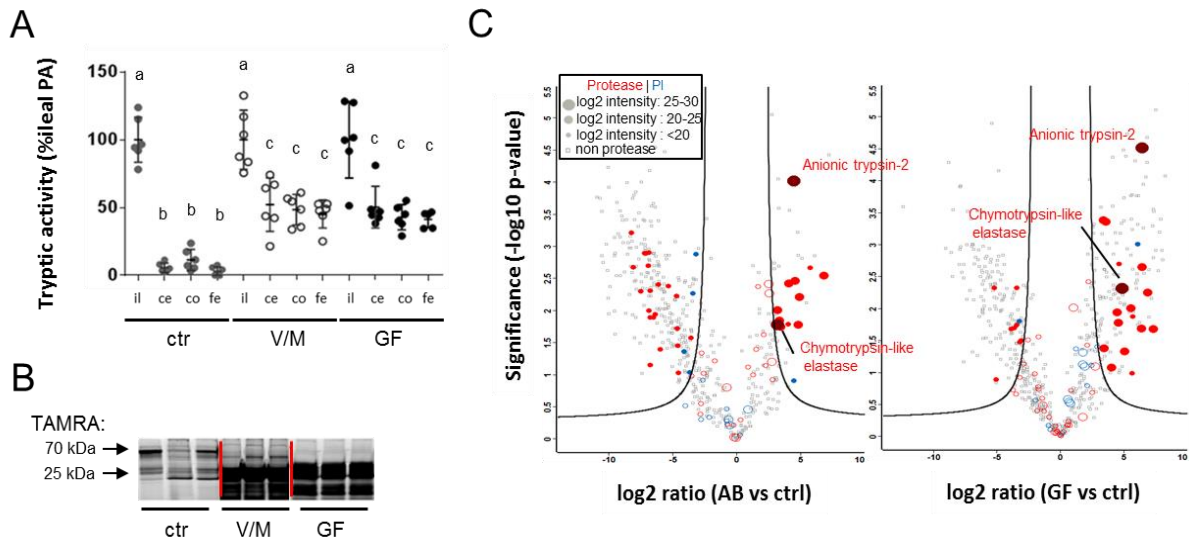

Figure 11: V/M treatment results in a serine protease dependent increase in PA to the level of GF mice.
(A) The intestinal transit PA in the different region of gut contents from untreated (ctr), V/M-treated (V/M) and germfree (GF) mice (il: ileum, ce: cecum, co: colon, fe: feces). (B) The active serine protease pattern stained by TAMRA-FP in cecal supernatant (cecal_sup) of ctr, V/M or GF mice. (C) The proteases and protease inhibitors in cecal_sup of ctr mice compared to V/M mice or GF mice were determined by LC-MS/MS analysis (n=3/group). The abundance of proteases and protease inhibitors are given as log2 intensity which are indicated by the size of circles. Abundance of proteases and protease inhibitors in ce_sup of ctr, V/M-treated and GF mice are listed in Supplementary Table 1.

The strong increase in protease activity is associated with the eradication of specific microbiota which have anti-proteolytic property. [144] We transferred fresh cecal bacteria from untreated and V/M-treated mice to GF mice in order to confirm that the absence of specific micro-organisms is causally linked to the increase in proteolytic activity. We investigated cecal protease activity in GF mice colonized with microbiota from either untreated mice (ctr->GF) or V/M-treated mice (V/M->GF). The ctr->GF mice showed normal cecal protease activity, similar to the level in untreated mice, whereas V/M->GF mice showed strongly increased cecal protease activity similar to the level in GF mice (Figure 12A). Analogous to the observation in V/M-treated and GF mice (Figure 11B), TAMRA staining revealed that V/M->GF mice showed strong increase in low molecular weight of active serine proteases (<25 kDa) (Figure 12B). However, ctr->GF mice showed an active serine protease pattern similar to untreated mice. These findings indicate that V/M treatment eradicates specific commensal bacteria

which physiologically inactivate pancreatic serine proteases in the large intestine. To confirm these findings (Figure 11A&B), cecal supernatant of GF mice was pre-incubated with fresh or heat-killed cecal microbiota from either SPF mice or V/M-treated mice. Protease activity in the GF cecal supernatant incubated with SPF microbiota was significantly decreased compared to the level of normal GF cecal supernatant (Figure 12C). However, the pre-incubation of GF cecal supernatant with heat-killed cecal microbiota of SPF mice showed a high protease activity. Whereas incubation with SPF microbiota decreased protease activity, the cecal microbiota in V/M-treated mice did not reduce protease activity. Therefore, we confirmed that anti-proteolytic bacteria, eradicated by V/M treatment, are essential for the reduction of protease activity.

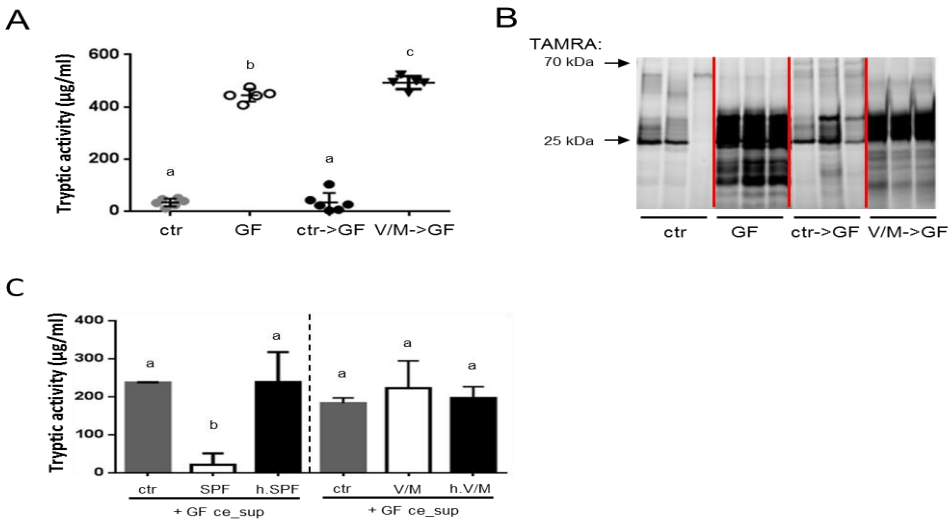

Figure 12: Proteases are inactivated by specific intestinal microbiota eradicated by V/M.
(A & B) The PA and the pattern of serine proteases in cecal_sup of ctr, GF, GF mice colonized with microbiota from ctr mice (ctr->GF) or V/M treated mice (V/M->GF) (A: the PA, B: TAMRA-FP staining). (C) The PA in cecal_sup from GF mice pre-stimulated with buffer ctr, fresh bacteria from SPF or V/M treated mice, and heat-killed respective bacteria.

To identify anti-proteolytic strains of bacteria, we analyzed protease activity in the cecal supernatant of GF mice associated with single bacteria. Cecal contents of GF mice colonized with single strains was provided by our colleagues as described in Table 2. Colonization of GF mice with *Bacteroides sartorii* did not show a reduction of cecal protease activity compared to the level in GF mice (Figure 13A). Colonization of GF mice with *Alistipes sp.* showed a slight decrease in cecal protease activity compared to the level in GF mice but not a statistically significant difference. Mono-association of GF mice with *Lactobacillus murinus* showed a high protease activity similar to that in GF mice. Colonization of GF mice with respective single bacteria showed still strong patterns of active serine proteases in the low

molecular weight similar to the pattern in GF mice (Figure 13B). None of the single bacteria was able to decrease protease activity. To increase the possibility to identify anti-proteolytic strains of bacteria in cecal content of SPF mice, I and my master students, Carina Deli and Tabea Moll, performed *in vitro* screenings of putative anti-proteolytic candidates. We identified 18 strains with anti-proteolytic property *in vitro*. GF mice were colonized with the candidate strains in order to determine whether the putative anti-proteolytic candidate strains reduce a high protease activity *in vivo*. Also, our colleagues and collaborators provided cecal contents of GF mice colonized with different consortia (Table 3). None of OligoMM, B6.ASF, MiBC and Corio consortia showed anti-proteolytic property (Figure 13C). Also, colonization of GF mice with Anti-PA1 or Anti-PA2 consortia did not decrease cecal protease activity, even though these consortia comprised several different bacteria which showed anti-proteolytic property *in vitro*. The patterns of active serine proteases in cecal supernatant of the associated mice were similar to the observations in GF mice (Figure 13D). Taken together, neither the single bacterial strains nor respective bacteria in different consortia were able to inactivate digestive proteases *in vivo*.

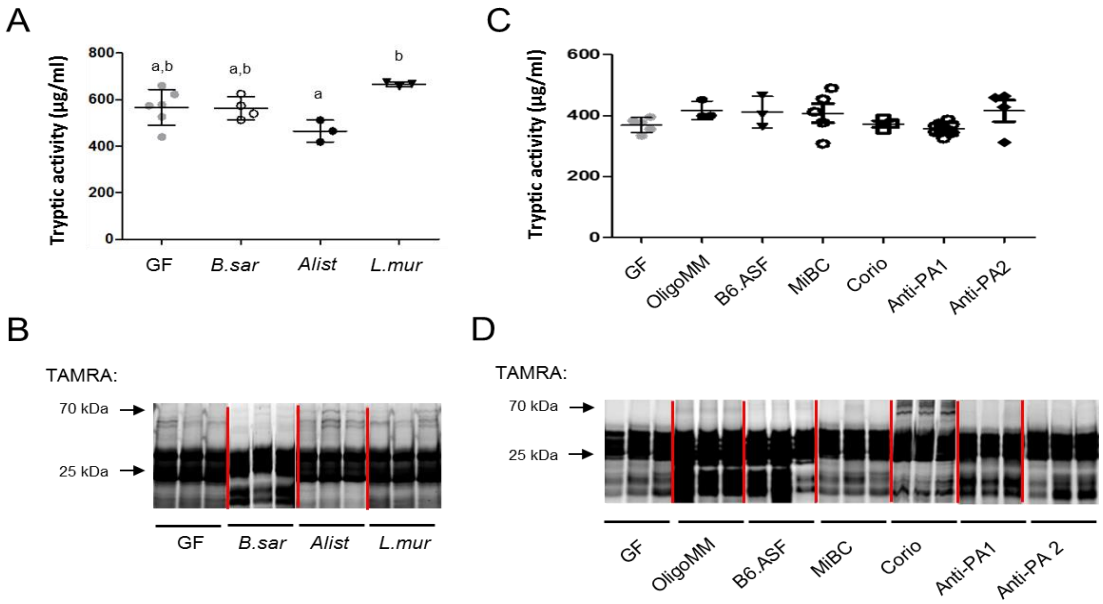

Figure 13: Neither single bacteria nor consortia are able to regulate PA.
(A & B) The PA and the pattern of serine proteases in cecal_sup of respective single bacteria colonized into GF mice (*B.sar*: *Bacteroides sartorii*, *Alist*: *Alistipes sp.*, *L.mur*: *Lactobacillus murinus*) compared to GF mice (A: the PA, B: TAMRA-FP staining). (C & D) The PA and the pattern of serine proteases in cecal_sup of different simplified consortia colonized into GF mice. The composition of respective simplified consortia is described in Table 4. The Anti-PA 1 and 2 consortia were isolated from fresh human stool and mice cecal contents by the master students, Carian Deli and Tabea Moll. The Anti-PA 1 and 2 consortia were composed of different bacterial strains having anti-PA *in vitro*. The PA and pattern in cecal_sup of GF mice associated with the respective microbial consortia were compared to GF mice.

4.2.2 Enhanced protease activity impairs barrier functions *in vitro* and *ex vivo*

The elimination of specific intestinal bacteria in response to V/M treatment increased protease activity in the large intestine. Since it is known that high luminal serine protease activity is detrimental to the intestinal barrier *ex vivo*, [201] the effect of enhanced protease activity on barrier function in the large intestine was further analyzed.

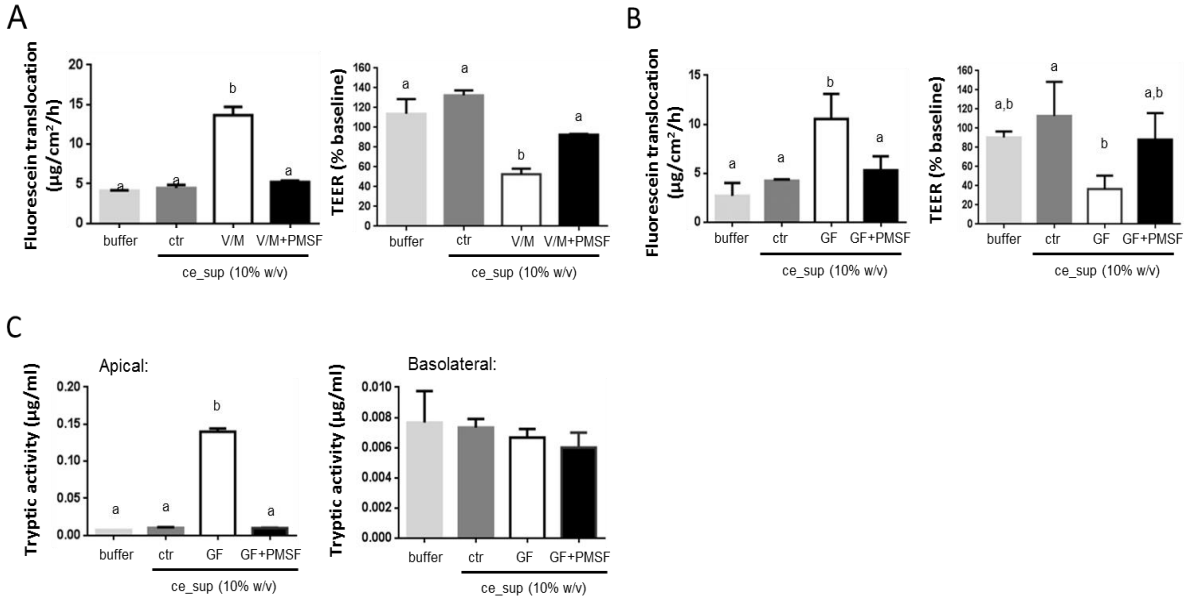

Figure 14: Enhanced PA in response to antibiotics increases epithelial permeability to the level in PTK 6 cells treated with cecal supernatant of GF mice.

(A & B) The TEER and the translocation of fluorescence were measure in the PTK6 cells apically stimulated with either cecal_sup (10% w/v) of V/M treated mice and GF mice, or the respective cecal_sup pre-incubated with PMSF for 4 hours (A: V/M treated mice, B: GF mice). (C) The PA was measured in apical and basolateral sides of transwell permable system after apical stimulation cecal_sup (10% w/v) of GF mice under the same conditions as in Figure 9B.

We hypothesized that the increased protease activity is potentially harmful to the physiological barrier in the large intestine. PTK6 cells were apically stimulated with the cecal supernatant (10 % w/v) of GF or V/M-treated mice in transwell permeable system. Fluorescence translocation to the basolateral side and TEER were analyzed in PTK6 cells in order to confirm the hypothesis. TEER was significantly decreased in PTK6 cells stimulated with cecal supernatant of V/M-treated mice (Figure 14A). However, pre-incubation of the cecal supernatant of V/M-treated mice with the serine protease inhibitor PMSF did not change the TEER, suggesting that increased protease activity is detrimental to the epithelial membrane integrity of PTK6 cells. Importantly, the stimulation of PTK6 cells with cecal supernatant of untreated mice tended to increase TEER slightly. The high protease activity in cecal supernatant of

V/M-treated mice highly increased fluorescence translocation, while apical exposure of PTK6 cells to pre-incubation with PMSF did not affect the epithelial barrier. To consolidate the findings, we repetitively performed stimulation of PTK6 cells with the cecal supernatant (10 % w/v) of GF mice. TEER in PTK6 cells stimulated with the cecal supernatant (10 % w/v) of GF mice was significantly reduced (Figure 14B). Interestingly, the reduction of TEER was completely reversed by the pre-incubation with PMSF. A slight increase in TEER was observed in the stimulation with cecal supernatant of SPF mice. The high protease activity in cecal supernatant of GF mice significantly increased fluorescence translocation, whereas pre-incubation with PMSF did not induce a permeable barrier. We measured apical and basolateral protease activity after the stimulation with the cecal supernatant of GF mice in the transwell permeable system in order to confirm whether the proteases are able to be translocated into the epithelium. Protease activity on the apical side of the transwell was significantly increased after the stimulation with the cecal supernatant of GF mice (Figure 14C). Importantly, protease activity on the basolateral side of the transwell did not change in the stimulation with the GF cecal supernatant, suggesting that the proteases do not translocate through the epithelium. Altogether, the enhanced proteases impaired barrier function and localized in the epithelium.

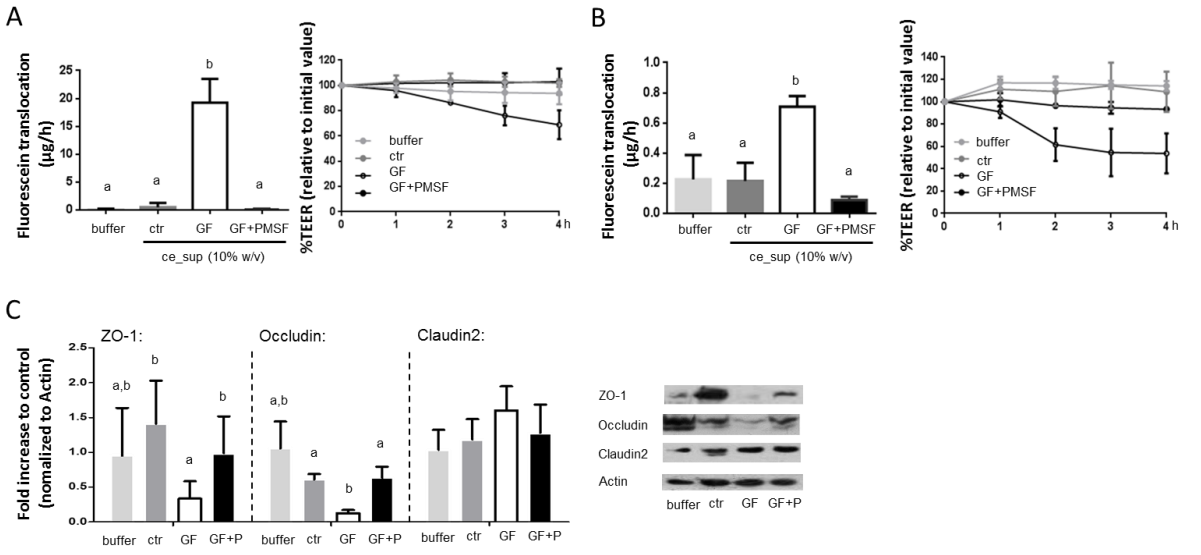

Figure 15: Increased PA in response to antibiotics affects intestinal epithelial barrier function through dysregulation of tight junctions.

