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Effects of microbiota modulations on host intestinal epithelial defense responses

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2 Abstract

The intestinal microbiota plays a major role in modulating the interaction between the epithelium and the immune system. Several gastrointestinal disorders like intestinal graft-versus-host disease or inflammatory bowel disease are suggested to be mediated by microbial dysbiosis. Furthermore, antibiotics are capable of changing the microbial composition and diversity in the gut leading to alterations of host immune response. However, the impact of antibiotic-related microbiota disturbances on epithelial defense parameters is not finally elucidated. In the present project, we investigated intestinal epithelial and immunological responses of C57BL/6 mice after inducing altered microbial states by either antibiotic treatment or fecal material transfer. First, mice were treated orally with antibiotics, either rifaximin (RFX) or a combination of ampicillin, metronidazole, vancomycin and neomycin (AMT). 16S rRNA sequencing of feces revealed dysbiosis-like microbiome in RFX-treated rodents. Analysis of cytokine and chemokine expression profiles of the innate and adaptive immune system in the caecum revealed an increase of IFNγ and MCP1 after AMT, whereas RFX induced a downregulation of Reg3b, a lectin with antimicrobial activity against pathogens and also anti-inflammatory properties. Reg3b was also found to be secreted into animal's feces. IL-22 was observed to rescue dysbiosis-induced Reg3b changes. MyD88, an essential adaptor protein in Toll-like receptor signaling, was observed not to be involved in Reg3b downregulation. DSS in combination with antibiotic treatment induced Reg3b downregulation and predisposed to intestinal inflammation and destruction of histological architecture. In summary, experimental manipulations of the gut microbiota induce distinct host responses with Reg3b as a highly interesting factor of epithelial homeostasis.

3 Zusammenfassung

Das intestinale Mikrobiom spielt eine große Rolle bei der Modulation der Interaktion zwischen dem Epithel und dem Immunsystem. Mehrere gastrointestinale Störungen wie die graft-versus-host Erkrankung oder die chronisch entzündliche Darmerkrankung werden vermutlich durch eine mikrobielle Dysbiose vermittelt. Außerdem sind Antibiotika in der Lage, die mikrobielle Zusammensetzung und Diversität im Darm zu verändern und führen somit zu Änderungen der Immunantwort des Wirtes. Jedoch ist die Auswirkung von antibiotikabedingten Störungen auf die epithelialen Abwehrparameter noch nicht gänzlich aufgeklärt. Im vorliegenden Projekt untersuchten wir die epithelialen und immunologischen Antworten im Intestinum von von C57BL/6 Mäusen, nachdem deren mikrobielle Beschaffenheit entweder durch antibiotische Behandlung oder Fäkaltransfer verändert worden war. Zunächst wurden die Mäuse oral mit Antibiotika, entweder mit Rifaximin (RFX) oder einer Kombination aus Ampicillin, Metronidazol, Vancomycin und Neomycin (AMT), behandelt. 16S rRNA Sequenzierung der Fäzes ergab Dysbiosis ähnliches Mikrobiom in Nagetieren, die mit RFX behandelt worden waren. Die Expressionsanalyse der Zytokin- und Chemokin-Profile des angeborenen und adaptiven Immunsystems im Zökum offenbarte einen Anstieg des IFNγ und MCP1 nach AMT. RFX hingegen induzierte eine Herabregulation des Reg3b, ein Lectin mit antimikrobieller Aktivität gegen Pathogene und auch antiinflammatorischen Eigenschaften. Ferner stellte sich heraus, dass Reg3b auch in den Tierfäzes sezerniert wird. Bei IL-22 wurde beobachtet, dass es Reg3b Veränderungen, die durch Dysbiosis verursacht worden sind, auffangen kann. MyD88, ein essentielles Adapterprotein im Toll-like Rezeptor Signalweg, ist beobachtet worden, nicht in der Herabregulation des Reg3b involviert zu sein. Kombinierte Behandlung von DSS und Antibiotika induzierte eine Herabregulation des Reg3b, eine Prädisposition für intestinale Entzündungen und Zerstörung der histologischen Architektur. Zusammenfassend lässt sich sagen, dass experimentelle Manipulationen des Darmmikrobioms distinkte Antworten beim Wirt auslösen, wobei Reg3b einen hoch interessanten Faktor der epithelialen Homöostase darstellt.

4 Abbreviations

µl	Microliter
16S rRNA	16S ribosomal RNA
AMT	Antimicrobial treatment
Bm1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenic protein
CBC	Crypt base columnar
cDNA	complementary deoxyribonucleic acid
Cq	Quantification cycle
CRAMP	Cathelicidin-related antimicrobial peptide
Crypt-4	Cryptdin 4
Ctrl	Control
CXCL1	CXC-chemokine ligand 1
CXCL2	CXC-chemokine ligand 2
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotides
DOC	Deoxycholate
DSS	Dextran sodium sulfate
E. coli	Escherichia coli
EGF	Epidermal growth factor
ELISA	Enzyme-linked Immunosorbent Assay
FcRγ	Fc receptor γ-chain
FMT	Fecal microbiota transfer
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase

GAPs	Goblet cell-associated antigen passages
GCs	Goblet cells
GF	Germ-free
GULDA	Gut Low-Density Array
HDAC	Histone deacetylase
HIF-1	Hypoxia-inducible factor 1
HIP	Hepatocarcinoma-intestine-pancreas
HMA	Human microbiota-associated
i.p.	Intraperitoneal
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFNγ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Lgr5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LPS	Lipopolysaccharide
MCP1	Monocyte chemoattractant protein-1
MDR-1-proteins	Multi-drug-resistance-1-proteins
MHC II	Major histocompatibility complex class II
MHC	Major histocompatibility complex
MIH	Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Munich, Germany
Mincle	Macrophage inducible C-type lectin

MLNs	Mesenteric lymph nodes
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MUC2	Mucin 2
MyD88	Myeloid differentiation primary response 88
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK	Natural killer
NORA	New-onset rheumatoid arthritis
PAP	Pancreatitis-associated proteins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
Reg	Regenerative islet-derived protein
Reg3b	Regenerative islet-derived protein 3 beta
Reg3g	Regenerative islet-derived protein 3 gamma
RFX FMT	Rifaximin-treated fecal microbiota transfer
RFX	Rifaximin
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
RORγt	Retinoic-acid-receptor-related orphan receptor-γt
rpm	Revolutions per minute
SAP130	Spliceosome-associated protein 130
SCFA	Short-chain fatty acid
SCFAs	Short-chain fatty acids
SGLT1	Sodium-glucose transporter protein

SOCS3	Suppressor of Cytokine Signaling 3
SPF	Specific pathogen free
Ssp.	Several species
STAT3	Signal Transducer and Activator of Transcription 3
Syk	Spleen tyrosine kinase
TGF- β	Transforming growth factor beta
TGF- β1	Transforming growth factor beta 1
TGFα	Transforming growth factor alpha
T_H	T helper
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNFα	Tumor necrosis factor alpha
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
VRE	Vancomycin-resistant enterococci
Wnt	Wingless type MMTV integration site family
Wnt3	Wingless-related MMTV integration site 3

5 Introduction

5.1 Microbiota

Approximately 100 trillion bacterial cells inhabit our bodies outnumbering eukaryote cells ten-fold. A community of microbes, that is present at a given environment, is called microbiota, whereas its genetic profile is termed microbiome (Becattini, Taur, & Pamer, 2016). With the introduction of metagenomics sequencing and sequence analysis of cloned microbial small-subunit ribosomal RNA genes (16S rRNA) the bacterial composition of the microbiota in the feces could be explored (Cho & Blaser, 2012). At least 1000 different species could be identified with *Firmicutes* and *Bacteroidetes* as the most abundant phyla constituting over 90% of the known population. Significantly differences in microbial diversity among healthy individuals could be detected (Qin et al., 2010). Since the lumen is an oxygen-deficient area, the gastrointestinal tract is mainly inhabited by anaerobic bacterial strains. Gram positive bacterial strains such as *Clostridia* are prominent members of the *Firmicutes* phyla, whereas *Bacteroidetes* phyla consists of gram negative strains (Becattini et al., 2016). Different studies have shown differences between stool and mucosal-adherent communities, possibly indicating further important interactions with the host that alter the intestinal environment (Eckburg et al., 2005; Zoetendal et al., 2002). Further, research on mucosa-associated subpopulations revealed a dispersed distribution pattern along the longitudinal colon axis (Eckburg et al., 2005), contrary to the belief of a linear gradient over the colon.

Due to the overwhelming number, the microbial genome possesses immense potential and opens new possibilities to the host to expand its own genetic repertoire (Qin et al., 2010). Commensal bacteria in the gut interact with the host as symbiotic partners. They are essential for the synthesis of vitamin groups B and K and are even capable of converting primary bile acids into secondary bile acids (Becattini et al., 2016), thereby being involved in the enterohepatic circulation. Under anaerobic conditions plant-derived fibers such as cellulose can be fermented by gut microorganisms, especially by *Clostridia clusters IV* and *XIVa* bacteria, and are transformed into short-chain fatty acids (SCFAs) including butyrate, propionate and acetate (Brestoff & Artis, 2013). Notably, these are functions not encoded by human enterocytes. Butyrate has been reported to ameliorate the inflammatory response in DSS colitis models (Maslowski et al., 2009). It is the preferred metabolic substrate for the colonic epithelium and, for instance, stabilizes hypoxia-inducible factor 1 (HIF-1) (Kelly et al., 2015). HIF-1 is a major cellular mechanism mediating the switch from oxidative to glycolytic metabolism under hypoxic conditions. It also regulates angiogenesis promoting vascular endothelial growth factor (VEGF) and erythropoietin (Semenza, 2011). Since the intestinal mucosa is constantly exposed to a physiological hypoxia (Suski, Zabel, Levin, Scheuenstuhl, & Hunt, 1997), low

levels of oxygen stimulate expression of HIF-1 augmenting barrier integrity and inhibiting colitis-associated mucosa damages (Keely et al., 2014). Due to the correlation of inflammatory responses with oxygen-deprivation (Frick et al., 2009; Karhausen et al., 2004), HIF-1 plays a further crucial role in ensuring the survival and functionality of recruited immune cells during inflammation (Keely et al., 2014). Reduced amounts of SCFAs and subsequently diminished expression of HIF-1 could be shown after antibiotic administration, thus illustrating the interaction between microbiota-derived metabolites and cellular signaling pathways (Kelly et al., 2015). Antibiotics are powerful agents capable of eliminating bacterial pathogens, while the human tissue remains unaffected by reason of structural differences between eukaryote and prokaryote cells. Since antibiotics cannot distinguish between beneficial and deleterious microbes, they induce long-lasting changes in the composition of the gut flora. Antibiotic treatment disrupts microbial homeostasis and permits *Clostridium difficile*, a toxin producing gram positive bacteria that is able to form endospores, to proliferate causing pseudomembranous colitis. However, under physiological conditions commensal bacteria compete against new proliferating strains resisting any foreign colonization (van der Waaij, Berghuis-de Vries, & Lekkerkerk-van der Wees, J. E. C., 1971). For example, the vaginal epithelium is colonized with *Lactobacillus* bacteria acidifying the lumen with lactic acid, and thereby protecting against bacterial species, susceptible to an acidic milieu. The concept of colonization resistance playing a critical role in *Clostridium difficile*-associated infections has been verified, since the establishment of fecal microbiota transfer (FMT) from healthy individuals can successfully cure recurrent *Clostridium difficile* infections (Hirsch et al., 2015). Furthermore, *Clostridium scindens* has been reported to synthetize secondary bile acids deoxycholic acid and lithocholic acid (Buffie et al., 2015). These metabolites inhibit proliferation of *Clostridium difficile* and emphasize the remarkable synergy of the microbiota and the enterohepatic circulation contributing to the therapeutic efficacy of FMT. Another mechanism of colonization resistance is the production of antimicrobial peptides. Bacteriocin 21 synthesizing *Enterococcus faecalis* strains have been shown to eliminate multidrug-resistant *Enterococci* from the gastrointestinal tract (Kommineni et al., 2015), proving the bactericidal potential of the microbiota. The microbiota is not only involved in infectious diseases, but also has been correlated with chronic illness. For instance, in obese mice, the altered microbiome is enriched for enzymes rendering indigestible dietary polysaccharides accessible and increases the absorption of energy-rich substrates (Turnbaugh et al., 2006). Additionally, a shift from *Bacteroidetes* towards *Firmicutes* with subsequent increased production of butyrate indicates the contribution of the bacterial community on obesity (Turnbaugh et al., 2006). By transferring the fecal microbiota of obese human into germ-free mice, they exhibit an obese phenotype emphasizing the transmissibility and importance of diet-induced microbial alterations in the pathogenesis of obesity (Ridaura et al., 2013b). Type 2 diabetes has also been linked to intestinal dysbiosis due to similar changes in the