(A & B) The TEER and basolateral fluorescence were measured in the cecal and the colonic tissue of SPF mice pre-stimulated with cecal_sup (10% w/v) of the GF mice in an *Ussing* chamber setup (A: cecum, B: colon). (C) The protein expression of tight junctions such as ZO-1, Occludin and Claudin2 was detected in the cecum after stimulation under the same conditions as in Figure 10A. Intensity of each blot in tight junctional proteins was measured and normalized to Actin (n=4).

Tight junctions are located at the lateral side of IEC and anchor the cells to maintain barrier integrity. [229] A few studies showed that high levels of active proteases break down the epithelial barrier in the intestine. [201, 226] To confirm the hypothesis that the increase in protease activity is detrimental to barrier function, we stimulated cecal and colonic tissue *ex vivo* with the cecal supernatant of GF mice (10% w/v) and measured the fluorescein translocation and TEER using the *Ussing* chamber system. The luminal stimulation of cecum with the cecal supernatant of GF mice strongly elevated fluorescein translocation (Figure 15A). This stimulation for 4 hours resulted in ~30% reduction in TEER. However, pre-stimulation with PMSF did not show a penetrable barrier and reduction in TEER. In the colon, the stimulation with the cecal supernatant of GF mice significantly increased fluorescein translocation and reduced TEER (~40%) (Figure 15B). The pre-incubation with PMSF abrogated this detrimental effect, suggesting the enhanced protease activity in the cecal supernatant of GF mice induced epithelial barrier dysfunction in the large intestine. We analyzed the protein expression of tight junctions after luminal stimulation with the cecal supernatant of GF mice in order to investigate the detrimental impact of high protease activity on junctional proteins. Claudin-2 was not changed by the stimulation with the cecal supernatant of GF mice (Figure 15C). Importantly, junctional proteins such as ZO-1 and occludin tended to decrease by the stimulation with the cecal supernatant of GF mice, compared to the stimulation with either buffer control or the cecal supernatant of SPF mice. The pre-incubation with PMSF partially reversed the protein expression of ZO-1 and occludin. These results indicate that **the rise in protease activity impaired the epithelial barrier in the large intestine through the dysregulation of tight junctional proteins such as ZO-1 and occludin.**

4.3 Impact of enhanced protease activity on barrier function and susceptibility to colitis in WT mice

A recent study revealed that antibiotic therapy, especially with fluoroquinolones and metronidazole, increases the risk of developing CD. [102] In spite of the high clinical relevance, the causality of antibiotic therapy and pathophysiological mechanisms underlying this observation are still unknown. Previous results suggest that the rise in protease activity in response to antibiotic treatment disrupts the intestinal barrier, which may be causal for accelerating colitis development. Therefore, we analyzed the impact of the enhanced protease activity in response to antibiotic on barrier functions and proinflammatory responses in WT mice as well as their relevance in disease development in DSS treated WT mice.

4.3.1 Increased protease activity transiently influences barrier function in the cecum of WT mice

To test, whether the enhanced protease activity is detrimental to barrier function *in vivo*, WT mice were sacrificed also at 2 days of V/M treatment (V/M) or at 7 days after discontinuation of V/M treatment (post_V/M) as described in Figure 16A. We investigated protease activity and barrier functions in V/M and post_V/M mice. Fecal protease activity was analyzed in post_V/M mice at day 0, 2, 7 and 14. At day 2, fecal protease activity was strongly increased more than 10 times compared to the level at day 0 (Figure 16B). Post_V/M mice showed sustained high protease activity in the feces during V/M treatment. Interestingly, the high level in fecal protease activity was maintained until day 14, even though inter-individual variability in fecal protease activity was observed during discontinuance of the V/M treatment. Similar to the high level in fecal protease activity, V/M treatment significantly increased cecal protease activity in V/M and post_V/M mice (Figure 16C), demonstrating that the V/M treatment caused a sustained rise in protease activity.

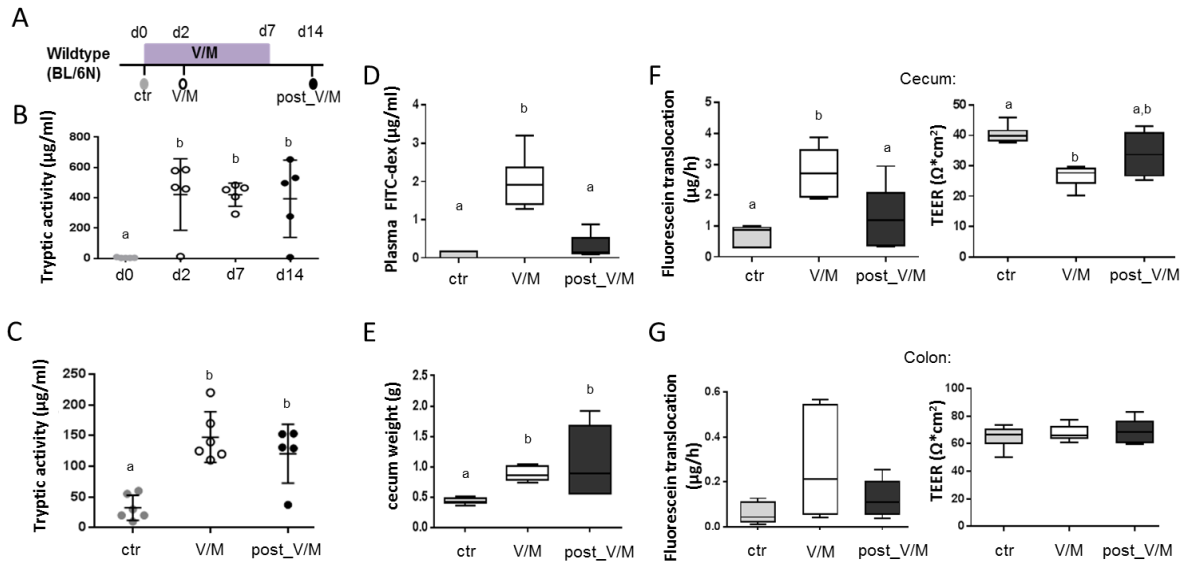

Figure 16: Increased PA in response to antibiotics transiently impairs barrier functions in the cecum of WT mice but not in the colon.

(A) The wildtype (WT) mice were treated with the V/M for 2 days and sacrificed (V/M, n=6). In another group, the WT mice were treated with the V/M for 7 days and the treatment discontinued for 7 days (post_V/M). The post_V/M mice were orally administrated 4kDa of FITC-dextran and were sacrificed at day 14. Feces were collected at day 0, 2, 7 and 14. (B) Fecal PA was measured at the respective day. (C) Cecal PA was increased in V/M and post_V/M mice compared to untreated mice (ctr). (D) Systemic permeability was increase in V/M mice and was normalized to ctr mice in post_V/M mice. (E) Cecum was enlarged in V/M and post_V/M mice. (F) Cecal permeability was increased in V/M mice only. (G) Barrier functions in the colon were not changed in V/M and post_V/M mice. Fluorescein translocation of the colon showed inter-individual variability in V/M mice. One-way ANOVA was conducted to compare a statistical significance. Post comparisons were carried out using Tukey's post-test. Different superscripts indicated a significant difference ($P < 0.05$).

FITC-dextran was orally administered to V/M and post_V/M mice 4 hours before mice were sacrificed in order to assess the role of increased protease activity in barrier functions. We measured systemic translocation of FITC-dextran. TEER and permeability of cecal and colonic tissue were analyzed in the *Ussing* chamber setup. V/M mice showed significant increase in plasma FITC-dextran compared to untreated mice, whereas post_V/M mice showed a normal level of FITC-dextran in plasma such as the untreated mice (Figure 16D). Increased protease activity might be connected with transiently elevated permeability in the cecum. Cecum weight was significantly increased in V/M and post_V/M mice. (Figure 16E) Cecum and distal colon of V/M and post_V/M were mounted in the *Ussing* chamber in order to analyze TEER and tissue permeability. V/M mice showed increased level of translocation of fluorescein and reduced TEER in the cecum (Figure 16F). Interestingly, post_VM mice increased fluorescein translocation and decreased TEER compared to V/M mice. In contrast to the level in the cecum, colonic permeability and TEER were not different among the three groups (Figure 16G). An individual difference in fluorescein translocation was observed in the colon of V/M mice. These findings revealed that the rise in protease activity was associated with transiently impaired barrier function in the cecum but not in the colon. The transient increase in plasma levels of orally administered FITC dextran was associated with barrier defect in the cecum.

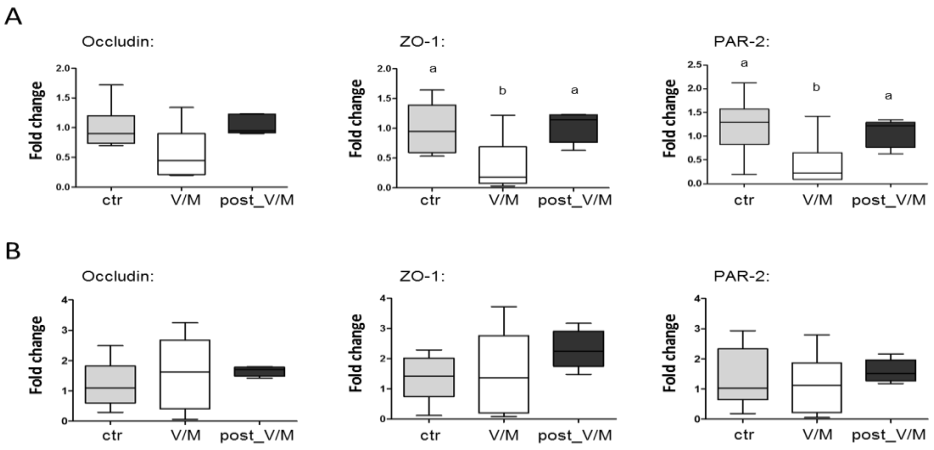

Figure 17: V/M treatment only affects the mRNA level of ZO-1 and PAR-2 in the cecum tip of WT mice.
(A & B) There was no difference in the mRNA level of tight junctions such as occludin and ZO-1, and PAR-2 in cecum tip (A) and pC (B) of V/M and post V/M mice. The mRNA levels of ZO-1 and PAR-2 were significantly decreased in only cecum tip of V/M mice. All target genes were normalized to GAPDH (n=6). One-way ANOVA was followed by Tukey's post-test. Values with different superscripts (a, b and c) were significantly different between groups ($P < 0.05$).

As shown above, the increase in protease activity was closely connected to barrier impairment in V/M-treated mice. However, causality of the permeable intestinal barrier in excess levels of pancreatic

proteases was still unclear. To assess the role of increased protease activity in disruption of tight junctions, the mRNA level of tight junctions and PAR-2 was analyzed in the cecum tip and proximal colon of V/M and post_V/M mice. Several studies revealed that tight junctional proteins are regulated by PAR-2 activation. [185, 188] One study showed that the rise in protease activity is associated with PAR-2 activation and its relevant junctional protein regulation. [201] The occludin gene expression was not changed in the cecum tip (CT) of V/M and post_V/M mice (Figure 17A). While V/M mice showed a significant decrease in the mRNA level of ZO-1, post_V/M mice showed restored ZO-1 expression. The mRNA level of PAR-2 in the CT of V/M and post_V/M mice was similar with the ZO-1 expression. The PAR2 mRNA was absent during antibiotic treatment and was strongly upregulated after antibiotic treatment. Similar to the occludin mRNA expression in the CT, the mRNA level of occludin in the proximal colon (pC) of V/M and post_V/M mice was unchanged (Figure 17B). No change in the ZO-1 and PAR-2 gene expression was shown in the pC of V/M and post_V/M. Overall, increased protease activity in response to V/M treatment is associated with transient impairment of barrier functions in the cecum via downregulation of the mRNA expression of ZO-1 and PAR-2.

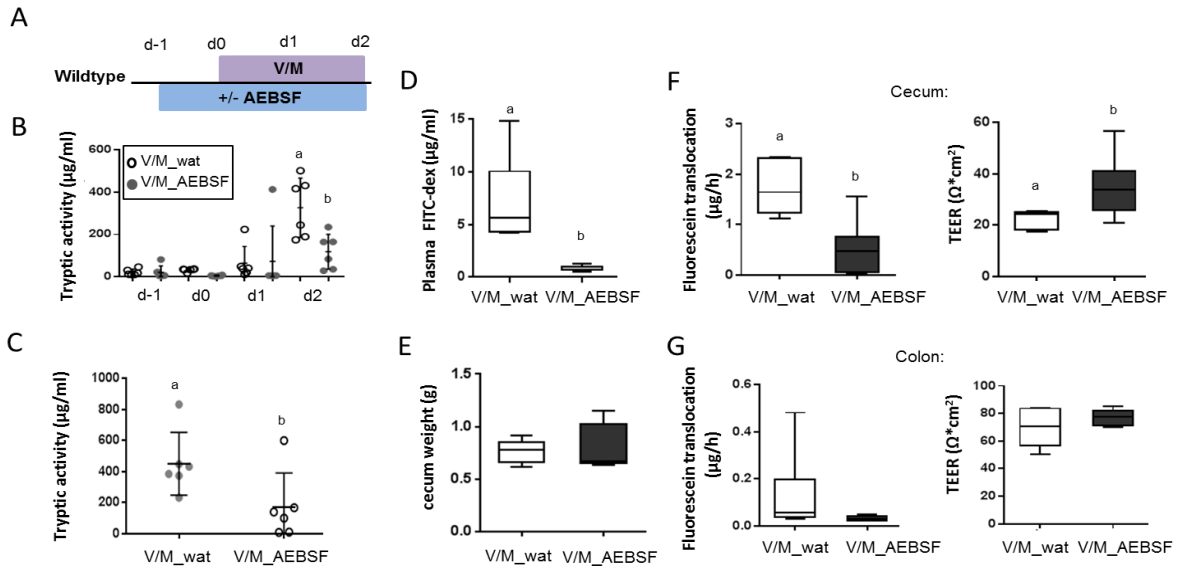

Figure 18: Serine protease inhibitor antagonizes barrier impairment in the cecum of WT mice.
(A) The WT mice were treated with AEBSF, a serine protease inhibitor for 3 days, starting one day before the V/M treatment (V/M_AEBSF). Mice only treated with V/M are referred to as V/M_wat. Feces were collected every day for 4 days (n=6). Cecal PA, cecum size and barrier function were analyzed in V/M_wat and V/M_AEBSF mice. (B) Increased PA in feces were partially reduced in V/M_AEBSF mice compared to V/M_wat mice at day 2. (C) Cecal PA was partially decreased in V/M_AEBSF mice. (D) The 4 kDa of FITC dextran in serum was decreased in V/M_AEBSF mice. (E) Cecum size was not different in both groups. (F & G) The V/M_AEBSF mice decreased fluorescein translocation and increased TEER only in the cecum. The fluorescein translocation of the colon showed inter-individual variability in V/M_wat mice.

We orally administrated the serine protease inhibitor AEBSF one day before V/M treatment in order to confirm that the increase in protease activity in response to the V/M treatment is causal for impairment of barrier in WT mice. The experimental design was described in Figure 18A. Feces were collected daily to confirm whether the serine protease inhibitor could reduce the rise in protease activity in the large intestine. The tissue permeability and TEER as well as FITC-dextran translocation were analyzed in V/M-treated mice (V/M_wat) and AEBSF co-treated (V/M_AEBSF). While fecal protease activity was strongly increased in V/M_wat mice, V/M_AEBSF mice showed partial reduced fecal protease activity at day 2 (Figure 18B). Importantly, AEBSF co-treatment did not completely normalize fecal protease activity compared to the level before V/M treatment. Also, cecal protease activity showed the same tendency with fecal protease activity at day 2 (Figure 18C), suggesting that AEBSF co-treatment reversed the detrimental impact of V/M treatment.

To access the causal role of increased protease activity in the impairment of barrier functions, systemic translocation of FITC-dextran was investigated in V/M_wat and V/M_AEBSF mice. AEBSF co-treatment significantly reduced FITC-dextran in plasma compared to the level in V/M_wat mice (Figure 18D). Translocation of FITC-dextran in V/M_AEBSF mice was similar to the level observed in untreated mice (Figure 16D). AEBSF co-treatment did not change cecum weight compared to V/M_wat mice (Figure 18E). These results demonstrate that the rise in protease activity is causal for increasing permeability. To investigate the effect of AEBSF co-treatment in tissue permeability, TEER and fluorescein translocation were measured in the cecum and colon of V/M_wat and V/M_AEBSF mice using the *Ussing* chamber setup. AEBSF co-treatment strongly reduced fluorescein translocation and significantly increased TEER in the cecum compared to only V/M treatment (Figure 18F). However, there were huge individual differences in fluorescein translocation and TEER in V/M_AEBSF mice. In the colon, fluorescein translocation and TEER were not different between V/M_wat and V/M_AEBSF mice (Figure 18G). Interestingly, V/M_wat mice showed huge variation in colonic permeability such as V/M mice (Figure 16G), whereas AEBSF co-treatment completely reduced fluorescein translocation. These findings demonstrated that the increase in protease activity in response to V/M treatment plays a causal role for transient impairment of barrier in the cecum of WT mice. Additionally, the serine protease inhibitor co-treatment is able to protect barrier function against high protease activity.

4.3.2 Metronidazole is a major cause of the intestinal barrier dysfunction

In order to test which antibiotic affected protease activity and barrier function, WT mice received either metronidazole or vancomycin only for 2 days (Figure 19A&G). Protease activity and barrier functions were analyzed in metronidazole treated and vancomycin treated mice. Fecal protease

activity was significantly increased during metronidazole treatment (Figure 19B). Also, the cecal protease activity was significantly elevated in metronidazole treated mice compared to untreated mice (Figure 19C).