microbial composition resulting in augmented energy metabolism (Karlsson et al., 2013; Qin et al., 2012). However, regarding these observations, the gut flora altering effect of metformin (Forslund et al., 2015), a drug commonly used in the treatment of type 2 diabetes, has to be considered. Furthermore, the intake of non-caloric artificial sweeteners, contrary to its intention of fighting obesity and diabetes, is controversially discussed to induce glucose intolerance (Suez et al., 2014). It is hypothesized that non-caloric artificial sweeteners enhance the energy utilization of the gut microbiota (Suez et al., 2014). These multiple aspects of microbial modulation highlight the complex interactions between gut flora alterations and disease manifestations and emphasize the impact of life-style and medication on the delicate intestinal homeostasis. Besides the microbiota located at the gastrointestinal tract, the cutaneous flora also modulates the emergence and development of skin diseases. Atopic dermatitis episodes are characterized with skin lesions colonized with *Staphylococcus aureus*, frequently a multidrug-resistant pathogen, which can be also found in surrounding unaffected skin areas (LEYDEN, MARPLES, & KLIGMAN, 1974). In contrast, skin commensals such as *Staphylococcus epidermidis* possess anti-inflammatory properties and are thought to control the growth of *Staphylococcus aureus* (Laborel-Preneron et al., 2015). Therefore, the cutaneous microbiota plays a vital role in augmenting, eliminating and suppressing the rise of pathogenic bacteria. Whether microbial alterations render the proliferation of foreign bacterial strains possible or they emerge consequently after the infection needs to be ascertained.

During the uterine development and growth, the human fetus is encapsulated by the amniotic sac, dwelling in a sterile environment and thereby possessing no microbiota. Due to the vaginal delivery mode, the neonate inherits the maternal flora of the birth canal consisting mainly of *Lactobacillus*, *Bifidobacterium* and *Bacteroides* bacteria, representing the first commensals colonizing homogenously the whole body (Dominguez-Bello et al., 2010). This initial bacterial community prepares the gastrointestinal tract for following strains and is relatively stable for approximately one year. Then it is transformed into an adult-like pattern (Palmer, Bik, DiGiulio, Relman, & Brown, 2007). In contrast, infants delivered by C-section do not get in touch with the vaginal flora. Their microbiota resembles the skin flora of adults (Dominguez-Bello et al., 2010), correlating with an increased risk to develop atopic diseases (Bager, Wohlfahrt, & Westergaard, 2008). This confirms the importance of the physiological microbial transmission for establishing a resilient homeostasis.

5.2 Intestinal mucosa

With a mucosal surface of roughly 32 m² the digestive tract surpasses the size of the skin (Helander & Fandriks, 2014). Similar to the skin, the gastrointestinal tract is colonized with bacteria and therefore embodies an unsterile organ. It has to protect the host from intruders, but at the same time nutrients must be able to traverse the epithelium. Additionally, the digestive tract displays a compartmentalization of various segments with their own highly specialized environment: esophagus, stomach, small and large intestine. The small intestine can be further divided into duodenum, jejunum and ileum, while caecum, colon, sigmoid colon and rectum are part of the large intestine. Even though every compartment has its own unique epithelial characteristics to fulfill specific functions, all of them share a common histological structure. First, the one-layered columnar epithelium represents the border between the body and the lumen and is responsible for most digestive and secretory functions. It inserts in the lamina propria, a connective tissue, where small blood vessels and immune cells can be found. Furthermore, the muscularis mucosae follows as a thin smooth muscle layer. These three segments form the mucosa, and underneath there is a connective tissue with embedded blood vessels, the submucosa. It is surrounded by strong circular and longitudinal muscle fibers called tunica muscularis. The outermost layer depends on the peritoneal position. All intraperitoneal organs are covered by connective tissue and mesothelium, the serosa. In contrast, organs with no connection to the abdominal cavity are not covered by the serosa. Additionally, the mucosa forms invaginations called crypts over the whole length of the gastrointestinal tract, whereas villi, finger-like extensions into the lumen, are only encountered in the small intestine.

The compartmentation of the gastrointestinal wall goes along with the presence of highly specialized cell types (Figure 1 for schematic overview). Since the mucosal epithelium is in close contact with the lumen, it has to fulfill many tasks at the same time. First, it has to loosen the epithelial barrier function in order to allow the absorption of energy sources and water, while preventing bacterial translocation. Absorptive enterocytes located at the surface of the crypt and villus express various transporter proteins to facilitate the uptake of essential nutrients. Sodium-glucose transporter protein (SGLT1) is one of these transporter units, which is capable of transporting glucose in the presence of sodium. In addition, the luminal side reveals extrusions called microvilli amplifying the absorption area. Furthermore, the tight junction between enterocytes allows the passive transition of water following an osmotic gradient. Especially the small intestine is responsible for the main nutrients and water absorption of up to 8 liters per day and therefore increases the luminal surface area by villi maximizing the efficiency. However, hydrophobic and potential deleterious molecules are still able to diffuse into the enterocytes. Therefore, ATP-consuming multi-drug-resistance-1-

proteins (MDR-1-proteins) can carry these substrates back into the lumen. Besides MDR-1-proteins there are also other means, by which the epithelium is protected from hazards. Strikingly, enterocytes possess the ability to release antimicrobial factors such as Reg3g (Cash, Whitham, Behrendt, & Hooper, 2006) and Reg3b (Burger-van Paassen et al., 2012), both structurally related to the C-type lectin family. Therefore, enterocytes could be referred to as part of the innate immune system and contribute to mucosal homeostasis. In addition to absorptive enterocytes, the mucosal epithelium harbors goblet cells that are predominantly found in the large intestine. They are spread between enterocytes with a main function in the production of mucus, a multifunctional exudate. Since it consists of glycoproteins such as MUC2, the mucus can bind water easily and is highly hydrated creating two mucus layers in the large intestine. The inner mucus layer is dense and devoid of bacteria outlining a physical barrier between luminal microbial invaders and colonic tissue. On the contrary, the outer mucus layer is infiltrated by bacteria, thereby losing its dense structure (Johansson et al., 2008). In this way, the mucus covers membranes and keeps the moisture preventing erosion of the epithelium. An epithelium without mucus is highly susceptible to microbial pathogens and cannot fulfill its defensive purpose leading to infection and inflammation (Sovran et al., 2015). The mucus of the small intestine only forms a soluble gel and is not firmly attached to the underlying epithelium (Atuma C, Strugula V, Allen A, Holm L, 2001). Concerning immune defense mechanisms, goblet cells interact with the adaptive immune system. Dendritic cells (DCs) residing at the lamina propria acquire luminal antigens from goblet cells via goblet cell-associated antigen passages (GAPs) (McDole et al., 2012). Therefore, the goblet cells do not only protect the epithelium passively by mere preservation, but in addition by actively facilitating bacterial antigen presentation.