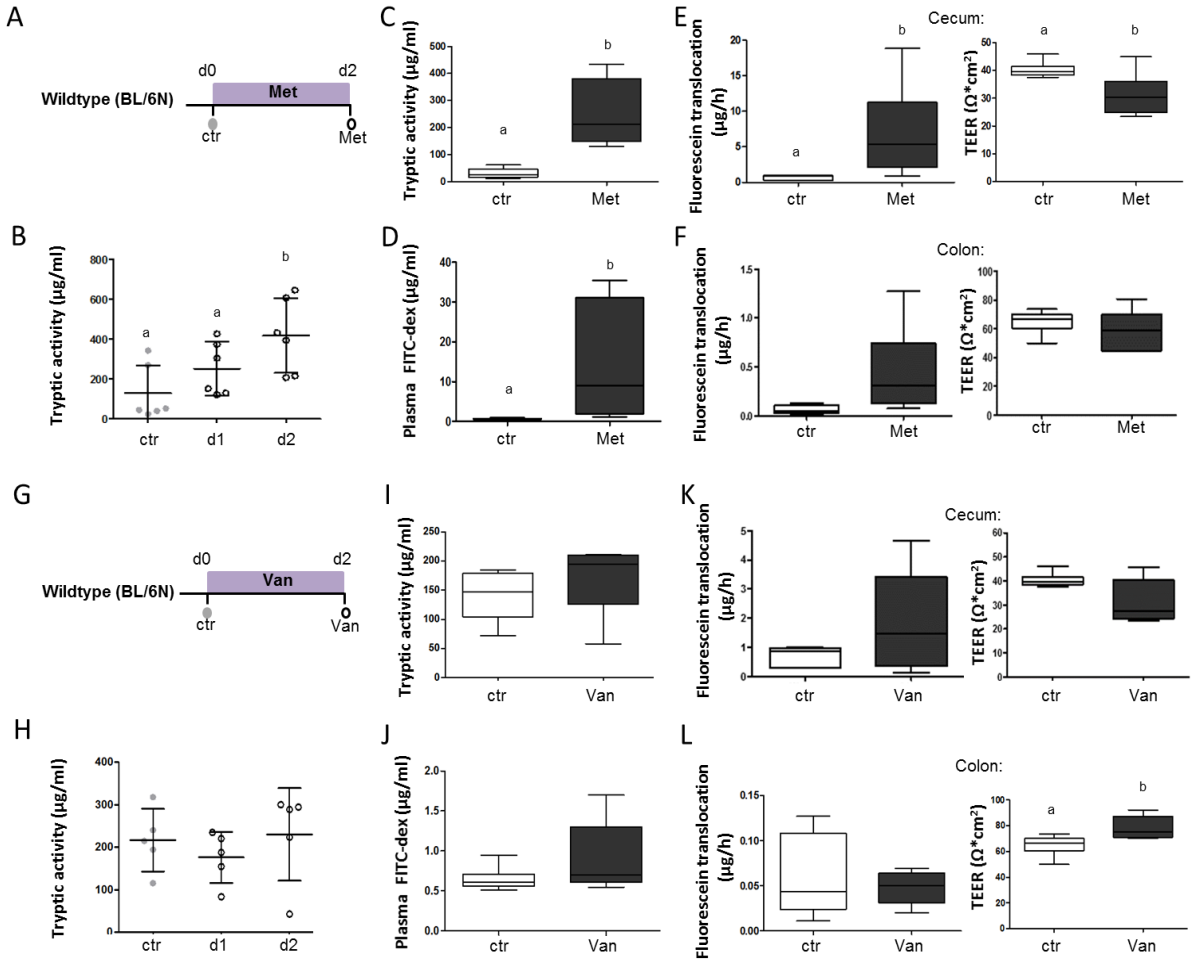

Figure 19: Metronidazole treatment increases PA and impairs epithelial barrier in the large intestine.
(A) The WT mice received metronidazole (Met) at the age of 8 weeks for 2 days (n=6). Feces were collected for 2 days. PA and barrier functions were analyzed in ctr and Met mice. (B & C) Kinetic fecal and cecal PA were increased in Met mice. (D) Systemic permeability was increased in Met mice. (E & F) Cecal permeability was increased in Met mice. (G) The WT mice were treated with the vancomycin for 2 days (Van) (n=5). Analysis of the PA and of barrier functions in Van mice was the same as in Met mice. (H & I) Fecal and cecal PA was not changed in Van mice. (J to L) The systemic and local barrier in the cecum and colon was not affected in Van mice. Unpaired t-test. Different characters (a and b) showed a difference in significance ($p<0.05$).

To consolidate the hypothesis of metronidazole treatment being detrimental to protease activity and barrier functions in the large intestine, tissue permeability and TEER in the cecum and colon, and systemic translocation of FITC-dextran were analyzed in metronidazole treated mice. Metronidazole

treatment strongly increased plasma levels of orally administered FITC-dextran compared to non-treatment (Figure 19D). While metronidazole treated mice showed a significant increase in fluorescein translocation and a decrease in TEER of the cecum (Figure 19E), barrier functions in the colon was not affected by metronidazole treatment (Figure 19F). However, high inter-individual differences in colonic permeability were observed in metronidazole treated mice. In contrast to metronidazole treatment, fecal protease activity was not affected by vancomycin treatment (Figure 19H). Similar to the fecal protease activity, cecal protease activity was not different between untreated and vancomycin treated mice (Figure 19I). Vancomycin treatment slightly increased FITC-dextran in the plasma due to the individual differences (Figure 19J). Levels of fluorescein translocation and TEER in the cecum of vancomycin treated mice were similar to observation in untreated mice (Figure 19K). In vancomycin treatment, there was no difference in colonic permeability compared to untreated mice. However, TEER in the colon was significantly increased in vancomycin treated mice (Figure 19L). These results suggested that enhanced protease activity and its relevant impairment of the cecal barrier in WT mice was mainly caused by metronidazole treatment rather than vancomycin treatment.

4.3.3 Enhanced protease activity does not affect intestinal homeostasis and inflammation

Analysis of gene expression related to intestinal homestasis and inflammation was performed in the proximal colon (pC) of untreated, V/M and post_V/M mice in order to evaluate the hypothesis of whether the enhanced protease activity in response to V/M treatment affects the cellular proliferation and differentiation, and triggers inflammation in WT mice (Figure 20A). The mRNA level of Ki67, the proliferation marker, intestinal alkaline phosphatase (ALPI) and intestinal fatty acid binding protein (IFABP) as markers of cellular differentiation were analyzed. The mRNA level of Ki67 was not different among all three groups (Figure 20B). However, inter-individual differences were observed in pC of V/M mice. ALPI and IFABP in the mRNA level were not changed in V/M and post_V/M mice compared to those in untreated mice (Figure 20C). These results indicated that the enhanced protease activity in response to V/M treatment was not associated with cellular proliferation and differentiation.

The mRNA levels of TNFα and IL1β, which are proinflammatory cytokines, were measured. Neither TNFα nor IL1β gene expression was changed in V/M and post_V/M mice (Figure 20D). However, there was a high inter-individual variability in TNFα gene expression of V/M mice and IL1β gene expression of post_V/M mice. V/M and post_V/M mice showed no difference in the mRNA level of CD3 (Figure 20E). Foxp3 gene expression was slightly but not significantly increased in V/M mice compared to untreated mice. These results suggested that increased protease activity is not associated with triggering the proinflammatory response in WT mice during or after V/M treatment. In addition, these

findings support the hypothesis that increased protease activity might not be associated with susceptibility towards DSS-induced colitis in WT mice.

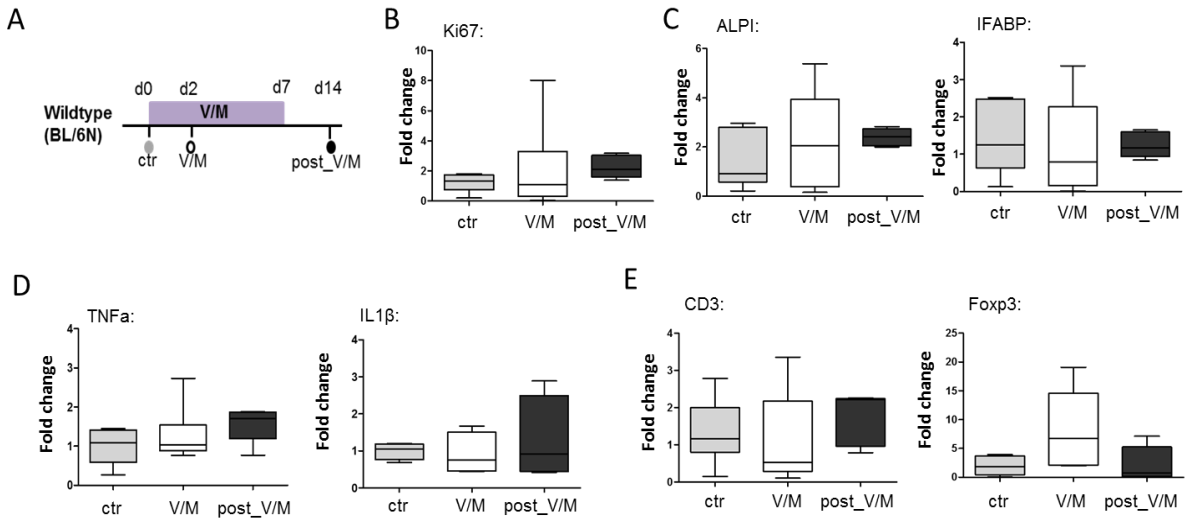

Figure 20: V/M treatment has no effect on the mRNA level of proinflammatory cytokines, T cells, proliferation and cellular differentiation in the proximal colon of WT mice.
(A) The experimental design was the same as in figure 11A. (B) The mRNA level of Ki67, which is a proliferation marker, did not differ in pC of V/M and post_V/M mice compared to ctr mice. (C) The mRNA level of cellular differentiation such as ALPI and IFABP was not altered in pC of V/M and post_V/M mice. (D) The mRNA levels of proinflammatory cytokines such as TNFα and IL1β were not altered in the proximal colon of V/M and post_V/M mice compared to ctr mice. (E) The mRNA level of CD3 and Foxp3 did not differ in the proximal colon of V/M and post_V/M mice. All target genes were normalized to GAPDH (n = 6/group). One-way ANOVA; Tukey's post-test. All values are mean ± SD.

4.3.4 Increased protease activity does not subsequently affect DSS-induced colitis in WT mice

WT mice were repetitively administered V/M for 7 days at the age of 4 and 8 weeks in order to evaluate the hypothesis whether enhanced protease activity exacerbates susceptibility towards DSS-induced colitis in WT mice (Figure 21A). The V/M-treated WT mice received DSS for 7 days at the age of 12 weeks. Feces were collected to analyze protease activity and the composition of intestinal microbiota before and after V/M and DSS treatment.

Repetitive V/M treatment did not affect the development of body weight (Figure 21B). Cecal weight was not different in repetitive V/M treated mice compared to untreated mice (Figure 21C). Colon length did not shrink in repetitive V/M treated mice (Figure 21D), suggesting that repetitive V/M treatment might not be associated with triggering inflammation in the cecum and colon. Repetitive V/M treated mice did not show any change in MLN and spleen weight (Figure 21E&F). Similar to the

observation of organ weight, the level of serum amyloid A (SAA), a systemic inflammation marker, was not changed in plasma of repetitive V/M treated mice (Figure 21G). The first cycle of V/M already induced an increase in fecal protease activity (Figure 21H). Importantly, enhanced protease activity consistently lasted until discontinuance of the second cycle of V/M. At the age of 12 and 13 weeks, high inter-individual differences in fecal protease activity were observed in repetitive V/M treated mice. DSS treatment did not affect fecal protease activity in untreated and repetitive V/M treated mice. These findings indicated that repetitive V/M treatment resulted in a long-lasting increase in protease activity. There was no difference in DAI between untreated and repetitive V/M treated mice after DSS treatment (Figure 21I). Similar to DAI score, the histopathological score did not change in repetitive V/M treated mice compared to untreated mice. All in all, long-lasting increase in protease activity upon V/M treatment did not disturb the intestinal immune homeostasis in WT mice.

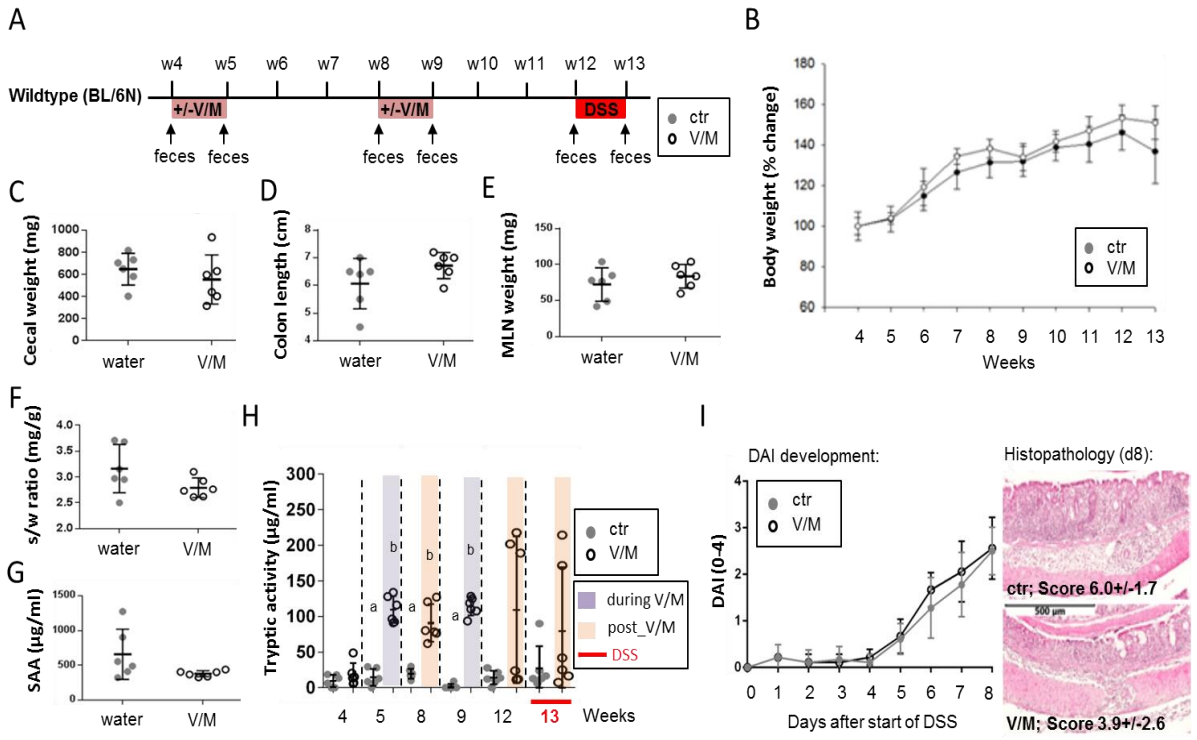

Figure 21: Repetitive antibiotic treatments result in a long-lasting increase in PA but does not affect subsequent DSS-induced colitis in WT mice.

(A) Scheme for repetitive V/M treatment is given. WT mice were treated with antibiotic two times for 7 days at the age of 4 and 8 weeks and induced acute colitis by 1.5% DSS at the age of 12 weeks. Feces were collected at the age of 4, 5, 8, 9, 12 and 13 weeks. (B) The body weight did not change during repetitive V/M treatment. (C to F) Macroscopic markers such as cecum weight (C), colon length (D), MLN weight (E) and spleen weight (F) were not affected in repetitive V/M treated (V/M) mice. (H) The kinetic fecal PA was increased from first course of V/M onwards and was partially normalized at 12 weeks in V/M mice. (G & I) The histopathological inflammation (I) as well as plasma SAA levels (a systemic inflammation marker, G) upon DSS treatment did not differ in V/M mice compared to ctr mice.

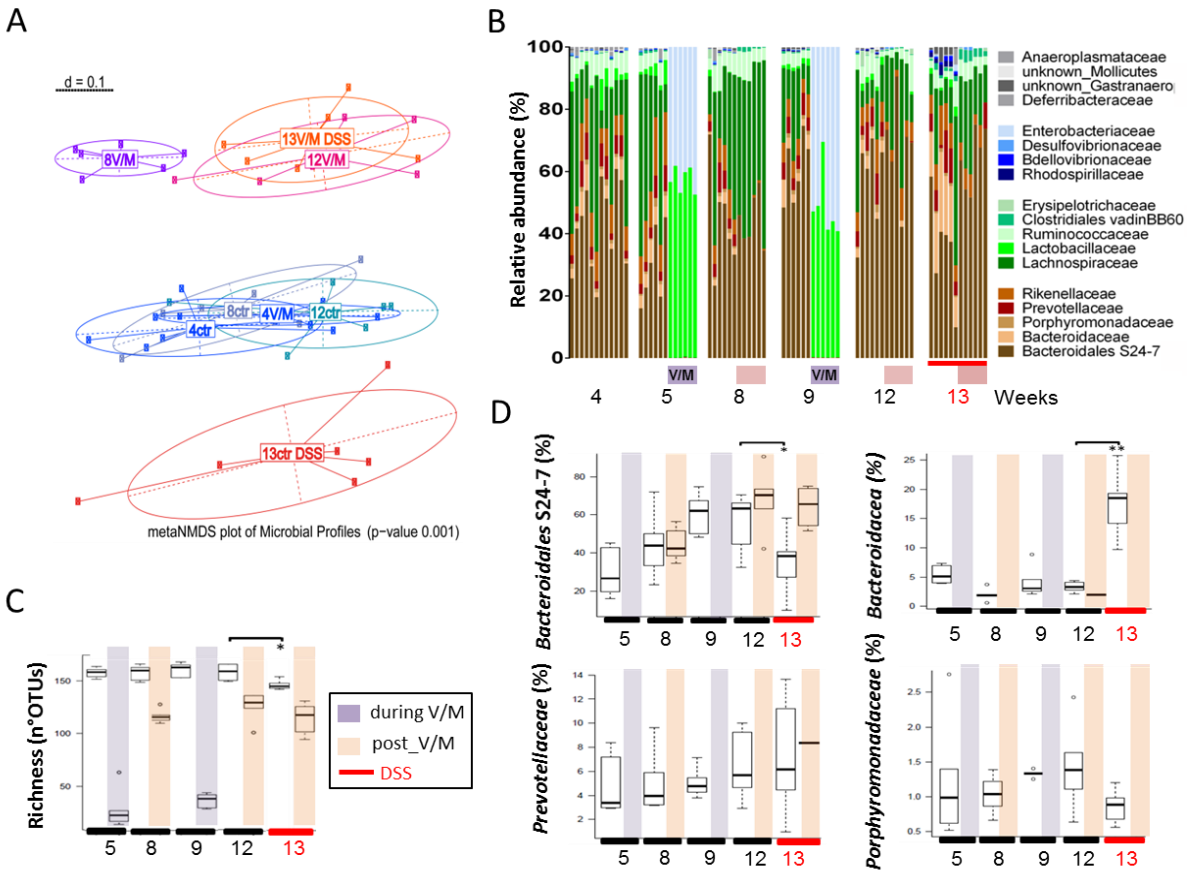

Figure 22: Repetitive antibiotic treatments promote lasting compositional dysbiosis.
(A) MetaNMDS analysis showed repetitive V/M treatment completely shifted microbial diversity in V/M mice. (B) Relative abundance of bacterial taxa in ctr mice compared to V/M mice at the family level. (C) Richness was decreased during repetitive V/M treatments and DSS treatment. (D) Some taxa were lastingly changed by repetitive V/M treatment. DSS treatment changed some taxa in V/M mice.