Furthermore, the small intestinal immune system is supported by Paneth cells, a specialized small intestinal epithelial lineage residing at the base of the crypts. They have been shown to secret a complex repertoire of antimicrobial peptides such as lysozyme, Reg3b, Reg3g (Cash et al., 2006) and members of the α-defensin family (Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008) via MyD88-dependent toll-like receptors (TLR) microbial sensing. An activation of the MyD88 signaling cascade limits the translocation of enteric microbes from the mucosal surface to underlying mesenteric lymph nodes (MLNs) (Vaishnava et al., 2008). Thereby, Paneth cells play a crucial role in the innate immune system. However, in a transgenic mouse model, in which a diphtheria toxin fragment expression selectively ablated Paneth cells, the luminal density of commensal bacteria showed no differences in comparison to wild-type mice (Vaishnava et al., 2008). Besides protective and digestive functions, the intestine also represents an endocrine organ. In the gut epithelium enteroendocrine cells are situated at the crypts or the base of the villi, which are responsible for the release of peptide hormones and neurotransmitter. A stimulation at their apical pole induces the

transportation of hormone vesicles to the basal pole and subsequent exocytosis. By accessing blood vessels, interstitial tissue and neurons, these hormones modulate the gastrointestinal motility and secretion of enzymes in the gastrointestinal tract (Takashima, Gold, & Hartenstein, 2013). All these highly specialized gastrointestinal cells are constantly exposed to toxic content and mechanical shear stress resulting in a high demand of a tremendous cellular turnover capacity. Thus, the intestine epithelium renews every five to seven days.

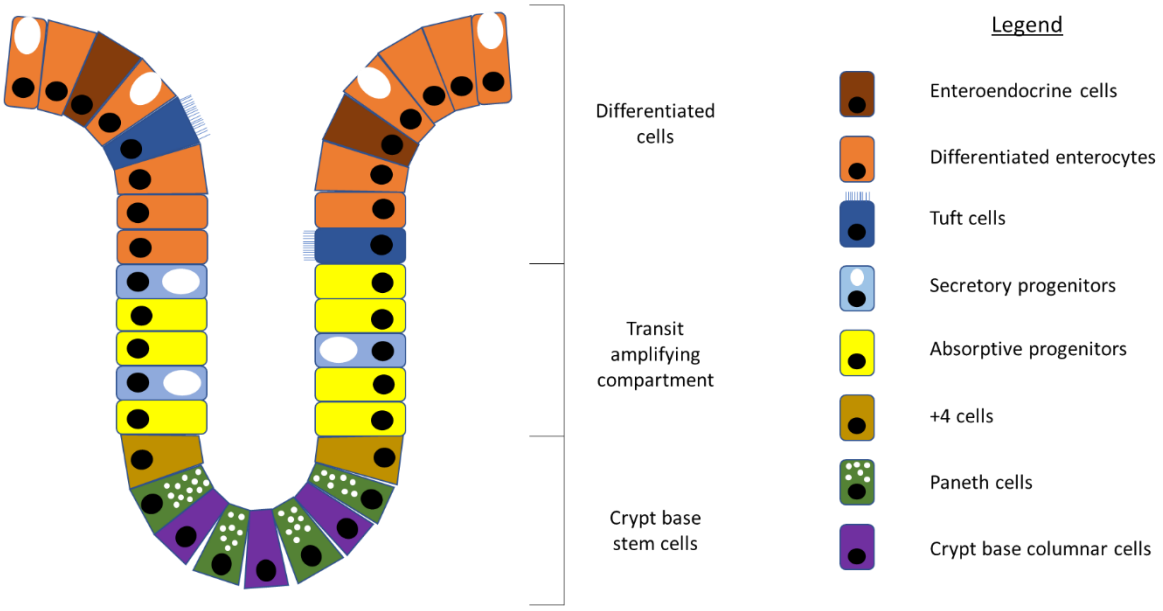


Figure 1 - Intestinal crypt architecture and cell types.

Crypt base columnar cells divide into one parent stem cell and one transit amplifying cell. Transit amplifying cells migrate towards the luminal pole and terminally differentiate after leaving the crypt zone.

Only intestinal stem cells endowed with longevity and multipotency to differentiate into the four cell types can supply the required cells in such a short time. Leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) is known to be a marker gene for stem cells in the small and large intestine and is also a target of the Wnt signaling cascade (Barker et al., 2007). It has been shown that Lrg5 is exclusively expressed by cycling crypt base columnar (CBC) cells. Approximately four to six independent stem cells reside near every crypt bottom in the stem cell niche and divide asymmetrically into one daughter cell, that replaces the parent stem cell and one transit amplifying cell (Barker, van de Wetering, & Clevers, 2008). The transit-amplifying cell undergoes a limited number of cell divisions while migrating along the vertical villus-luminal axis (Biswas et al., 2015). After reaching the crypt-villus junction it terminally differentiates. During the migration and maturation process, different signaling cascades are activated and influence the cell development. For instance, Wnt signaling supports stem cell function, self-renewal and drives transit amplifying cell proliferation by increasing cytosolic levels of β-catenin, which leads to transcription of proliferation