To investigate the effect of repetitive V/M treatment on compositional change in intestinal microbiota and on eradication of anti-proteolytic taxa, we analyzed microbial composition, *beta* diversity, species richness and regulated bacterial taxa in collected feces using 16S rRNA gene-based sequence analysis. *Beta*-diversity analysis revealed that repetitive V/M treatment showed clear separation between untreated and V/M treated groups (Figure 22A). The bacterial community structure was not clustered at 3 weeks after discontinuance of the second course of V/M. DSS treatment also showed separation of the bacterial community structure between both groups. The structure of the bacterial community in first and second V/M treatment was differentiated from that in untreated mice (Figure 22B). The bacterial composition from the first course of V/M to the second course of V/M did not return to the pre-treatment state. Repetitive V/M treated mice showed acute compositional alteration at the age of 5 and 9 weeks. Additionally, repetitive V/M treatment resulted in long-term compositional changes in

the intestinal microbiota. *Lactobacillaceae* and *Enterobacteriaceae* were dominant families during V/M treatment. Certain taxonomic groups did not come back to normal status at 3 weeks after the cessation of the second V/M treatment. Repetitive V/M treatment significantly reduced microbial richness (Figure 22C). Repetitive V/M treatment even diminished microbial richness upon DSS treatment. Some taxa such as *Bacteroidaceae*, *Prevotellaceae* and *Porphyromonoadaceae* significantly diminished due to V/M and DSS treatment (Figure 22D). These findings suggested that repetitive V/M treatment resulted in compositional disturbances in the intestinal microbiota.

Taking all findings together, repetitive V/M treatment led to a long-lasting increase in protease activity and alteration in the intestinal microbiota but not to the development of subsequent DSS-induced colitis.

4.4 Increased protease activity accelerated colitis in disease-susceptible hosts

Previous results revealed that V/M treatment exerted detrimental effects on the large intestinal barrier in WT mice but did not exacerbate subsequent DSS-induced colitis. Despite the high clinical relevance of antibiotic therapy aggravating colitis, [103, 104] it is unknown whether the impact of any specific antibiotic therapy is associated with protease activity and a subsequent development of colitis. To address the question of whether increased protease activity in response to repetitive V/M treatment is associated with impairment of intestinal barrier and acceleration of colitis in the susceptible host, IL10-/- mice, being a model of chronic inflammation-induced colitis development, received antibiotics.

4.4.1 Enhanced protease activity consistently impairs barrier function in IL10-/- mice

An identical treatment with V/M and analysis in WT mice (Figure 16) was performed in IL10-/- mice in order to investigate the impact of V/M treatment on the increase in protease activity and its relevant barrier impairment in IL10-/- mice (Figure 23A). IL10-/- mice were sacrificed at day 2 of V/M treatment (V/M) or at day 7 after discontinuation of V/M treatment (post_V/M). V/M treatment increased fecal protease activity rapidly (~3-fold), and the protease activity remained constant (Figure 23B). V/M treatment significantly increased cecal protease activity of V/M and post_V/M mice (Figure 23C). Systemic translocation of FITC-dextran was significantly increased in V/M mice and did not diminish in post_V/M mice (Figure 23D). V/M and post_V/M mice showed an enlarged cecum compared to untreated IL10-/- mice (Figure 23E). While cecal permeability was not significantly increased in V/M mice due to the inter-individual variability, post_V/M mice showed a permeable barrier in the cecum (Figure 23F). TEER of the cecum, analyzed with the *Ussing* chamber system, was unchanged in both

V/M and post_V/M mice. Surprisingly, both V/M and post_V/M mice showed an increased colonic permeability, however huge inter-individual differences were measured in post_V/M mice (Figure 23G). These results suggested that V/M treatment led to an increase in protease activity and impaired barrier in the cecum and colon of IL10-/- mice. Interestingly, whereas V/M treatment in WT mice showed a transient barrier impairment only in the cecum but not in the colon, V/M treatment in IL10-/- mice showed a consistent barrier impairment both in the cecum and colon.

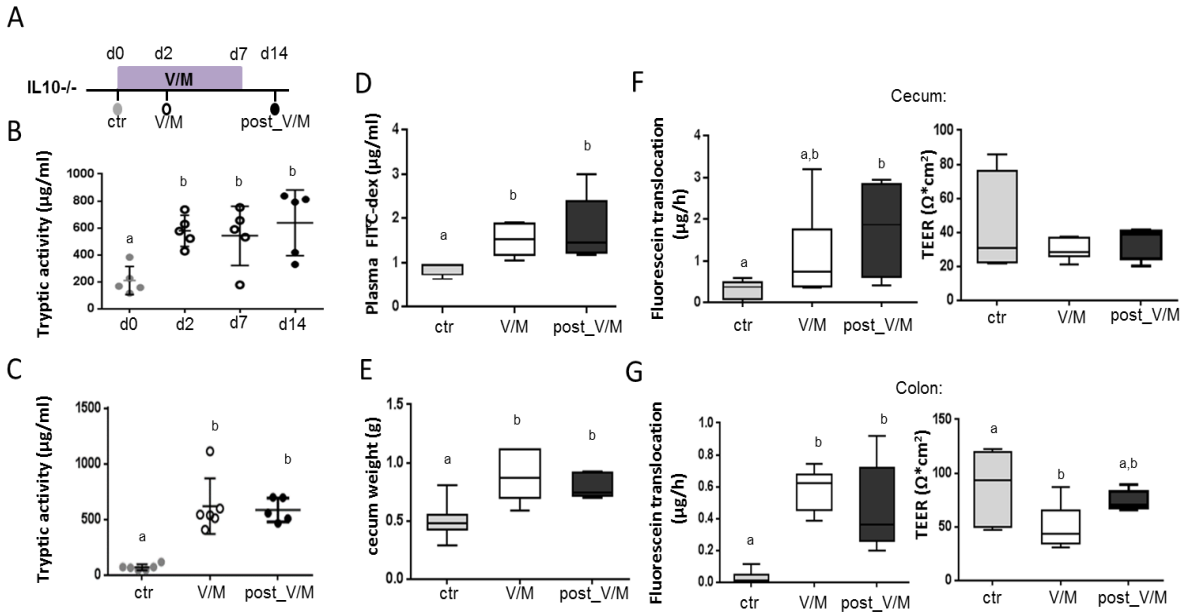

Figure 23: Enhanced PA in response to antibiotics results in persistent impairment of the large intestinal barrier in IL10-/- mice.

(A) The experimental design of the V/M treated IL10-/- mice was the same as in figure 11A. IL10-/- mice were treated with the V/M for either 2 days (V/M, n=6) or 7 days and sacrificed at day 14 (post_V/M, n=5). (B) Kinetic fecal PA was strongly increased from day 2 of the V/M treatment. (C) Cecal PA was increased in V/M IL10-/- mice and was persistently maintained in post_VM IL10-/- mice. (D) Systemic permeability was strongly increased in V/M and post_V/M IL10-/ mice. (E) Enlarged cecum at day 2 of the V/M treatment was not reduced at day 14. (F & G) Cecal permeability was increased only in post_V/M IL10-/- mice, however colonic barrier was impaired in both V/M and post_V/M IL10-/- mice. Different characters (a and b) showed a difference in significance ($p<0.05$); One-way ANOVA, Tukey's post-test..

4.4.2 Impaired barrier is associated with dysregulation of tight junctional protein through PAR-2 activation and inflammation in IL10-/- mice

It has been a long known fact that PAR-2 activation promotes disruption of tight junctions related to barrier dysfunction in the intestine. [194] To confirm the hypothesis that increased protease activity activates PAR-2, we measured the mRNA level of PAR-2 in the cecum tip, proximal and distal colon of

V/M and post_V/M mice. Immunofluorescence staining and protein level of PAR-2 were analyzed in the proximal and distal colon of V/M and post_V/M mice.

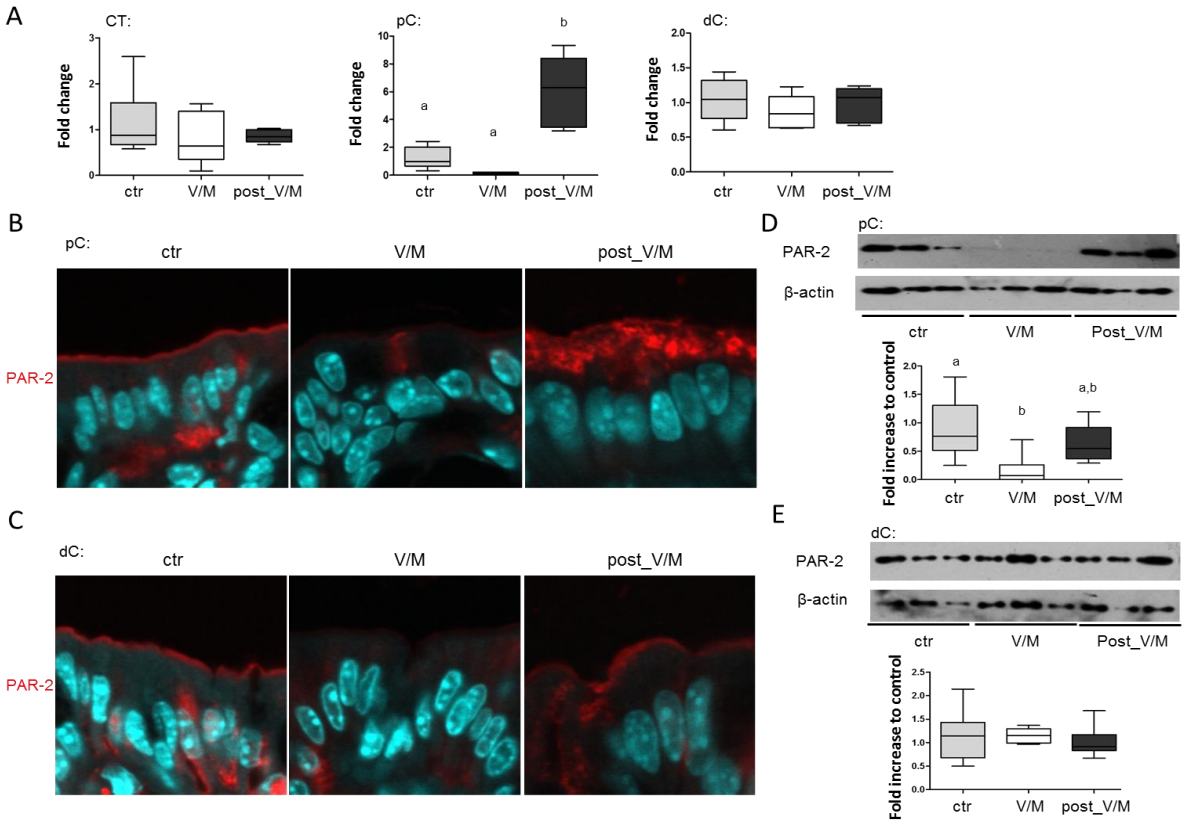

Figure 24: PAR-2 is absent during V/M treatment and is restored at the cessation of V/M in the proximal colon of IL10-/- mice.
(A) The mRNA level of PAR-2 was decreased in pC of V/M IL10-/- mice and was increased in pC of post_V/M IL10-/- mice. However, the mRNA level of PAR-2 was not altered in other parts of tissue in V/M and post_V/M IL10-/- mice. (B & C) PAR-2 disappeared in both proximal and distal colon of V/M IL10-/- mice and recovered in post_V/M IL10-/- mice (magnification: 180X, proximal colon: upper panel, distal colon: lower panel). (D) The western blot of PAR-2 in pC showed that the expression of PAR-2 was decreased in V/M treatment and partially recovered in post_V/M IL10-/- mice. (E) The expression of PAR-2 in dC was not altered in V/M and post_V/M IL10-/- mice. All tight junctional proteins were normalized to Actin (n = 5-6/group). One-way ANOVA; Tukey's post-test ($P < 0.05$). All values are mean ± SD.

There was no difference of PAR-2 at the mRNA level in the cecum tip and distal colon of V/M and post_V/M mice compared to untreated IL10-/- mice (Figure 24A). Interestingly, transcript level of PAR-2 was elevated in the proximal colon of post_V/M mice. Immunofluorescence staining revealed that PAR-2 completely disappeared in the proximal colon of V/M mice and was restored in the pC of post_V/M mice (Figure 24B). While the location of PAR-2 was in apical membrane of epithelial cells in

untreated mice, PAR-2 was observed in intracellular vesicles of epithelial cells in the proximal colon. Immunofluorescence staining of PAR-2 in the proximal colon showed the same tendency with the transcript level of PAR-2. Loss of PAR-2 was observed in the distal colon of V/M mice (Figure 24C). However, PAR-2 was restored in the distal colon of V/M mice. Western blot analysis clearly demonstrated an absence of PAR-2 in the proximal colon of V/M mice but not in post_V/M mice (Figure 24D). In contrast to immunofluorescence staining, the PAR-2 protein level was not reduced in the distal colon of V/M mice (Figure 24E). These findings suggested that PAR-2 was activated by increased protease activity in the proximal colon of V/M mice and was relocated to the proximal colon of post_V/M mice.

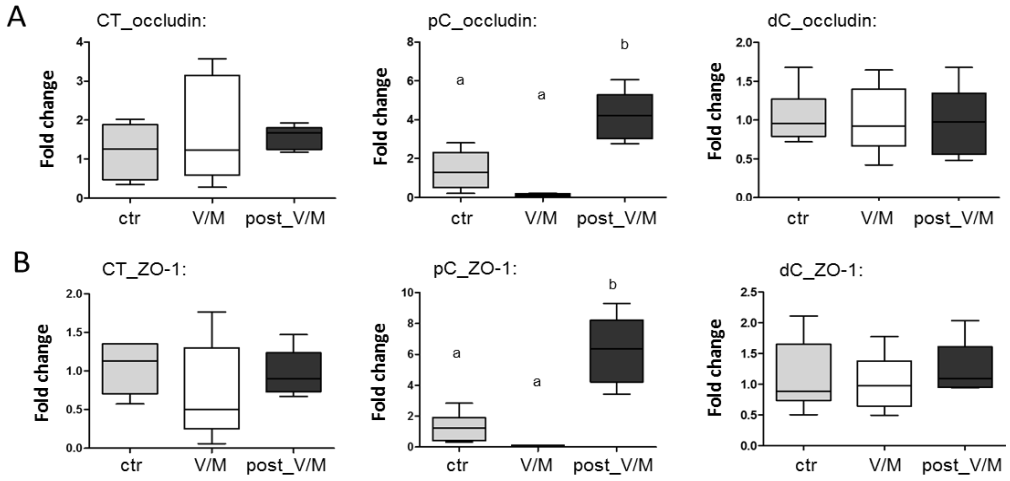

Figure 25: V/M treatment shows an increase in mRNA levels of occludin and ZO-1 only in the proximal colon of post_V/M mice.
(A & B) The mRNA levels of occludin and ZO-1 were decreased in pC of V/M IL10-/- mice and were restored in pC of post_V/M IL10-/- mice. However, other parts of tissue showed no change in mRNA level of tight junctional proteins (A: Occludin, B: ZO-1). Experiment of target genes were normalized to GAPDH. (n = 6/group). One-way ANOVA was followed by Tukey's post-test. Different superscripts (a, b and c) showed statistical significances between groups ($P < 0.05$). All values are mean ± SD.

Tight junction proteins were analyzed in the colon of V/M and post_V/M mice in order to consolidate the hypothesis that enhanced protease activity impairs barrier function proteins through PAR-2 activation. Whereas occludin gene expression was not changed in the cecum tip and distal colon of both V/M and post_V/M mice, the expression pattern of occludin diminished only in the proximal colon of V/M mice and was highly elevated in the proximal colon of post_V/M mice (Figure 25A). Interestingly, the transcript level of ZO-1 gene expression in each tissue showed the same tendency as the occludin expression (Figure 25B). These findings suggested that downregulation of occludin and ZO-1

expression only in the proximal colon is associated with PAR-2 activation and is caused by dysfunction of the colonic barrier during V/M treatment. Importantly, however, profiling of tight junctions mRNA expression is not associated with consistent impairment of the colonic barrier in post_V/M mice.