genes (Biswas et al., 2015). Therefore, Wnt signaling is restricted to the bottom third of the crypt, where most of cell proliferation takes place (Biswas et al., 2015). The initiation of the terminal differentiation of mature intestinal cells is dependent on bone morphogenic protein (BMP) signaling as its activation opposes the effect of Wnt (Auclair, Benoit, Rivard, Mishina, & Perreault, 2007). Since the concentration of BMP ligands is highest in cells at the surface (He et al., 2004), the proliferation sustaining Wnt signal is more and more repressed during migration along the crypt – luminal axis. Paneth cells form an exception, since they stay at the bottom of the crypt contrary to the absorptive, secretory and enterendocrine cell types. In addition to Wnt and BMP signaling, Paneth cells are essential for stem cell maintenance since they express essential signals such as EGF, TGFα and Wnt3 (Sato et al., 2011). Furthermore, their ablation in genetic mouse models results in concomitant loss of stem cells (Sato et al., 2011). However, the necessity of Paneth cells is controversially discussed as an unimpaired growth of stem cells has been shown in absence of Paneth cells (Kim, Escudero, & Shivdasani, 2012). A possible explanation could be the existence of a second stem cell pool. These cells express Bmi1 and reside at the fourth position relative to the crypt base or +4 position (Barker et al., 2008; Sangiorgi & Capecchi, 2008). Therefore, they are upstream of the Lgr5+ CBC cells and the Paneth cells. In case of elimination of Lgr5 expressing cells, these cells are able to compensate for their loss and even to give rise to Lgr5+ cells (Tian et al., 2011). These findings contradict the unidirectional hierarchy, where the degree of differentiation correlates with the upward progression and rejects the idea of a dedifferentiation towards the crypt base.

5.3 Carbohydrate-binding proteins: the lectin family

Towards the end of the 19th century, researchers discovered proteins derived from plants with erythrocyte-agglutinating properties (Sharon & Lis, 2004). After discovery of their ability to distinguish between different blood types, they were referred to as lectins, derived from the Latin word “lego” (to choose, to pick out) (Boyd & Shapleigh, 1954). Due to the high carbohydrate-specificity of lectins detecting glycoconjugates on cell surfaces, lectins represent a way of mediating cell adhesion and reading information encoded by complex carbohydrate structures (van Kooyk & Rabinovich, 2008). Therefore, lectins played a major role in the identification of the ABO blood group system (Morgan & Watkins, 2000). Later, this term was generalized to include all carbohydrate-binding proteins independent of origin and specificity (Sharon & Lis, 1972) embracing now more than a thousand members. Historically, lectins were divided into Ca^{2+}-dependent (C-type) and Ca^{2+}-independent carbohydrate-binding proteins. An important feature of C-type lectins is their carbohydrate-recognition domain. The carbohydrate-recognition domain is an unique and highly

conserved module mediating the carbohydrate-binding activity and is a defining characteristic of C-type lectins (Zelensky & Gready, 2005). It contains four Ca^{2+}-binding sites with the second one being crucial for the carbohydrate binding. The remaining sites stabilize the molecular structure (Zelensky & Gready, 2005). However, proteins, which do not fulfill the criteria of lectins but resemble the structure of the carbohydrate-recognition domain of C-type lectins, are also found (Hurtado et al., 2004; Plougastel, Dubbelde, & Yokoyama, 2001). Therefore, all C-type lectin-like domain containing proteins form the C-type lectin-like domain superfamily. Additionally, vertebrate C-type lectin-like domains can be divided into 14 groups considering biochemical architecture and phylogenetic analysis (Drickamer, 1988; Drickamer & Fadden, 2002). In addition, three additional groups found in *Fugu rubripes* were proposed (Zelensky & Gready, 2004).

Due to their ability to discriminate between foreign and host carbohydrate structures, lectins play an important role in the immune defense. Complementing the adaptive immune system, represented by lymphoid and plasma cells, the evolutionary ancient innate immune system serves as a fast reacting first-line host defense (Hoffmann, 1999). The innate immune system can be activated by three different pathways and induces chemotaxis of macrophages, opsonization and lysis of pathogens. The classical pathway is antibody-dependent and triggers the complement system leading to the recruitment of the membrane attack complex. The alternative pathway involves the covalent binding of C3 to the cell surface of microorganisms. Finally, the lectin pathway relies on pattern-recognition receptors such as mannose-binding lectin and the subsequent activation of mannose-binding lectin-associated serine proteases 1 and 2 (Fujita, 2002). These cleave and activate complement factors C2, C3 and C4. Soluble mannose-binding protein belongs to the C-type lectin receptor family, and its involvement in the detection of pathogenic glycoconjugates must not be underestimated. Thus, a lack of mannose-binding lectin impairs opsonization (Super, Lu, Thiel, Levinsky, & Turner, 1989) and phagocytosis resulting in severe infections (Summerfield et al., 1995) and even clinical symptoms of immunodeficiency in children (Sumiya et al., 1991). It is hypothesized that the lectin pathway is necessary to bridge the period between maternal antibody depletion and the maturation of the adaptive immune repertoire (Walport, 2001). Therefore, this time period depicts a vulnerable phase where the infant is solely protected by the innate immune system.

Pathogen recognition and subsequent antigen presentation are crucial for the induction of T cell differentiation, which would not be possible without numerous and diverse pattern-recognition receptors including the archetypical Toll-like receptors as well as C-type lectin receptors (Akira, Uematsu, & Takeuchi, 2006). This supports the relevance of C-type lectins in both the innate and the adaptive immune system. Besides soluble proteins, transmembrane C-type lectins also function as pattern-recognition receptors and can be divided into two groups. The mannose receptor family and

the asialoglycoprotein receptor family (Geijtenbeek & Gringhuis, 2009). Macrophage inducible C-type lectin (Mincle) is an important representative of the latter. Mincle is a type II transmembrane protein anchored in the surface of macrophages and possesses an extracellular C-type lectin domain (Graham & Brown, 2009). After ligand binding, the associated immunoreceptor tyrosine-based activation motif (ITAM), containing adaptor molecule Fc receptor γ-chain (FcR γ), is phosphorylated recruiting spleen tyrosine kinase (Syk), which activates the Card9-Bcl10-MALT1 signaling axis (Smith & Williams, 2016). As a result, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is upregulated and mediates the expression of CXC-chemokine ligand 2 (CXCL2), Interleukin 10 (IL-10) and tumor necrosis factor α (TNFα) (Geijtenbeek & Gringhuis, 2009). This signaling cascade is activated by spliceosome-associated protein 130 (SAP130). This protein is localized in the nucleus and it is released by necrotic cells, inducing neutrophil recruitment to damaged tissue and aiding in clearance of cell debris (Yamasaki et al., 2008). Furthermore, Mincle can recognize mannose on the fungal surface of *Malassezia* independently of Toll-like receptors (Yamasaki et al., 2009) and therefore plays an essential role in eliciting anti-fungal immunity. In terms of anti-fungal immune responses, Mincle is supported by the lectin dectin-1. Dectin-1 is specialized in recognizing β-1,3-glucan, an essential part of the yeast cell wall component zymosan (Di CARLO & FIORE, 1958). Dectin-1 is a type II transmembrane protein localized on the surface of macrophages and dendritic cells in the spleen and lymph nodes (Reid, 2004) and activates NF-κB via recruitment of Syk dependent on Card9 (Gross et al., 2006). Dectin-1 deficiency can lead to impaired host defense responses and enhanced fungal dissemination (Taylor et al., 2007). Therefore, binding of β-1,3-glucan is hypothesized to be involved in antigen presentation and T_H1 and T_H17 cell differentiation by augmenting expression of TNF, interleukin 6 and 23 (LeibundGut-Landmann et al., 2007). However, lectins do not only promote pro-inflammatory responses, but can also induce counter-regulatory pathways. The immunoreceptor tyrosine-based inhibitory motif (ITIM) serves as the antagonist of ITAM and is part of a suppressing signaling cascade in natural killer (NK) cells. After ligand binding and subsequent phosphorylation of ITIM, cytoplasmic tyrosine phosphatases SHP-1 and the inositol phosphatase SHP-2 are recruited. These two molecules modulate various activation signals, in the end leading to cell division arrest and inhibition of chemokine release and Ca^{2+} mobilization (Ravetch, 2000). NK cells are known to induce apoptosis in cells that present foreign antigens as major histocompatibility complex (MHC) products at the surface or which do not express MHC. Therefore, cell surface receptors are needed for the initiation of ITIM phosphorylation in order to prevent autolytic activities and auto aggression. Various Ly49 proteins and type II transmembrane lectins represent such inhibitory receptors (Lanier, 2005).

Another distinct group of mammalian C-type lectins is constituted by the regenerative islet-derived protein (Reg) family, which can be further classified into three subgroups (Gironella et al., 2005). First

discovered during a study investigating experimental pancreatitis in rats, pancreatitis-associated proteins (PAP) (Keim, Rohr, Stöckert, & Haberich, 2004) serve as acute-phase proteins responding to pancreatic injuries. PAPs are involved in the prevention of subsequent complications (Zhang, Kandil, Lin, Levi, & Zenilman, 2004) and are hypothesized to be involved in the regeneration of β-cells (Xia et al., 2016). However, the expression of PAPs is not restricted to pancreatic tissue. Murine PAP II (equivalent to Reg3a) is specifically expressed in the pancreas, whereas murine PAP I (alternatively Reg3b) and III (also known as Reg3g) expression is localized in the small intestine (Zhang et al., 2004), with Paneth cells as the key production site (Cash et al., 2006) and in the colonic enterocytes (Matsumoto, Konishi, Maeda, Kiryu-Seo, & Kiyama, 2012). In contrast, human PAP is also expressed in the pancreas as well as in the small intestine. In addition, it has been even detected in hepatocellular carcinoma, leading to its full term hepatocarcinoma-intestine-pancreas/pancreatitis associated protein (HIP/PAP) (Demaugre et al., 2004). It represents the human counterpart of Reg3 and is 65% identical with murine Reg3g (Cash et al., 2006). Furthermore, PAPs have anti-inflammatory properties as they provide protection against leukocyte-induced lung injuries, as an example (Zhang et al., 2004). This anti-inflammatory effect is attributed to the inhibition of the NF-κB pathway through a Janus kinase/Signal Transducer and Activator of Transcription 3-mediated (JAK/STAT3) mechanism counteracting the activation of TNFα and IL-6 (Folch-Puy, Granell, Dagorn, Iovanna, & Closa, 2006). Similar to IL-10, phosphorylation of STAT3 by PAP I leads also to induction of Suppressor of Cytokine Signaling 3 (SOCS3), thus downregulating inflammatory responses as a feedback inhibitor of the JAK/STAT3 signaling pathway (Folch-Puy et al., 2006).

The expression of Reg3b and Reg3g is mediated through MyD88-dependent TLRs microbial sensing of Paneth cells. There is even evidence that enterocytes are capable of secreting antimicrobial proteins of the Reg family via their own TLR-MyD88 signaling cascade (Vaishnava et al., 2008). Additionally, IL-22 has been reported to be directly involved in the release of Reg3b and Reg3g in colonic epithelial cells, while IL-23 seems to be necessary for the early induction of IL-22 (Zheng et al., 2008). The study of Reg3 and HIP/PAP protein is an important research focus, since their epithelial secretion is upregulated during inflammation in the gastrointestinal tract. Thus, patients suffering from inflammatory bowel disease (IBD) reveal elevated mRNA levels of HIP/PAP in the colonic epithelium, while bacterial reconstitution in germ-free mice strongly upregulates Reg3 expression (Ogawa et al., 2003). This supports their contribution to maintenance of mucosal homeostasis. Reg3g and HIP/PAP show high binding affinity towards peptidoglycan (Cash et al., 2006), a molecule forming the bacterial cell wall. Since peptidoglycan of gram-negative bacteria is buried in the periplasmic space, Reg3g and HIP/PAP are only effective against gram-positive microbes. Additionally, they lack collagenous domains necessary for complement recruitment, emphasizing their solely direct bactericidal potential (Cash et al., 2006). In contrast, Reg3b is equipped with antimicrobial properties against

gram-negative strains due to its affinity towards lipopolysaccharide (LPS) by recognizing the carbohydrate moiety of lipid A. This carbohydrate moiety constitutes the bulk of the outer leaflet of the outer membrane of gram-negative bacteria shielding the peptidoglycan from antimicrobial peptides (Miki, Holst, & Hardt, 2012). Like Reg3g and IHP/PAP, Reg3b also does not possess an activation site to recruit the complement system. A further member of the Reg3 class is Reg3a, which mainly participates in intracellular signaling cascades. It regulates pancreatic cell growth by activating proliferative pathways and protooncogenes (Xu et al., 2016) and can even lead to exacerbation of pancreatic malignancies. In addition, colorectal cancer risk has been linked to increased expression of Reg3a (Ye et al., 2016), emphasizing its involvement in angiogenesis and proliferation of various cell types. Therefore, the Reg3 peptides represent a diverse protein class, whose entire functions have not been definitely elucidated and remain to be ascertained.