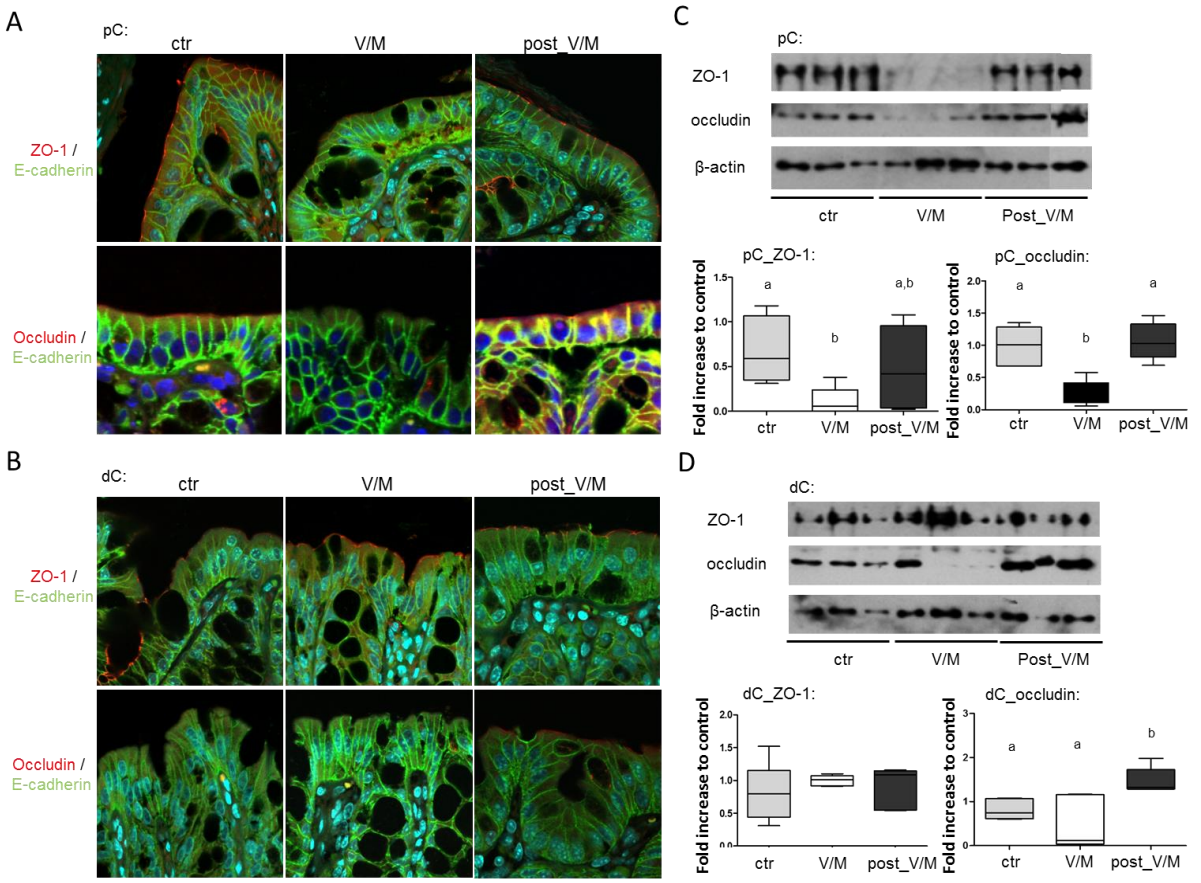

Figure 26: Occludin and ZO-1 expression is transiently reduced in the proximal colon of V/M treated IL10-/- mice.
(A) Immunofluorescence (IF) staining showed the absence of occludin and ZO-1 in the proximal colon of V/M IL10-/- mice and the restoration of occludin and ZO-1 in post_V/M IL10-/- mice (120X, ecadherin: green, Occludin: red, DAPI: Blue). (B) The IF stating of occludin and ZO-1 was not different in dC of V/M and post_V/M IL10-/- mice (120X, ecadherin: green, ZO-1: red, DAPI: Blue). (C) The Occludin and ZO-1 expressions were decreased in pC of V/M mice and recovered in post_V/M mice. (D) The expression levels of Occludin and ZO-1 were not decreased in distal colon (dC) of V/M and post_V/M mice. All tight junctional proteins were normalized to Actin. (n = 5-6/group). One-way ANOVA; Tukey's post-test. All values are mean ± SD.

To investigate the impact of increased protease activity on tight junction proteins, we measured the occludin and ZO-1 protein levels using the western blot and immunofluorescence analysis. Occludin and ZO-1 disappeared in the epithelial cells of the proximal colon in V/M mice, while post_V/M mice showed restoration of these tight junction proteins (Figure 26A). A western blot analysis confirmed

that downregulated tight junction proteins ZO-1 and Occludin in response to V/M treatment were almost restored in post_V/M mice (Figure 26C). However, there was no difference of ZO-1 on protein expression level in the distal colon (Figure 26B). Occludin was not clearly observed in the distal colon of untreated and V/M mice, but post_V/M mice showed a presence of occludin. The protein level of ZO-1, analyzed by the western blot, showed a similar tendency with that shown in the immunofluorescence staining (Figure 26D). Unexpectedly, post_V/M mice showed a significant increase on the protein level of occludin.

All in all, these results indicated that the significant loss of occludin and ZO-1 in response to V/M treatment was causal for the colonic barrier impairment in IL10-/- mice. However, the cause of barrier dysfunction in the colon after the cessation of V/M treatment remains to be found.

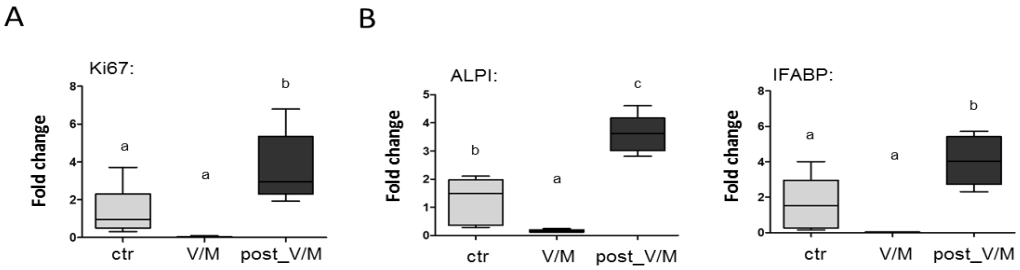

Figure 27: V/M treatment transiently reduces the mRNA level of cellular proliferation and differentiation in proximal colon of IL10-/- mice.
(A) The mRNA level of Ki67, which is a proliferation marker, was downregulated in pC of V/M IL10-/- mice and was upregulated in pC of post_V/M IL10-/- mice. (B) The mRNA levels of ALPI and IFABP, both markers of cell differentiation, showed the same tendency as in Figure 22 A. Experiment of target genes were normalized to GAPDH (n = 6/group). One-way ANOVA was followed by Tukey's post-test. Different superscripts (a, b and c) showed statistical significances between groups ($P < 0.05$). All values are mean ± SD.

Since the barrier impairment in the colon of post_V/M mice was not associated with the tight junctional proteins, we investigated other markers associated with the impaired barrier in the large intestine. Except for defects in tight junction proteins, epithelial proliferation and differentiation are important factors for epithelial integrity and homeostasis of the intestinal barrier. [199] We measured the mRNA levels of ALPI and IFABP, a cellular differentiation marker, as well as Ki67, a cellular proliferation marker. The mRNA expression of Ki67 was reduced in the proximal colon of V/M mice and was significantly increased in post_V/M mice (Figure 27A). The qPCR profiling of cellular differentiation genes revealed no difference in ALPI and IFABP (Figure 27B), suggesting that the observed gene expression levels of cellular proliferation and differentiation were not associated with impairment of barrier functions in post_V/M.

It has been long known that proinflammatory response leads to an increase in intestinal permeability. [230] Thus, we investigated the gene expression of proinflammatory cytokines such as TNFα, IL1β and IL6, and the mRNA levels of proinflammatory chemokine IP10, as well as T cell markers such as CD3 and Foxp3. Downregulation of proinflammatory cytokines expression was observed in the proximal colon of V/M mice, whereas post_V/M mice showed a significant increase in the mRNA levels of TNFα, IL1β and IL6 (Figure 28A). The gene expression of IP10 at the transcript level presented also the same tendency as proinflammatory cytokines (Figure 28B). The mRNA level of CD3 was downregulated during V/M treatment and was significantly increased at the cessation of V/M treatment (Figure 28C). Interestingly, Foxp3 expression was also elevated in post_V/M mice. These results suggested that the proinflammatory response was associated with barrier impairment at the cessation of V/M treatment in L10-/- mice.

Altogether, the increased protease activity causes the loss of tight junction proteins during V/M treatment. The proinflammatory response at the cessation of V/M leads to consistent impairment of barrier in the colon of IL10-/- mice. Additionally, it is predicted that the triggered proinflammatory response and consistent barrier impairment may accelerate colitis in V/M-treated IL10-/- mice.

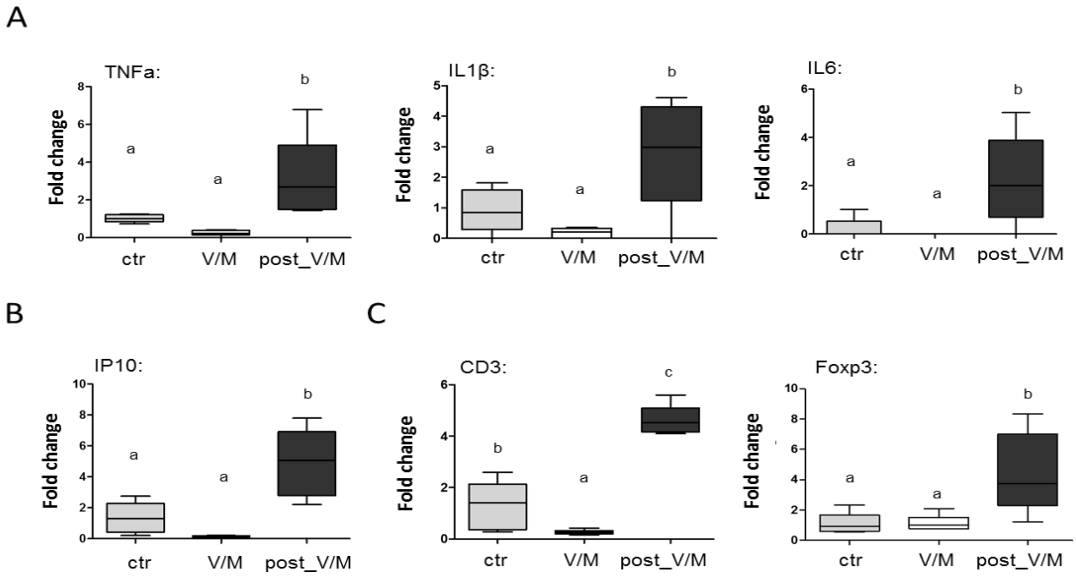

Figure 28: Proinflammatory response causes impairment of barrier function in V/M treated IL10-/- mice at the cessation of the V/M treatment.
(A) The mRNA level of proinflammatory cytokines such as TNFα, IL1β and IL6 were upregulated in post_V/M IL10-/- mice. (B) The mRNA level of IP10, a proinflammatory chemokine, was similar to figure 23A. (C) The mRNA level of CD3 was reduced in V/M mice and was increased in post_V/M IL10-/- mice (left panel). The mRNA level of Foxp3 was increased in only post_V/M IL10-/- mice (right panel). All target genes were normalized to GAPDH ((n = 6/group). One-way ANOVA; Tukey's post-test. a, b and c showed statistical significances between groups ($P < 0.05$).

4.4.3 Increased protease activity is a prerequisite for barrier dysfunction in IL10-/- mice

We orally administered the serine protease inhibitor AEBSF to IL10-/- mice in order to confirm the hypothesis that V/M treatment increases protease activity and impairs barrier functions. IL10-/- mice received V/M one day after either AEBSF (V/M_AEBSF) or water treatment (V/M_wat) as illustrated in Figure 29A. We measured the tissue permeability of the cecum and colon in the *Ussing* chamber setup and the systemic translocation of FITC-dextran.

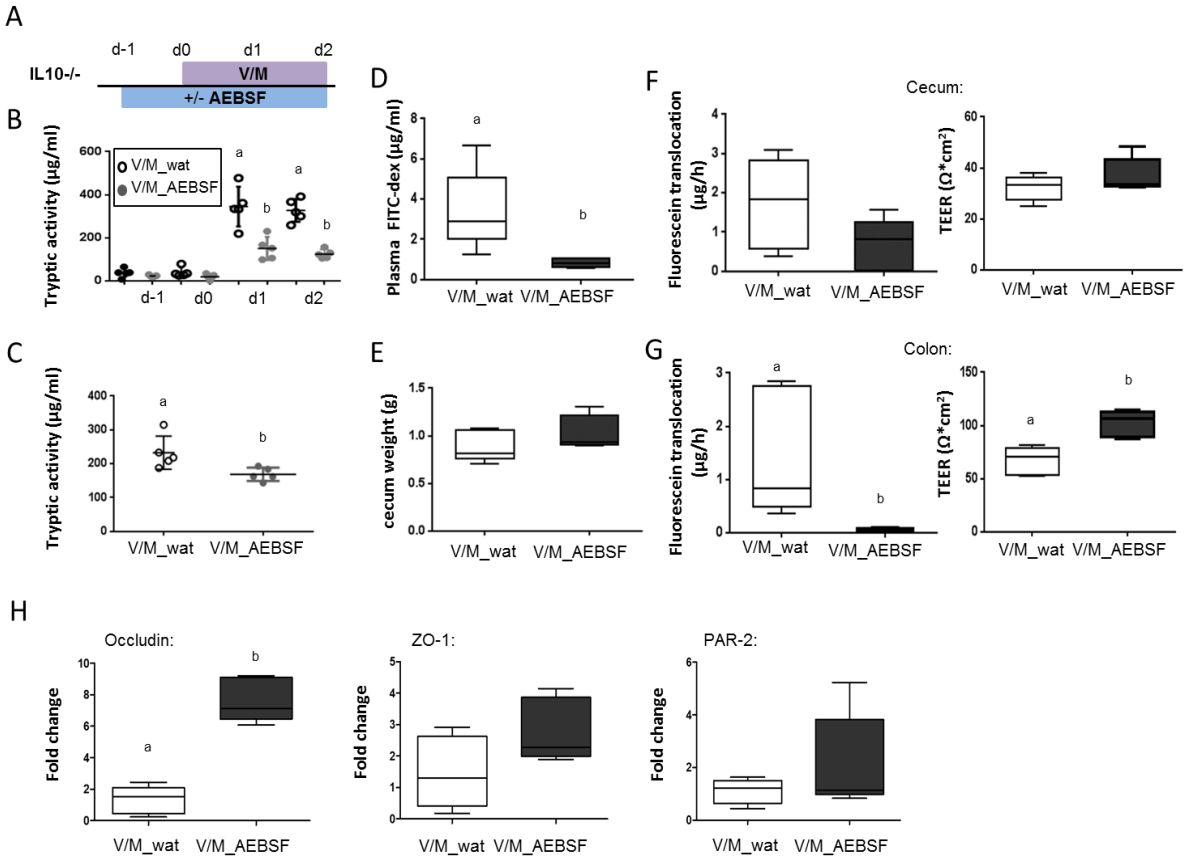

Figure 29: Enhanced PA in response to V/M causes impairment of barrier function through dysregulated occludin in IL10-/- mice.

(A) Experimental design and analysis in IL10-/- mice was the same as in figure 14A. IL10-/- mice were orally administrated either water or AEBSF for 3days from the day before the V/M treatment (water: V/M_wat, AEBSF: V/M_AEBSF). (B & C) V/M_AEBSF IL10-/- mice showed partial decrease in fecal and cecal PA compared to V/M_wat. (D) Plasma FITC-dextran was reduced in V/M_AEBSF IL10-/- mice. (E) Cecum size was not different between both groups. (F & G) Colonic barrier functions were restored in V/M_AEBSF IL10-/- mice (F: cecal barrier, G: colonic barrier). (H) The V/M_AEBSF IL10-/- mice showed an increase in mRNA level of the occludin and no alteration in the mRNA levels of ZO-1 and the PAR-2 in pC. Experiment of target genes were normalized to GAPDH (n = 5/group), and followed by the Unpaired t-test. Different character (a and b) were significant difference ($p<0.05$).

Analysis of fecal protease activity revealed that V/M treatment rapidly increased protease activity at day 1 (Figure 29B). Co-treatment of IL10-/- mice with AEBSF partially decreased protease activity compared to V/M_wat mice. AEBSF co-treatment partially reduced protease activity in the cecum (Figure 29C). The systemic translocation of FITC-dextran was significantly decreased in V/M_AEBSF mice compared to the level in V/M_wat mice (Figure 29D). Cecal weight was not changed in V/M_AEBSF mice (Figure 29E). AEBSF co-treatment did not affect tissue permeability in the cecum (Figure 29F). Importantly, however, AEBSF co-treatment dramatically diminished the colonic permeability. TEER of the colon was ameliorated in V/M_AEBSF (Figure 29G). To investigate whether the gene expression of tight junctions and PAR-2 were affected by reduction in protease activity, the mRNA level of occludin, ZO-1 and PAR-2 was measured in AEBSF co-treated IL10-/- mice. While co-treatment of IL10-/- mice with AEBSF showed a high-level expression of occludin, the mRNA level of ZO-1 was not significantly elevated in V/M_AEBSF mice (Figure 29H). PAR-2 expression was not different between the groups, however high inter-individual variability was observed in V/M_AEBSF mice. These results suggested that the enhanced protease activity plays a major role in barrier impairment in V/M-treated IL10-/- mice.

4.4.4 Enhanced protease activity aggravates colitis in IL10-/- mice

IL10-/- mice repetitively received V/M with and without AEBSF for 7 days at the age of 4 and 8 weeks and were sacrificed at the age of 16 weeks in order to evaluate the hypothesis that the accelerated colitis in V/M-treated IL10-/- mice is caused by the enhanced protease activity (Figure 30A). Feces were collected to analyze protease activity, microbial composition and complementary component 3 (C3). Similar to the observations in repetitive V/M-treated WT mice, repetitive V/M treatment showed a long-lasting increase in fecal protease activity from the age of 5 weeks until the age of 9 weeks (Figure 30B). AEBSF co-treatment partially decreased fecal protease activity. Alteration of microbial composition was observed in repetitive V/M-treated IL10-/- mice (Figure 30C). This alteration did not recover until the age of 16 weeks. The bacterial families *Lactobacillaceae* and *Enterobacteriaceae* were dominant during the first and second V/M treatments (>99% relative abundance). Similar to the separation in the structure of the bacterial community in V/M-treated WT mice (Figure 22A), beta-diversity revealed a strong separation between V/M treated and untreated IL10-/- mice during repetitive V/M treatment (Figure 30D). The alteration of the microbial structure in repetitive V/M treated mice did not return to the normal structure until they reached 16 weeks of age. During repetitive V/M treatment, microbial richness significantly diminished in V/M treated IL10-/- mice (Figure 30F). However, the differences of microbial richness were not detected at the age of 16 weeks between V/M treated and untreated IL10-/- mice. Repetitive V/M treatment led to a reduction of

Bacteroidaceae, *Bacteroidales* S24-7 and *Prevotellaceae* (Figure 25F). Therefore, repetitive V/M treatment resulted in a long-lasting rise in protease activity and compositional disturbances in the intestinal microbiota. Although the increased protease activity in response to the V/M treatment was normalized at 4 weeks after second V/M treatment, the altered bacterial composition did not fully recover.