5.4 Host – microbiota interaction

The interplay between the microbiota and the host is a coevolved homeostasis. Despite many benefits for both, this coexistence constitutes a potential hazard for the host, since pathogenic microbes are present in the gastrointestinal tract as well. Therefore, maintaining harmless communities in the gastrointestinal tract, while preventing deleterious strains from flourishing, represents a challenging task. However, the gut flora represents a competitive environment, where rapid-growing strains can outcompete their rivals. To evade the growth of unwanted species and to promote the establishment of slow-proliferating adjuvant bacteria, the host intervenes as a selectivity amplifier by secreting antimicrobial peptides and providing nutrients on the epithelial cell surface (Schluter & Foster, 2012). The generation of complex glycoconjugates on mucosal cells demonstrates an elegant way to modulate the emergence of epithelium-associated microbial communities. Desired microbial species can convert these carbohydrate structures and are promoted in their growth as a result (Schluter & Foster, 2012). Even in times of starvation due to infections, the host mobilizes its energy resources to shed fucosylated proteins into the gastrointestinal lumen to sustain beneficial bacteria (Pickard et al., 2014). This prevents the growth of harmful strains. Regarding antimicrobial mechanisms, immunoglobulin A and Reg3g are released into the lumen and represent powerful molecules to interfere with microbial proliferation. Commensal flagellin is sensed by toll-like receptor 5 (TLR5) on $CD103^{+}CD11b^{+}$ DCs in the lamina propria, initiating the expression of IL-23 via the TLR-MyD88 mediated signaling cascade. Upon release of IL-23 by DCs, the production of IL-22 in innate lymphoid cells (ILCs) is activated, and its release leads to phosphorylation of STAT3 (Pickert et al., 2009). Finally, Reg3g expression is promoted in intestinal epithelial cells (IEC) and

Paneth cells (Kinnebrew et al., 2010; Kinnebrew et al., 2012a). However, this axis needs to be well regulated, as overexpression of IL-23 can drive chronic immune pathology in the intestine by activating an autocrine feedback loop that serves as local amplifier of cytokines such as TNF-α (Hue et al., 2006). Besides, IL-22 is involved in the constitution of the mucus layer, and its deficiency alters the microbiota and leads to increased susceptibility to experimentally induced colitis (Zenewicz et al., 2013b). Strikingly, CD103 also marks a subset of DCs that incorporate orally administered antigens in the lamina propria. They subsequently migrate to draining mesenteric lymph nodes (MLNs) and present the antigens to naïve T cells. These naïve T cells differentiate to Foxp3$^+$ T regulatory cells in presence of TGF-β1 (Coombes et al., 2007). Furthermore, CD103$^+$ DCs can imprint gut-homing phenotype on CD8$^+$ T cells (Annacker et al., 2005). Since CD103$^+$ DCs do not constitutively produce pro-inflammatory cytokines and are rather associated with the expression of anti-inflammatory IL-10, they are proposed to be the underlying mechanism for the induction of oral tolerance (Chirdo, Millington, Beacock-Sharp, & Mowat, 2005). Therefore, the CD103$^+$ subgroup represents interesting immune cells equipped with both tolerogenic and inflammatory properties. During intestinal homeostasis CD103$^+$ DCs induce the differentiation towards dietary proteins tolerating T cells, whereas inflammation in the gastrointestinal tract and the associated pro-inflammatory milieu favor pathways responding to the environmental cues (Laffont, Siddiqui, & Powrie, 2010). In addition, specialized strains of the microbiota are involved in the development of Foxp3$^+$ T regulatory cells. *Clostridium spp.* belonging to cluster IV and XIVa *Clostridia* are known to induce the release of TGF-β from IEC, increasing the number of regulatory T cells and strengthening resistance to DSS-induced colitis (Atarashi et al., 2011).

A possible explanation for these immunomodulatory characteristics of *Clostridal cluster* IV and XIVa bacteria could be their ability to produce short-chain fatty acids (SCFAs) (Brestoff & Artis, 2013). SCFAs have beneficial effects on the proliferation of colonic regulatory T cells and can even directly influence them through GPCR43, encoded by the FFar2 gene (Smith et al., 2013). GPCR43 is the G-protein-coupled receptor 43 interacting with SCFAs and is part of immunosuppressive pathways, as its deficiency leads to dysregulation of inflammatory responses (Maslowski et al., 2009). Binding of SCFAs inhibits the histone deacetylase (HDAC) and subsequently increases the gene expression and suppressive capacity of regulatory T cells (Smith et al., 2013). Furthermore, SCFAs can affect DCs by conferring a higher potential to modulate differentiation of naïve T cells into regulatory T cells (Becattini et al., 2016). Besides anti-inflammatory properties in the gastrointestinal tract, microbiota-derived SCFAs also promote protective mechanisms in extra-intestinal tissues. As an example, pancreatic β-cells have been found to express cathelicidin-related antimicrobial peptide (CRAMP), which reduces the pro-inflammatory activities of macrophages, while increasing the number of