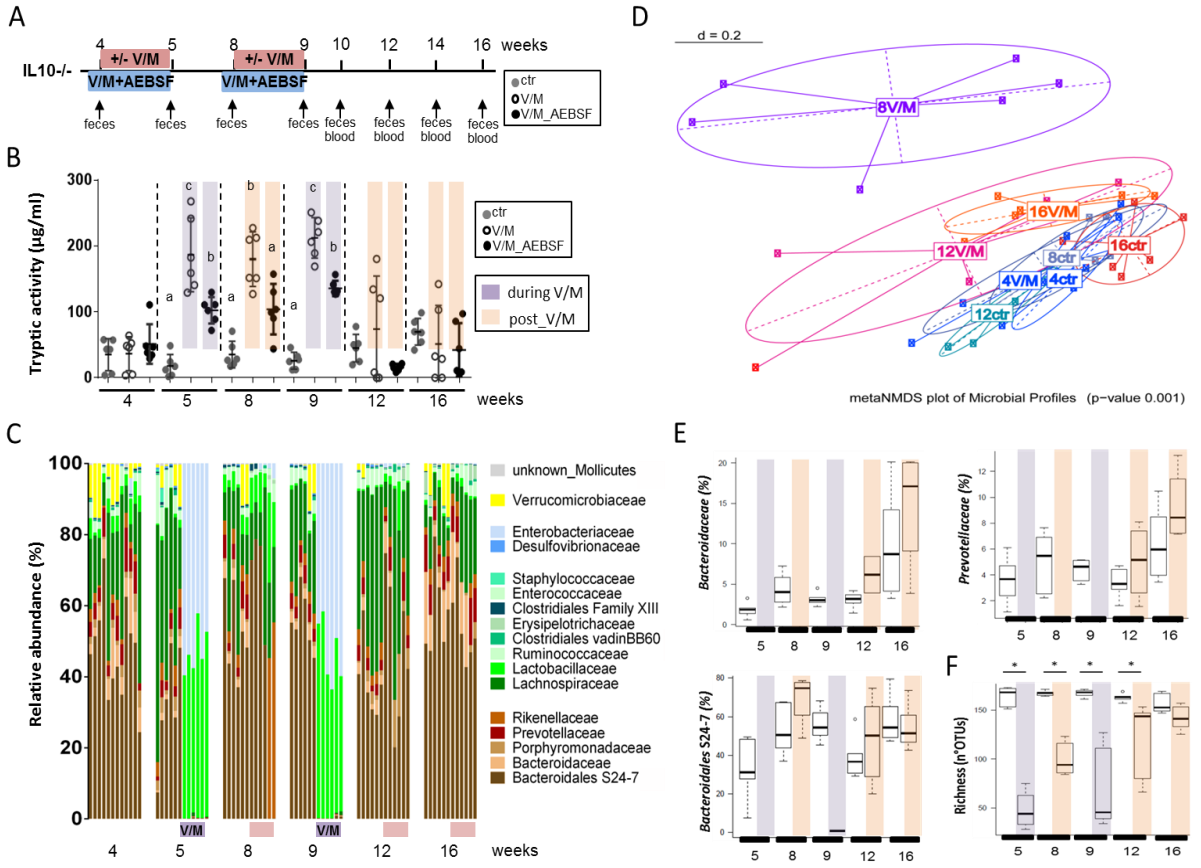

Figure 30: Repetitive V/M treatment persistently increases PA and changes the intestinal microbial ecosystem in IL10-/- mice.
(A) IL10-/- mice received repetitive V/M treatment for 1 week at the age of 4 and 8 weeks (V/M). IL10-/- mice received serine protease inhibitor for 1 week from the day before repetitive V/M treatment (V/M_AEBSF). (B) Fecal PA was lastingly increased in V/M IL10-/- mice and was partially decreased in V/M_AEBSF IL10-/- mice. (C & D) Microbial composition and beta diversity were totally shifted during repetitive V/M treatment in IL10-/- mice. (E & F) Microbial richness and taxa were altered during repetitive V/M treatment in IL10-/- mice (n = 6/group). Data are given as mean ± SD; unpaired t-test. *p<0.05.

The increase in protease activity was shown to be associated with triggering a proinflammatory response in IL10-/- mice upon short-term V/M treatment. To investigate whether enhanced protease activity exacerbates colitis development in repetitive V/M-treated IL10-/- mice, a staining of CD 3 (T-

cells) was performed in the cecum tip and the colon of V/M treated (V/M) and of AEBSF co-treated (V/M_AEBSF) IL10-/- mice. A number of T cell infiltrations appeared in the cecum tip and the colon of V/M IL10-/- mice compared to untreated mice (Figure 31A). In contrast, V/M_AEBSF IL10-/- mice showed a partially diminished number of T cell infiltrations in the cecum tip and the colon. The increase in protease activity led to an infiltration of T cells in the large intestine of repetitive V/M-treated IL10-/- mice.

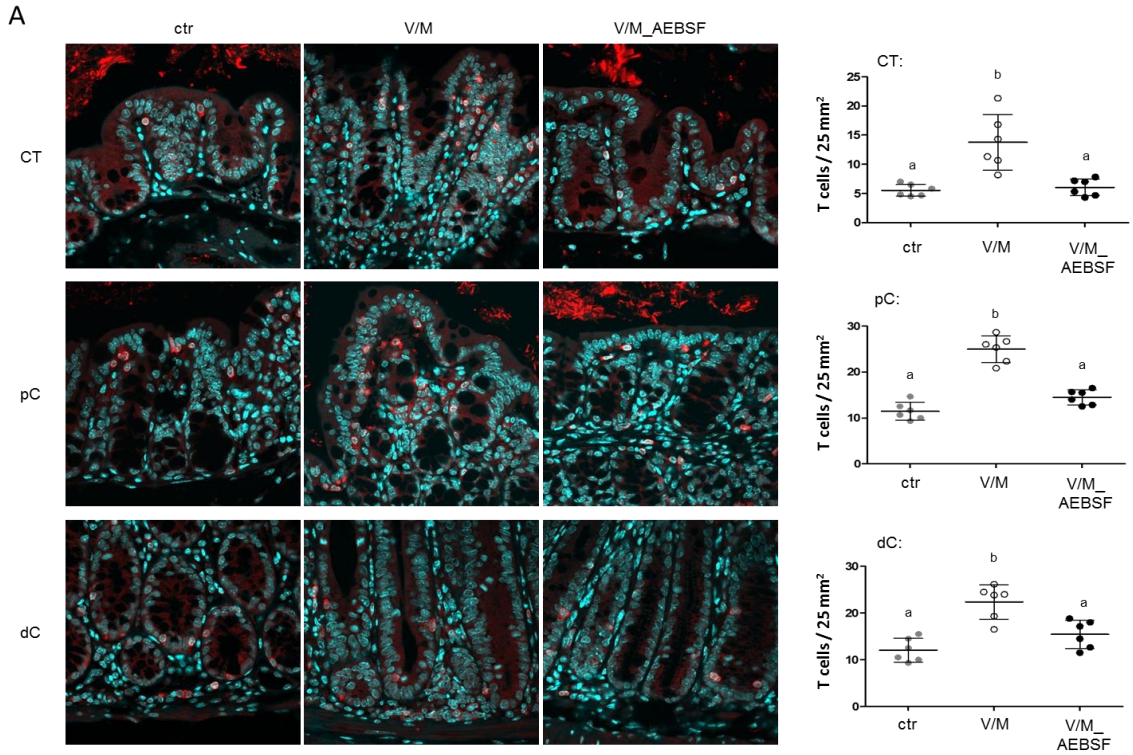

Figure 31: The number of infiltrated T cells is increased in the large intestine of V/M-treated IL10-/- mice.
(A) Number of T cells in lamina propria was strongly increased in the large intestine of V/M IL10-/- mice and was decreased in V/M_AEBSF IL10-/-mice (right panel: immunofluorescence staining of CD3, left panel: counting of infiltrated T cell, Red: CD3, Blue: DAPI). Data are given as mean ± SD; One-way ANOVA, Tukey's post-test.

The above results hint at a triggering proinflammatory response in IL10-/- mice being caused by the increased protease activity. We observed changes in tissue morphology, different inflammation markers and histopathological scores in V/M and V/M_AEBSF IL10-/- mice. MLN weight was significantly elevated in V/M IL10-/- mice, whereas co-treatment of IL10-/- mice with AEBSF partially reduced MLN weight (Figure 32A). Spleen weight showed the same tendency with MLN weight (Figure 32B). Colon length was not changed in V/M and V/M IL10-/- mice (Figure 32C). While a rise in crypt depth was observed in V/W IL10-/- mice, AEBSF co-treatment significantly reduced crypt depth (Figure

32D). SAA level in the plasma was significantly increased in V/M IL10-/- mice from the age of 14 weeks onwards (Figure 32E). V/M_AEBSF IL10-/- mice showed a partial decrease in SAA. The C3 level, a local inflammation marker, showed the same tendency as the SAA level (Figure 28F). The mRNA levels of C3 and proinflammatory cytokines such as IL1β and IFNγ were dramatically increased in V/M IL10-/- mice, whereas co-treatment of IL10-/-mice with AEBSF significantly decreased these mRNA expressions (Figure 32G). Importantly, the histopathology score revealed that repetitive V/M treatment elevated colonic inflammation, while AEBSF co-treatment significantly alleviated colitis development (Figure 32H). Surprisingly, V/M treatment induced early tumor development (Figure 32I). However, V/M_AEBSF IL10-/- mice showed alleviated tumor development at the age of 16 weeks.

These observations demonstrated that the increase in protease activity in response to repetitive V/M treatment accelerated colitis in IL10-/- mice. Additionally, co-treatment with protease inhibitor ameliorated the detrimental effects of repetitive treatment of V/M in IL10-/- mice.

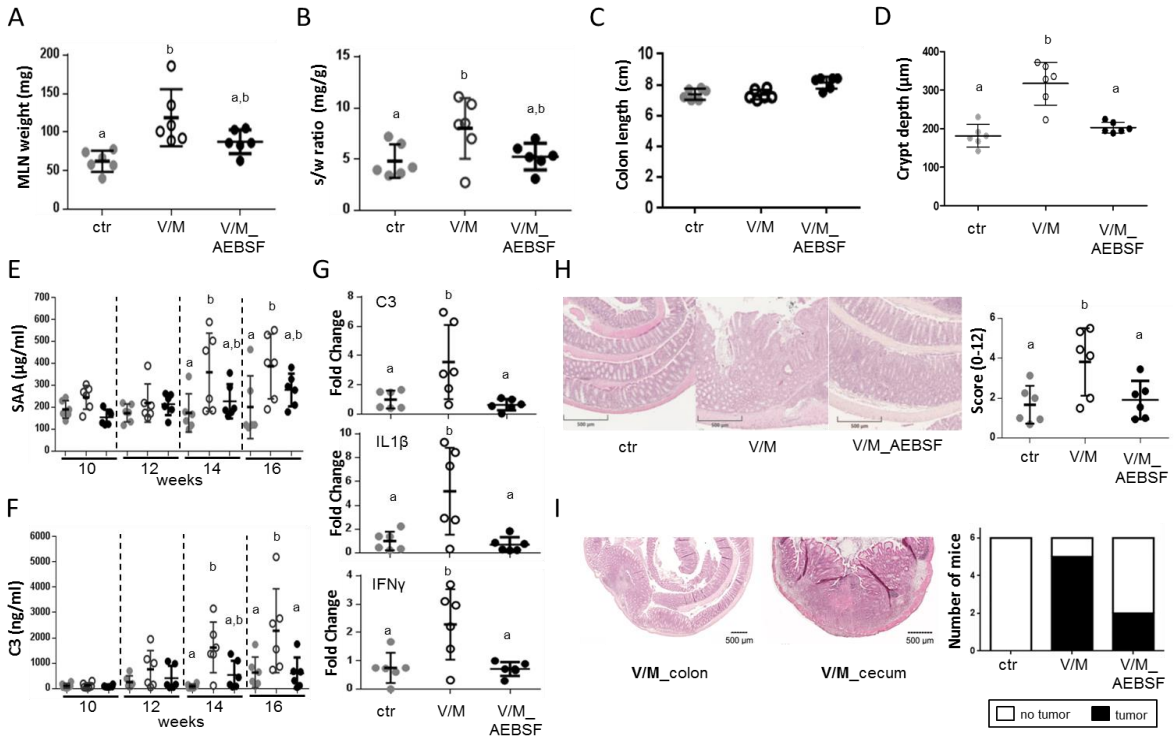

Figure 32: Repetitive V/M treatments result in acceleration of colitis and tumor development in IL10-/- mice. (A to D) Tissue morphology such as MLN weight (A), spleen weight (B) and crypt depth in pC (D) was altered in repetitive V/M treated IL10-/- mice. Colonic length (C) was not different among the three groups. (E & F) Kinetic SAA in plasma and C3 in feces were increased from age of 14 weeks in V/M IL10-/- mice and were partially decreased in V/M_AEBSF IL10-/- mice. (G) mRNA levels of C3, IL1β and IFNγ were increased in V/M IL10-/- mice and were normalized to the level of untreated (ctr) IL10-/- mice in V/M_AEBSF IL10-/- mice. (H & I) Histopathological score and the tumor development were significantly increased in the large intestine of V/M IL10-/- mice and were decreased in V/M_AEBSF IL10-/- mice.

5 Discussion

5.1 Microbial inactivation of protease activity

In the present work, we investigated the influence of antibiotics on the protease activity in the large intestine. We found that the inactivation of digestive enzymes was abrogated by V/M treatment. One of the adverse effects of antibiotics, namely increased protease activity, is thought to be due to eradication of intestinal microbes, highly associated with the efficient degradation of pancreatic proteases in the large intestine. The intestinal microbiota is necessary for the ability to inactivate the high amount of pancreatic proteases in the large intestine. Consistently, the anti-proteolytic property of intestinal bacteria was confirmed by colonizing GF mice with cecal microbiota from SPF mice. Several studies provided confirming results demonstrating that the enteric microbiota is essential for the inactivation of pancreatic proteases in the large intestine. [134, 135, 137] The incompetence of the cecal microbiota from V/M-treated mice to degrade pancreatic proteases upon transfer into GF mice supports the fact that V/M treatment eradicates anti-proteolytic bacteria. Mice treated with only vancomycin still possessed the abililty to inactivate digestive enzymes, indicating that anti-proteolytic microorganisms are resistant to vancomycin. Since it is well known that vancomycin treatment particularly reduces the abundance of *Firmicutes,* [231] *Firmicutes* might not be associated with anti-proteolytic property. In contrast, the increased protease activity in metronidazole-treated mice points toward eradication of anti-proteolytic bacteria. In the present study, analogous to the publication results by others, [144, 232] Bacteroidales were associated with the inactivation of protease activity. One study revealed that *C. perfringens* and *Bacteroides,* which might be connected to regulating PA, are inhibited by metronidazole. [233] In the patients, fluoroquinolones (e.g. levofloxacin) inhibiting bacterial DNA replication were used primarily for treating complicated infections in the respiratory tract, urine and GI tract against pathogens. [234–237]

Since the increased protease activity was observed in treatment of fluoroquinolone/imidazole, further analysis of microbial alterations correlated to protease activity in patients receiving fluoroquinolone/imidazole is necessary. One study reported that not all enteric bacteria in the large intestine are able to regulate protease activity. [139] Gnotobiotic mice monoassociated with a specific *Bacteroides distasonis* strain show normal low protease activity whereas the ones monoassociated with another *Bacteroides distasonis* strain did not, [144] clearly demonstrating that the anti-proteolytic property is bacterial strain-specific effects. In V/M-treated mice, *Bacteroidaceae*, *Ruminococcaceae* or *Prevotellaceae w*ere strongly inhibited, even though protease activity was strongly increased again 3 weeks after the second V/M treatment, indicating that these taxa were not associated with the anti-proteolytic property.

It is a well-known fact that bacterial inactivation of pancreatic proteases contributes to the maintenance of appropriate protease activity in the large intestine, however the identification of anti-proteolytic bacteria and the respective inactivation mechanisms are insufficient. For this reason, the anti-proteolytic property of consortia was investigated. *In vitro,* several bacterial strains of the genera *Bacteroides, Parabacteroides* or the order *Clostridiales* were found to reduce the tryptic activity. However, we were not able to reproduce the anti-proteolytic effect of these candidate strains *in vivo*. Normalization of the increased protease activity in the large intestine seems to be a rare bacterial function as none of the investigated simplified microbial consortia (e.g. Corio, [238] oligoM, [239] altered Schaedler flora [145] or miBAC, [240]) were able to normalize the level of pancreatic protease activity. Two potential mechanisms of how the intestinal bacteria inactivate digestive proteases have been proposed but have yet to be proven: first, bacteria utilize digestive proteases as a nitrogen source or substrates for energy metabolism; [129] second, bacteria secrete protease inhibitors in order to degrade pancreatic proteases. [241] Due to the rare nature and strain-specificity of the anti-proteolytic activity, it might be impossible to identify these bacteria via correlation analyses between microbiome data and protease activity. Additionally, establishing new consortia capable of inactivating the pancreatic proteases, as the supplementary agents (e.g. genetically modified bacteria, antibiotic-insensitive anti-proteolytic bacteria and serine protease inhibitors) may be important to reduce protease activity in patients treated with antibiotics.

5.2 Clinical relevance of the antibiotic-specific increase in protease activity

We found that approximately 25% of patients treated with different antibiotics showed a major increase in stool protease activity. It is known that antibiotic treatments result in a disturbance in the inactivation of digestive proteases. [141, 142] Furthermore, the oral application of antibiotics in patients substantially increased fecal trypsin levels approximately 100-fold. [143] **This clearly demonstrates that the abrogation of inactivating digestive enzymes in response to antibiotic treatments is a major cause of the excessive protease activity in the large intestine.** Here, another purpose of the present study was to address the question of which antibiotics are associated with elevation of protease activity in the large intestine. The causative link between fluoroquinolone/metronidazole treatment and an increase in protease activity has not yet been properly addressed. Since fluoroquinolone and imidazole are commonly prescribed in severe clinical settings, research on a causal relationship between these antibiotics and increased protease activity is highly essential. In our patient cohort, fluoroquinolone/metronidazole treatment showed a strong rise in stool protease activity. Furthermore, in our mouse experiment, increased protease activity was observed only in the metronidazole treatment but not in the vancomycin treatment. Similar to our

findings, a mixture of 10 different antibiotics including metronidazole showed an increase in protease activity. [242, 243] However, certain antibiotics such as ampicillin and neomycin did not show an increase in protease activity, [191] illustrating that specific antibiotics e.g. fluoroquinolone/metronidazole and macrolide, but not all antibiotics, are associated with a high risk for an increase in protease activity.

In our clinical study, inter-individual variability in protease activity was rarely observed in patients receiving the same antibiotics. Apart from antibiotics, many other factors are capable of regulating protease activity. In the large intestine, protease activity can be adjusted by the level of secreted pancreatic proteases depending on the amount of dietary protein intake. [244] Gerber *et al*. showed that a lower than average level of pH in the large intestine reduced tryptic activity. [245] Therefore, the difference in the pH level in the intestine might be associated with the variance in individual protease activity. Also, the amounts of pre-enzyme and secreted protease inhibitors are connected to the inactivation of the digestive enzymes. [146] Therefore, inter-individual differences in protease activity might be caused by different factors. Interestingly, antibiotic-treated patients presenting symptoms of diarrhea showed a significant increase in protease activity. There is limited evidence for the causative relationship and mechanism between diarrhea and increased fecal protease activity. Tooth *et al.* reported that rapid transit of gut contents in the intestine reduced the inactivation time of digestive proteases, and consequently increased protease activity in feces. [246] This tendency was clearly demonstrated in patients with irritable bowel syndrome having diarrhea. [201] Accordingly, this indicates that diarrhea may represent a physiological scenario, in which the inactivation of digestive proteases by the intestinal microbiota is significantly decreased. All these points and the high inter-individual variability of the intestinal microbiota are important factors indicating why the baseline protease activity level may vary between patients, which may in turn affect the response towards a given antibiotics. Although the influence of antibiotics on the fecal protease activity varied considerably between individuals, detrimental effects of specific antibiotics on the protease activity were observed in patients receiving antibiotics. Thus, the analyses of a largescale cohort of patients treated with fluoroquinolone/imidazole with respect to the change in protease activity and intraindividual effect of antibiotics treatment are required.

Importantly, a major increase in fecal protease activity upon antibiotic treatment seems to be clinically relevant to the onset of diseases. One study by Gecse *et al*. reported that an increase in protease activity is correlated with pathogenesis of IBS and IBD. [201] Maeda *et al*. revealed that an increase in fecal serine protease activity elevates severity of IBD in dogs. [185] Similar to the correlation in dogs, IBD patients (both in CD and UC) showed enhanced fecal protease activity. [191, 247] Therefore, it can

be assumed that the increase in protease activity might be associated with the triggering of chronic inflammation in susceptible individuals.

5.3 Enhanced protease activity impairs the intestinal epithelial barrier

The impact of increased protease activity on barrier functions was assessed by the stimulation of PTK6 cells with high PA cecal supernatant *in vitro*. PTK6 cells are a conditionally immortalized cell line isolated from the mouse colonic mucosa, and are an excellent cell culture model system due to polarization, formation of tight junctions and development of an electrical resistance. [227] *In vitro* measurements of TEER, using the transwell permeable system, is a useful method to study integrity of the intestinal epithelial barrier measured by electrical permeability of epithelial monolayers. [248] In our experiments, increased permeability induced by the stimulation with cecal supernatant of GF mice *in vitro* illustrated the detrimental effects of excessive protease activity on the epithelial barrier. Analogous to these results, stimulation of PTK6 cells with high PA stool supernatant from patients after the treatment with antibiotics showed the same adverse effect on the barrier function. This adverse effect has already been demonstrated in that the excessive protease activity of stool from IBS patients has a detrimental effect on the colonic barrier *ex vivo*. [201] Pre-stimulation of PTK6 cells with the serine protease inhibitor negated the detrimental effects, demonstrating that increased protease activity is causal for the impairment of barrier functions. In the present work, additional *Ussing* chamber experiments using the large intestine showed that a high level of pancreatic protease activity is detrimental to the large intestinal barrier functions. This is in line with literature showing that the high PA fecal supernatants from UC patients induce the defective barrier in the colon *ex vivo*. [249] This clearly demonstrates the causal relationship between increased protease activity and barrier impairment.

Consistent with the present work *in vitro* and *ex vivo*, the impairment of barrier functions was observed in V/M-treated mice. Additionally, the observed increase in large intestinal permeability in V/M-treated mice was due to the excessive level of active digestive enzymes and decrease in protease inhibitor. Annaházi *et al.* showed that intracolonic infused cysteine proteases from constipated IBS patients increased colonic permeability in mice. [250] In other study, change in the colonic paracellular permeability caused by the bacterial protease*, E. faecalis* Gelatinase, displays signs of proteases regulating the barrier function. [226] Administration of pancreatic proteases into the intestinal wall in ischemic rats leads to disruption of mucin integrity and epithelial cells. [251] This literature supports our findings that increased protease activity in response to antibiotics causes the barrier dysfunction.

Prevention of detrimental effects by the oral co-administered serine protease inhibitor underlines that the rapid increase in protease activity causes the observed impairment of the intestinal barrier.

5.4 Mechanism of the increased protease activity-mediated barrier impairment

Increase protease activity in response to antibiotics induces impairment of the intestinal barrier. To study mechanisms underlying the defective barrier upon V/M treatment, PAR-2 activation and regulation of tight junctions were investigated in V/M treated WT and IL10-/- mice. The high protease activity in response to V/M treatment rapidly impaired barrier function in the cecum of WT and the colon of IL10-/- mice. While the V/M-treated WT mice showed transient increase in cecal permeability through downregulated ZO-1, increased protease activity impaired consistently the colonic barrier of IL10-/- mice through loss of ZO-1 and occludin and proinflammatory responses. Increased paracellular permeability is a consequence of loss of tight junctions. [252] ZO-1 is able to reorganize the cytoskeleton which plays an important role in the regulation of transcellular permeability. [253] Another study provided evidence that decreased levels of occludin result in a defective barrier. [254] Cenac *et al.* demonstrated that the increased paracellular permeability is linked to the phosphorylation of mucosal MLC and dysregulated tight junctions, dependent on serine protease signaling. [180] The dysregulated junctional proteins reduce strength of cellular contraction, resulting in luminal molecules being able to infiltrate into the epithelium. This might be explained by the fact that MLCK-dependent reorganization of tight junctions increases paracellular permeability. [190, 255] These factors point towards that V/M treatment rapidly impairs the barrier function of the intestine in WT and IL10-/- mice.

Interestingly, the cessation of V/M treatment induced a different integrity of the intestinal barrier between WT and IL10-/- mice. WT mice recovered barrier functions after the V/M treatment, whereas IL10-/- mice showed a consistent impairment of the colonic barrier. Since the cecal barrier rapidly recovered in WT mice despite constantly increased protease activity, it can be assumed that the epithelial barrier adapted to the enhanced protease activity. Relocation of occludin in WT mice after discontinuation of V/M treatment might be an effective feedback mechanism to restore the integrity of the intestinal barrier. An equivalent mechanism has been reported in one study stating that the MAPK signaling-dependent tight junctions, e.g. ZO-1 and occludin, were restored by bacterial metabolites. [254] Furthermore, microbial peptides have been shown to redistribute ZO-1. [256] Although the stimuli for redistribution of occludin were not investigated in our study, an unknown factor might be associated with a feedback mechanism of the maintenance of barrier homeostasis induced by the redistribution of junctional proteins.

The activation of PAR-2 showed a similar tendency with change in tight junctional proteins in V/M-treated mice. It is known that PAR-2 activation leads to dysregulation of tight junctions and increase in paracellular permeability. [184, 188] According to the literature, PAR2 agonists increase the permeability and subsequently induce redistribution of tight junctions as well as perijunctional F-actin, [190] suggesting that the loss and subsequent redistribution of tight junctions can be assumed to depend on PAR-2 activation. PAR-2, which is localized at the apical and basolateral membrane of intestinal epithelial cells, can be activated by serine proteases. [257] Rapidly increased protease activity at Day 2 of V/M treatment already activated PAR-2. Kong *et al.* showed that the luminal serine protease such as trypsin activates PAR-2. [258] Activated PAR-2 is endocytosed through early endosome and lysosomal degradation, and PAR-2 expression was inhibited during the endocytosis. [191, 194] This data supports our findings that the internalization of PAR-2 and decrease in PAR-2 mRNA expression in the colon of IL10-/- mice are associated with the activation of PAR-2 in response to the rapidly increased protease activity during V/M treatment. Interestingly, relocation of PAR-2 in the apical membrane in spite of the consistently high protease activity was unexpectedly observed in V/M-treated IL10-/- mice. One study demonstrates that receptor adaptation depends on calcium concentration in the cytoplasm. [259] Serine protease strongly increases calcium concentration and activates PAR. [260] This evidence hints at the calcium mobilization and high protease activity being associated with PAR-2 desensitization.

In IL10-/- mice, the barrier was found to be impaired even after the cessation of V/M treatment and despite tight junctions normalized. Regarding the cause of consistent barrier impairment in IL10-/- mice, proinflammatory mediators such as TNFα and IFNγ are also capable of influencing the integrity of intestinal barrier. [261] In the present work, proinflammatory cytokines such as IL6, IL1ß and TNFα and chemokine IP10 were found to be elevated in the colon of IL10-/- mice after V/M treatment. Inflammatory activation of the intestinal tissue and especially high expression of TNF and IFNγ are known to be detrimental to the intestinal barrier. [262–267] Several studies show that proinflammatory cytokines such as IFN-γ and TNF-α disturb intestinal homeostasis through a substantial reduction of IEC proliferation [268, 269] and barrier tightness. [266, 267] This data suggests that the early proinflammatory response is the driver for the consistent barrier impairment observed in IL10-/- mice. Since the gene expression levels of cellular proliferation and differentiation were increased after the cessation of V/M treatment, these were not associated with defective barrier in IL10-/- mice.

In summary, V/M treatment transiently impaired the cecal barrier of WT mice through PAR-2 activation and dysregulated ZO-1. In contrast, V/M treatment consistently increased colonic permeability of IL10-/- mice. However, activated PAR-2 and dysregulation of tight junctional proteins such as ZO-1 and

occludin temporally occurred during V/M treatment and normalized after V/M treatment. Co-treatment with the serine protease inhibitor AEBSF demonstrated that rapidly enhanced protease activity in response to V/M treatment causes constant impairment of barrier function in the large intestine of IL10-/- mice. The increased permeability could induce the penetration of microbial antigens, resulting in the early proinflammatory activation in the colon of IL10-/- mice.

5.5 Potential consequence of antibiotic treatment in colitis development

The impaired barrier and the subsequent proinflammatory response are thought to be the main causal factors for the acceleration of colitis development via the increased protease activity in V/M-treated IL10-/- mice. At the cessation of V/M treatment in IL10-/- mice, the consistently impaired barrier functions in the large intestine might induce the penetration of luminal antigens, resulting in triggering a proinflammatory response. The proinflammatory response at the cessation of V/M treatment, as well as the accumulation of CD3 T cells in repetitive V/M treatment, might be associated with the aggravation chronic inflammation in the large intestine. It has been a well-known fact that the mucosal immune response is triggered by the infiltrated luminal antigen in the genetically susceptible host. [270] The CD4+ T cells play a pivotal role in the initiation and persistence of chronic inflammation in the intestine. [271] The number of infiltrated immune cells is increased in the intestinal mucosa under inflammatory conditions such as IBD. [272] These are in line with the literature showing that the CD4+ T cells are activated in the appendix of humans and diffuse the inflammation to the entire colon and rectum prior to the onset of UC. [273] In repetitive V/M treated IL10-/- mice, co-treatment with the serine protease inhibitor AEBSF partially decreased the proinflammatory cytokines and chemokine as well as the infiltrated CD3 T cells, suggesting that the proteolytic enzymes aggravate colitis development in the genetically susceptible host. This might be explained by the fact that the PAR-2 activation significantly increases the mature DCs, and subsequently activates T cells. [274] The activated PAR-2 could lead to the intestinal inflammation [275], IBS [276] and cancer development. [277] As shown in the literature, PAR-2 activation triggered subsequently a proinflammatory response in the colon of IL10-/- mice after V/M treatment.

A defective barrier is not only predisposing for IBD but a pivotal characteristics of the chronic inflammation in UC and CD, [278] and it is thought to be of high relevance for the vicious circle of increased contact of the intestinal immune system with luminal antigens. Similar to the barrier dysfunction in CD patients, dysregulated tight junctions are frequently observed in the UC patients. [213, 279] This literature supports our finding that aggravated colitis is a consequence of the impaired barrier in V/M treated IL10-/- mice. Interestingly, the increase in protease activity and PAR-2 activation

are correlated with the development of colitis, [280, 281] indicating that the long-lasting increase in protease activity in response to V/M treatment is a risk factor for the acceleration of colitis. Furthermore, PAR-2 activation is observed in patients with IBD or colon cancer. [282, 283] This data is similar to our findings that the proinflammatory response and tumor development were increased in repetitive V/M-treated IL10-/- mice.

Importantly, V/M treatment showed transient impairment of barrier functions and did not induce the proinflammatory activation in WT mice. Repetitive V/M treatment did not show increased susceptibility towards DSS-induced colitis in WT mice despite the alteration in the intestinal microbiota and the increase in protease activity. One study demonstrates a reduced frequency of T-cells expressing IFNγ and IL17 in WT mice after antibiotic treatment. [284] This evidence explains our findings that the repetitive V/M treatment did not trigger a proinflammatory response and did not accelerate DSS-induced colitis in WT mice. The present work supports the assumption that the immune system in healthy organisms can adapt well to the antibiotics-mediated increase in protease activity and changes in the intestinal microbiota.

5.6 Clinical implications for antibiotic therapy in IBD patients

The experimental and clinical evidence in our study suggest that specific antibiotic treatments may be a risk factor for later pathogenesis of IBD in susceptible individuals. Several studies have already revealed that antibiotic treatments are correlated to the onset of IBD in patients and mice. [103, 285] A recent cohort study demonstrates that the antibiotic treated patients (66.3% of 718 patients) show more severe symptoms of UC and CD (an increase of approximately 10%). [92] Transferring CD4+ T cells from antibiotic-treated mice at an early age induces severe inflammation in Rag1-/- mice, [286] indicating that the antibiotic treatment in early-life accelerates the onset of IBD. Shaw *et al.* showed that the diagnosis of CD has increased by 20% in the patient treated with more than one antibiotic in the first year of life. [10] A population-based prospective cohort study revealed that the development of UC and CD is associated with the number and dose of antibiotic treatment (CD cases for ≥1~2 times of antibiotic and UC cases for more than 3 times). [105] These studies are in line with our findings that the repetitive V/M treatment accelerated colitis development in IL10-/- mice. Analogous to our findings, one study showed that neomycin/metronidazole treatment aggravated colitis in IL10-/- mice without decreasing in the mucosal adherent bacteria. [285] Additionally, Kronman *et al.* demonstrated that the pathogenesis of IBD depends on the age of patinets and the repetition time of antibiotic treatments as well as the specific type of antibiotic. [104] Regarding the impact of different antibiotic therapies, several clinical studies showed metronidazole or fluoroquinolones to be strongly associated

with the risk of the developing CD. [103, 105] This might be explained in part by our findings that fluoroquinolone treatment may confer a higher risk for an increase in protease activity compared to other antibiotics. Considering the causal relationship of antibiotic treatment in IBD patients, the observed adverse effects of the antibiotic treatment on the protease activity and colitis development suggest that the excessive proteases might be an independent variable for the triggering of intestinal inflammation in IBD susceptible individuals.

6 Conclusion and Perspective

In summary, the present study is the first to provide experimental evidence that antibiotic therapy has the potential to aggravate colitis development in genetically susceptible individuals via changes in protease activity. The increase in pancreatic proteases, which is similarly observed in patients receiving antibiotic therapy, was an observed primary side effect of antibiotic treatment in mice. We showed that the increased protease activity in response to V/M caused accelerated colitis development in susceptible IL10-/- mice, presumably due to the lasting impairment of the large intestinal barrier upon exposure to high loads of active pancreatic proteases. In contrast to the long-term detrimental effects of antibiotic therapy in genetically susceptible mice, the high protease activity induced transient barrier impairment in WT mice but did not result in increased susceptibility towards subsequently acute DSS-induced colitis. Therefore, the antibiotic treatment causes the aggravation of colitis in susceptible individuals. In the long term, the antibiotic therapy for patients might be detrimental to triggering chronic inflammation rather than being protective.

Considering the adverse impact of antibiotic therapy on the development of IBD, this study suggests that antibiotic therapy needs to be specifically implemented in a more restricted and targeted way, especially for IBD patients. Additionally, the finding that the excessive digestive proteases are detrimental to the large intestine emphasizes the importance of attempts to monitor and reduce the protease activity using serine protease inhibitors during antibiotic therapy. The study may provoke investigations about the impact of specific antibiotics on protease activity in large patient cohorts, which may lead to predicting the response of each individual to a given antibiotic therapy in the future. Furthermore, the isolation of anti-proteolytic bacterial strains is pivotal to enabling future studies on the anti-proteolytic mechanisms of these bacteria and to enabling their potential use as a protective supplement for patients.

7 Supplementary Table

Supplementary Table 1. Abundance of proteases and protease inhibitors in cecal supernatant of WT, V/M-treated and GF mice determined via LC-MS/MS analysis

The number in the columns with color code is mean intensity of proteins (log2 intensity Based Absolute Quantitation (iBAQ)) as described by the dot size in Figure 11C. The -log10 p-value is a significance level of abundant difference in proteases and protease inhibitors between respective two groups. p-value cutoff: 0.01. NaN: not detected, Color code: red = high intensity; green = low intensity.

Protein names	Gene names	Peptides for quantification	MS/MS Count	category	average intensity (log2 iBAQ)			-log10 p-value (AB vs WT mice)	-log10 p-value (GF vs WT mice)
					Ctrl mice	AB mice	GF mice		
Anionic trypsin-2	Prss2	11	6463	serine protease	23.5766	28.0432	29.9985	4.019	4.518
Chymotrypsin-like elastase family member 3B	Cela3b	14	5278	serine protease	23.5337	26.728	28.3561	1.776	2.314
Chymotrypsin-like elastase family member 2A	Cela2a	6	190	serine protease	21.7679	21.7117	20.39	0.017	0.482
Chymotrypsin B	Ctrb1	6	110	serine protease	19.5578	22.0543	20.5615	2.417	2.016
Dipeptidyl peptidase 4	Dpp4	28	536	serine protease	18.0997	19.6489	20.8236	1.393	1.097
Enteropeptidase;	Tmprss15	16	182	serine protease	13.9013	16.7161	19.5985	0.923	1.879
Serine protease 30	Prss30	3	33	serine protease	16.5288	16.8291	18.7442	0.294	2.426
Suppressor of tumorigenicity 14 protein homolog	St14	5	29	serine protease	15.0437	15.9482	15.8813	1.122	0.718
Heat shock protein HSP 90-beta	Hsp90ab1	4	16	serine protease	16.1568	12.6041	14.6636	#N/A	0.187
Lysosomal protective protein	Ctsa	6	31	serine protease	15.958	NaN	15.5355	1.031	0.064
Lysosomal Pro-X carboxypeptidase	Prcp	3	13	serine protease	16.0532	13.3089	13.4714	1.341	1.511
Haptoglobin	Hp	9	48	serine protease	15.8547	14.0443	NaN	0.981	1.133
Transmembrane protease serine 2	Tmprss2	3	10	serine protease	NaN	NaN	14.9942	#N/A	1.428
Kallikrein 1-related peptidase b22	Klk1b22	3	16	serine protease	14.6359	19.2748	NaN	#N/A	#N/A
Mast cell protease 2	Mcpt2	5	15	serine protease	17.6206	NaN	15.2794	1.394	0.732

Acylamino-acid-releasing enzyme	Apeh	1	7	serine protease	15.4951	12.0414	NaN	#N/A	#N/A
Mast cell protease 1	Mcpt1	6	22	serine protease	20.5828	NaN	NaN	1.151	0.890
Prolyl endopeptidase	Prep	6	24	serine protease	16.0909	NaN	NaN	1.453	1.480
Kallikrein 1-related peptidase b27	Klk1b27	2	5	serine protease	NaN	17.2671	NaN	#N/A	#N/A
Complement factor D	Cfd	1	8	serine protease	15.3681	NaN	NaN	#N/A	#N/A
Lactotransferrin	Ltf	7	18	serine protease	NaN	NaN	NaN	#N/A	#N/A
Proteasome subunit beta type-5	Psmb5	10	56	threonine protease	18.6519	14.1762	18.5673	2.000	0.029
Gamma-glutamyltranspeptidase 1	Ggt1	10	171	threonine protease	12.084	18.569	18.3859	2.669	0.991
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	Aga	5	50	threonine protease	14.4049	14.3463	18.5446	0.228	2.698
Proteasome subunit alpha type-1	Psma1	11	57	threonine protease	17.988	11.9031	17.5781	2.697	0.169
Proteasome subunit alpha type-3	Psma3	9	34	threonine protease	18.4944	9.14509	18.7178	2.674	0.073
Proteasome subunit alpha type-4	Psma4	6	48	threonine protease	18.3884	10.4219	18.4404	1.892	0.014
Proteasome subunit alpha type-2	Psma2	8	46	threonine protease	18.4408	11.0811	16.696	2.897	0.778
Proteasome subunit alpha type-5	Psma5	5	54	threonine protease	18.8371	NaN	18.77	2.892	0.403
Proteasome subunit beta type-6	Psmb6	3	39	threonine protease	18.2126	NaN	17.8972	1.892	0.084
Proteasome subunit alpha type-7	Psma7	9	64	threonine protease	19.2483	NaN	17.8216	2.303	0.599
Proteasome subunit beta type-2	Psmb2	6	42	threonine protease	17.9923	NaN	18.5744	1.940	0.174
Proteasome subunit beta type-1	Psmb1	6	34	threonine protease	18.9079	NaN	17.6095	2.906	0.393
Proteasome subunit alpha type-6	Psma6	9	69	threonine protease	19.1951	NaN	17.4419	3.215	0.739
Proteasome subunit beta type-3	Psmb3	3	12	threonine protease	17.1155	NaN	18.2711	#N/A	0.686
Proteasome subunit beta type-4	Psmb4	5	30	threonine protease	18.9379	NaN	NaN	2.310	2.327
Proteasome subunit beta type-8	Psmb8	4	11	threonine protease	15.4107	11.692	NaN	#N/A	#N/A

Proteasome subunit beta type-10	Psmb10	3	13	threonine protease	16.7471	NaN	NaN	2.405	1.747
Proteasome subunit beta type-9	Psmb9	1	9	threonine protease	16.5771	NaN	NaN	#N/A	#N/A
Proteasome subunit beta type-7	Psmb7	2	6	threonine protease	16.4413	NaN	NaN	1.720	1.680
Transthyretin	Ttr	12	785	metalloprotease	21.2916	24.025	24.7347	1.202	1.382
N-acetylated-alpha-linked acidic dipeptidase-like protein	Naaladl1	33	1930	metalloprotease	19.1751	23.2481	24.6997	2.422	2.003
Aminopeptidase N	Anpep	57	2258	metalloprotease	19.803	23.1391	24.3476	1.844	1.778
Carboxypeptidase A1	Cpa1	16	704	metalloprotease	22.9263	22.759	22.5157	0.030	0.080
Meprin A subunit beta	Mep1b	25	1168	metalloprotease	19.4138	22.6245	23.8723	2.007	1.943
Meprin A subunit alpha	Mep1a	18	687	metalloprotease	15.7532	20.5415	22.595	1.781	2.250
Glutamyl aminopeptidase	Enpep	49	1225	metalloprotease	15.6209	20.5377	21.9794	2.207	1.688
Xaa-Pro dipeptidase	Pepd	25	385	metalloprotease	18.531	21.0939	20.2736	2.266	0.303
Angiotensin-converting enzyme	Ace	43	532	metalloprotease	15.8097	20.3496	20.8612	2.462	1.327
Dipeptidase 1	Dpep1	12	165	metalloprotease	16.793	20.2423	20.7908	1.748	1.075
Carboxypeptidase Q	Cpq	10	116	metalloprotease	16.6141	16.3843	20.2002	0.219	3.360
Neprilysin	Mme	20	324	metalloprotease	14.3784	17.1243	20.4309	1.785	2.647
Angiotensin-converting enzyme 2	Ace2	27	472	metalloprotease	14.0885	20.9126	21.405	2.540	1.685
Carboxypeptidase A2	Cpa2	7	42	metalloprotease	21.0787	15.304	15.2301	0.385	0.666
Aspartyl aminopeptidase	Dnpep	6	17	metalloprotease	15.2966	17.4331	NaN	1.620	#N/A
Cytosol aminopeptidase	Lap3	10	36	metalloprotease	15.7985	17.3056	13.8277	1.140	0.981
Cytochrome b-c1 complex subunit 2, mitochondrial	Uqcrc2	1	1	metalloprotease	15.793	NaN	NaN	#N/A	#N/A
Dipeptidyl peptidase 3	Dpp3	3	5	metalloprotease	15.3036	NaN	NaN	1.571	1.504
Cathepsin S	Ctss	12	211	cysteine protease	18.1434	19.8341	21.5114	2.371	3.381

Gamma-glutamyl hydrolase	Ggh	7	108	cysteine protease	18.3017	16.6136	18.0305	1.216	0.073
Cathepsin B	Ctsb	9	36	cysteine protease	16.8027	14.0326	17.5193	0.546	0.178
Dipeptidyl peptidase 1	Ctsc	1	22	cysteine protease	16.4998	NaN	16.555	#N/A	#N/A
Bleomycin hydrolase	Blmh	3	15	cysteine protease	15.3392	16.5988	15.4948	0.993	0.301
Pro-cathepsin H	Ctsh	2	3	cysteine protease	NaN	12.7599	13.2319	#N/A	#N/A
Calpain-5	Capn5	4	5	cysteine protease	NaN	NaN	13.5846	#N/A	0.240
Caspase-7 subunit	Casp7	3	12	cysteine protease	16.5006	11.7026	NaN	2.225	2.323
Calpain-1 catalytic subunit	Capn1	6	29	cysteine protease	15.5826	NaN	NaN	1.018	0.923
Ubiquitin carboxyl-terminal hydrolase 14	Usp14	1	2	cysteine protease	16.1796	NaN	NaN	#N/A	#N/A
Prolactin-inducible protein homolog	Pip	4	63	aspartyl protease	21.4604	20.6779	19.7326	0.804	1.561
Cathepsin D	Ctsd	2	14	aspartyl protease	16.44	NaN	NaN	2.381	1.695
Alpha-1-antitrypsin 1-2	Serpina1b	24	1044	protease inhibitor	23.0819	22.4417	23.7386	0.304	0.544
Serine protease inhibitor A3K	Serpina3k	27	1256	protease inhibitor	22.3221	21.773	24.0954	0.219	1.130
Alpha-1-antitrypsin 1-4	Serpina1d	9	421	protease inhibitor	20.3095	21.2089	22.2327	0.456	1.093
Serine protease inhibitor A3M	Serpina3m	2	28	protease inhibitor	20.4737	21.3456	19.665	0.308	0.113
Leukocyte elastase inhibitor A	Serpinb1a	28	861	protease inhibitor	22.2774	19.0897	22.8749	2.877	0.583
Alpha-1-antitrypsin 1-3	Serpina1c	6	454	protease inhibitor	20.4958	19.7195	22.2703	0.277	1.315
Alpha-1-antitrypsin 1-5	Serpina1e	9	169	protease inhibitor	19.9406	18.0668	19.0202	0.519	0.176
Cystatin-B	Cstb	5	82	protease inhibitor	20.3827	16.9768	21.1659	2.271	0.513
Antithrombin-III	Serpinc1	15	170	protease inhibitor	18.1731	17.3909	19.4094	0.470	1.376
Alpha-2-macroglobulin	A2m	33	280	protease inhibitor	16.8397	16.1246	18.9413	0.338	0.797
Murinoglobulin-1	Mug1	21	243	protease inhibitor	15.742	16.0074	18.609	0.057	1.401

Serpin B12	Serpinb12	11	126	protease inhibitor	13.5918	12.7235	19.6256	#N/A	3.010
Serine protease inhibitor A3N	Serpina3n	5	43	protease inhibitor	17.1157	14.1584	17.2664	1.041	0.053
Alpha-2-antiplasmin	Serpinf2	3	12	protease inhibitor	15.065	14.3742	16.5981	#N/A	1.108
Complement C3 family	C3	22	101	protease inhibitor	15.5092	13.526	12.4006	1.364	1.801
Alpha-1-microglobulin	Ambp	2	12	protease inhibitor	NaN	13.8969	14.6954	#N/A	#N/A
Submaxillary gland androgen-regulated protein 3A	Smr3a	4	131	protease inhibitor	17.7611	18.1514	NaN	0.910	#N/A
Latexin	Lxn	2	1	protease inhibitor	16.2007	13.5171	NaN	0.919	0.566
Glia-derived nexin	Serpine2	1	4	protease inhibitor	NaN	NaN	14.5194	#N/A	#N/A

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Abbreviations

AB	Antibiotic
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
ALPI	alkaline phosphatase
Amox/Clav.	Amoxicillin Clavulanate
Azithro.	Azithromycin
B. fragilis	*Bacteroides fragilis*
B. subtilis	*Bacillus subtilis*
C. concisus	*Campylobacter concisus*
C. difficile	*Clostridium difficile*
C. albicans	*Candida albicans*
C. perfringens	*Clostridium perfringens*
C3	Complement C3
CD	Crohn's Disease
CD	cluster of differentiation
cecal_sup	cecal supernatant
Ciproflox.	Ciprofloxin
CO_2	carbon dioxide
CT	cecum tip
Ctr	Control
DAB	3,3'-Diaminobenzidine
DAI	disease activity index
DC	dendritic cell
dC	distal colon
DerP1	endopeptidase 1
DSS	dextran sulfate sodium
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1/2
F. prausnitzii	*Faecalibacterium prausnitzii*
FITC	Fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GF	germ free
GI	gastrointestinal
GWAS	genome-wide association studies
H. pylori	*Helicobacter pylori*
IBD	inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-D	irritable bowel syndrome with diarrhea
IEC	intestinal epithelial cell

IF	immunofluorescence
IFABP	intestinal fatty acid binding protein
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IHC	immunohistochemistry
IL	interleukin
JAM	junctional adhesion molecule
kDa	kilodlaton
Kgp	lys-gingipain
LC-MS/MS	liquid chromatography–mass spectrometry
Levo(Met.)	Levofloxacin Metronidazole
LPS	lipopolysaccharides
M. voltae	*Methanococcales voltae*
MLCK	myosin light chain kinase
MMLV	Moloney Murine Leukemia Virus
MUC	mucin
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	nucleotide-binding oligomerization domain containing protein 2
NSAID	non-steroidal anti-inflammatory drugs
P. aeruginosa	*Pseudomonas aeruginosa*
P. gingivalis	*Porphyromonas gingivalis*
PA	protease activity
PAR	protease activity receptor
pC	proximal colon
PCR	polymerase chain reaction
pH	potential of hydrogen
pMLC	*phosphorylation of myosin light chain*
PMSF	phenylmethane sulfonyl fluoride
PTK6	protein tyrosin kinase 6
rDNA	ribosomal deoxyribonucleic acid
Rgp	Arg-gingipain
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
S. pyogenes	*Streptococcus pyogenes*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. typhimurium	*Salmonella typhimurium*
SAA	serum amyloid A
SCFA	short chain fatty acids
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLIGRL	Ser-Leu-Ile-Gly-Arg-Leu-NH(2), PAR-2 activating peptide
SlyCEP	*S. pyogenes* cell envelope protease

stool_sup	stool supernatant
TEER	transepithelial electrical resistance
Th17	T helper 17
TLR	Toll-like receptors
TNFα	Tumor necrosis factor alpha
UC	ulcerative colitis
V/M	Vancomycin/metronidazole
w/v	weigh per volume
WT	wildtype
ZO-1	zonula occludens-1

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Publications and Presentations

Publications

1. **H. Yoon**, M. Schaubeck, H. Hahne, I. Lagkouvardos, T. Clavel, S. Panda, P. Kump, A. Blesl, B. Küster, C. Manichanh, D. Haller*, G. Hörmannsperger*, "Increased pancreatic protease activity in response to antibiotics Impairs gut barrier and triggers colitis" (Accepted to Cellular and Molecular Gastroenterology and Hepatology)
2. A. Kindt, G. Liebisch, T. Clavel, D. Haller, G. Hörmannsperger, **H. Yoon**, D. Kolmeder, A. Sigrüner, S. Krautbauer, C. Seeliger, H. Daniel, D. Helm, B. Küster, J. Krumsiek, J. Ecker*, "The gut microbiota promotes hepatic fatty acid desaturation and elongation" (Accepted to Nature Communications)
3. G. Hörmannsperger, **H. Yoon**, J. Calasan, D. Haller*, "Therapeutic implementation of encapsulated prtP-encoded lactocepin on experimental colitis" (in preparation)
4. N. Barki, **H. Yoon**, G. Hörmannsperger, D. Haller M. Schemann*, "Increase in neuronal activation and intestinal motility by serine protease" (in preparation)
5. **H. Yoon**, J. Ju, J. Lee, H. Park, J. Lee, H. Shin, W. Holzapfel, K. Park, M. Do*, "The probiotic Lactobacillus rhamnosus BFE5264 and Lactobacillus plantarum NR74 promote cholesterol efflux and suppress inflammation in THP-1 cells", J Sci Food Agric. 2013 Mar 15;93(4):781-7 (cited 7 times)
6. **H. Yoon**, J. Ju, H. Kim, H. Park, J. Lee, J. Lee, H. Shin, M. Do, W. Holzapfel*, "Reduction in cholesterol absorption in Caco-2 cells through the down-regulation of Niemann-Pick C1-like 1 by the putative probiotic strains Lactobacillus rhamnosus BFE5264 and Lactobacillus plantarum NR74 from fermented foods", Int J Food Sci Nutr. 2013 Feb;64(1):44-52. (cited 7 times)
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8. **H. Yoon**, J. Ju, H. Kim, J. Lee, H. Park, Y. Ji, H. Shin, M. Do, J. Lee, W. Holzapfel*, "Lactobacillus rhamnosus BFE 5264 and Lactobacillus plantarum NR74 promote cholesterol excretion through the up-regulation of ABCG5/8 in Caco-2 cells", Probiotics Antimicrob Proteins. 2011 Dec;3(3-4):194-203 (cited 11 times)
9. J. Ju, **H. Yoon**, H. Park, M. Kim, H. Shin, K. Park, J. Yang, M. Sohn, M. Do*, "Anti-obesity and antioxidative effects of purple sweet potato extract in 3T3-L1 adipocytes in vitro", J Med Food. 2011 Oct;14(10):1097-106 (cited 12 times)
10. H. Lee, **H. Yoon**, Y. Ji, H. Kim, H. Park, J. Lee, H. Shin, W. Holzapfel*, "Functional properties of Lactobacillus strains isolated from kimchi", Int. J. Food Microbiol. 2011 Jan 31;145(1):155-61 (cited 77 times)

Published Abstracts

1. UEG week 2016: ***Antibiotics-mediated increase in large intestinal protease activity is associated with impaired intestinal barrier functions and aggravation of spontaneous colitis***
   H. **Yoon**, M. Schaubeck, D. Haller, G. Hörmannsperger; United European Gastroenterology Journal; 2016: 2 (Supplement 1) October 16, 2016; pp. A157–A720
2. DDW 2016: ***Antibiotic Mediated Rise in Large Intestinal Proteolytic Activity Is Paralleled by Reduced Large Intestinal Barrier Function and May Be a Risk Factor for IBD Development in Susceptible Organisms***
   H. **Yoon**, M. Schaubeck, D. Haller, G. Hörmannsperger; Gastroenterology 150(4):S313 · April 2016

Oral presentations

1. **H. Yoon**, M. Schaubeck, P. Kump, D. Haller, G. Hörmannsperger, "Antibiotic-mediated change in gut microbiota increases protease activity and aggravates colitis", Plenary Presentation, **^{2th}IMKASID**, *Seoul, S. Korea*, 2018
2. **H. Yoon**, "Functionality of proteolytic bacteria: gut physiology and colitogenic mechanism", **Invited Seminar** in Seoul National University, *Pyeongchang, S. Korea* (16.04.2018)
3. **H. Yoon**, M. Schaubeck, P. Kump, C. Högenauer, C. Manichanh, D. Haller, G. Hörmannsperger, "Antibiotic Therapy and IBD Pathogenesis: Intestinal Proteases and Colitogenic Mechanisms", **^{5th}Asian Organization for Crohn's and Colitis**, *Seoul, S. Korea*, 2017
4. **H. Yoon**, M. Schaubeck, D. Haller, G. Hörmannsperger, "Antibiotic-mediated alterations in the intestinal microbiota affect large intestinal pancreatic protease activity and colitogenic mechanisms in IL10-/- mice", **5th Joint Conference of the DGHM & VAAM**, *Würzburg, Germany*, 2017
5. **H. Yoon**, J. Calasan, D. Haller, G. Hörmannsperger, "Therapeutic implementation of encapsulated *prtP*-encoded lactocepin on experimental colitis"**, 5th Joint Conference of the DGHM & VAAM**, *Würzburg, Germany*, 2017
6. **H. Yoon**, "Antibiotic treatment as risk factor for development of IBD", **DAAD Annual Scholarship meeting**, *Munich, Germany* (23.11.2016)
7. **H. Yoon**, "AB-mediated increase in liPA is associated with impaired intestinal Barrier Functions and Aggravation of Spontaneous Colitis", **Invited Seminar** in Handong University, *Pohang, S. Korea* (12.09.2016)
8. **H. Yoon**, M. Schaubeck, D. Haller, G. Hörmannsperger, "Antibiotic Therapy as Risk Factor for IBD: Are Intestinal Proteases a Missing Link?", **MICS 2016**, *Toronto, Canada*, 2016
9. W. Holzaphel, Y. Ji, H. Shin, **H. Yoon***, "Probiotics and Serum Cholesterol Reduction - Assessment of the Current Status", **IPC 2011**, *Kosice, Slovakia*, 2011

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