regulatory macrophages (Sun et al., 2015). Since these endocrine cells express GPCR43, the production of CRAMP in pancreatic islets is controlled by SCFAs (Sun et al., 2015). DCs transport β-cell antigens to draining lymph nodes and present them to autoreactive T cells, contributing to the pathogenesis of autoimmune diabetes (Ganguly, Haak, Sisirak, & Reizis, 2013). However, CRAMP induces regulatory DCs, which have the potential to initiate the differentiation into regulatory T cells, necessary for protection against autoimmune diabetes (Tang & Bluestone, 2008). These hypotheses are confirmed by the fact that non-obese diabetic mice show an impaired expression of CRAMP, and their diabetic disease can be ameliorated by microbial transfer (Sun et al., 2015). In contrast, antibiotic treatment favors diabetogenic T cells and promotes disease progression (Sun et al., 2015). Furthermore, SCFAs are involved in the prevention of allergic inflammation in the lung by suppressing T helper type 2 cells (Trompette et al., 2014). This emphasizes the systemic impacts of microbiota-derived metabolites. Therefore, the gut flora does not only have a restricted effect on the gastrointestinal tract, but plays a major role in the differentiation and regulation of the immune system in various organs, principally through the induction of regulatory T cells to maintain immune tolerance in the host. Regarding intestinal homeostasis, high level of CX_3CR1 expressing cells, sharing characteristics of both dendritic and macrophage cells, are endowed with the ability to transport bacterial antigen to MLNs activating an adaptive immune response. Under normal physiological conditions, the migration of CX_3CR1^{hi} cell is abrogated by a MyD88-dependent mechanism triggered by commensal-derived signals, which remain to be elucidated. Altering the commensal composition of the microbiota by antibiotics impairs this pathway and causes an increase in DCs and augmented bacterial trafficking to immune-priming sites (Diehl et al., 2013). Furthermore, bacteria are able to cross the epithelium through GAPs (Knoop, McDonald, Kulkarni, & Newberry, 2016a). In the presence of an undisturbed gut flora and an intact mucosal barrier, goblet cells (GCs) can sense commensal microbes and subsequently inhibit GAPs and bacterial translocation (Knoop et al., 2016a). This protective detection system is dependent on MyD88, which detects microbial signals via TLR. MyD88 deficiency aggravates the severity of experimentally induced colitis due to increased bacterial translocation (Knoop et al., 2016a). Antibiotic-induced dysbiosis also impairs the sensing abilities of GCs and subsequently renders the epithelium more susceptible to mucosal lesion and inflammation (Knoop et al., 2016a). Therefore, the symbiosis between gut flora and the host is enabled by the TLR-MyD88 signaling pathway, which crucially contributes to immunosuppressive processes to ensure the tolerance of the non-pathogenic communities in the gastrointestinal tract. However, the molecules or peptide patterns that distinguish harmless from deleterious strains are still unknown. Independently from IL-22 and IL-23, group 3 ILCs expressing retinoic-acid-receptor-related orphan receptor-γt (RORγt) are able to restrict adaptive immune responses targeting commensal bacteria (Hepworth et al., 2013). Thereby, ILC3s stabilize the symbiosis between the gut flora and the host. A

deficiency of RORγt$^+$ ILCs in mice results in spontaneous intestinal inflammation created by dysregulated commensal bacteria-dependent CD4$^+$ T cell. The presence of beneficial microbial strains is necessary, as the inflammatory disease can be ameliorated by antibiotic treatment. This emphasizes the immunogenic attributes of non-pathogenic microorganisms (Hepworth et al., 2013; Hepworth et al., 2015). By processing and presenting luminal antigens via MHC II to commensal bacteria-specific CD4$^+$ T cells, ILCs directly induce their apoptotic cell death. In this way, chronic immune activation against the gut flora is prevented. It is hypothesized that this safety mechanism could be impaired in patients suffering from IBD (Hepworth et al., 2015). Since this procedure resembles the negative selection in the thymus, the term intestinal selection has been proposed (Hepworth et al., 2015).

5.5 Animal models for microbiome research

Due to their low maintenance cost, high productive rate and short generation time, murine models have been widely used in scientific research, leading to many discoveries about human biology (Nguyen, Vieira-Silva, Liston, & Raes, 2015). Additionally, Knock-out mice can be easily generated to investigate the effects of impaired signaling pathways and molecules. A further advantage of mice as laboratory animals is their structural similarity in anatomy and physiology to humans. Nevertheless, some distinct morphologic differences between human and mice need to be considered. On a macroscopic level, the surface area of the human small intestine is 400 times bigger compared to the large intestine. In mice, the difference in surface area of the small intestine is only 18 times (Casteleyn, Rekecki, van der Aa, Simoens, & van den Broeck, 2010). Furthermore, the human caecum is small in comparison to its gastrointestinal tract, whereas the murine caecum misses an appendix and is relatively large, emphasizing its great importance in the fermentation of fibers and production of vitamin K and B (Nguyen et al., 2015). These anatomical characteristics underline the different gut compartmentalization between humans and mice, likely affecting the microbial composition in the gut. Furthermore, by creating ecological micro-niches transverse folds along the colon could be influencing the gut flora. In humans, transverse folds are featured along the whole colon, whereas in mice they are only present in the proximal colon and caecum (Nguyen et al., 2015). Thus, the distribution of transverse folds could be a further reason for differing commensal communities between humans and mice. Regarding the histological structure, mice express longer villi than humans. In addition, the distribution of Paneth cells differs between both species and could also explain discrepancies in the microflora. Besides their main abundance in the small intestine, Paneth cells are also localized in the human caecum and the proximal colonic mucosa, whereas they are not

found in the murine colon (Nguyen et al., 2015). When analyzing the gut microbiota, the most abundant phyla in both species are *Bacteroidetes* and *Firmicutes*, but only 15% of the bacterial genera has been identified in the microbiota of both species (Eckburg et al., 2005; Ley et al., 2005). The comparability is limited due to different material acquisition techniques. In mice, the caecum is usually harvested and its content is processed for 16S rRNA sequencing to determine the composition of the murine gut flora. In humans, stool samples are collected as it is practical and more comfortable for the patients. It has been already shown, that stool and mucosa-adherent communities differ from each other (Eckburg et al., 2005; Zoetendal et al., 2002), and could be an explanation for the discrepancy between human and murine results. Since laboratory animals live under strictly controlled conditions, husbandry practices and environmental factors have to be considered as major factors influencing the microbial compositions. To prevent contaminations of the experimental animals with pathogens, they are usually housed in specific-pathogen-free (SPF) conditions in contrast to humans, who are exposed to a vast abundance of microbes. Another factor to be considered is coprophagy. Mice consume fecal matter to obtain additional nutrients leading to a homogenization of gut microbiota between co-housed animals (Zenewicz et al., 2013a). Furthermore, laboratory animals are confined in a restricted space and regularly handled by animal care takers causing a certain amount of stress, which can affect the microbial composition (Ma et al., 2012).

Depending on the aim of the investigation, the choice of the most suitable experimental model is important. For research on one microbe or defined bacterial consortia in the gut, the colonization of germ-free (GF) mice with bacteria is an established method. Thus, any putative functions or impacts after the colonization can be linked to the particular bacterial species. In this way, it was discovered that the monocolonization of GF mice with segmented filamentous bacteria induces TH17 cell responses in the lamina propria of the gastrointestinal tract (Ivanov et al., 2009). However, a huge disadvantage of this model is the missing interaction between the investigated bacterial strains and the whole microbial community. On the other hand, the comparison of conventional and GF mice enables to correlate the microbiota with different physiological processes and demonstrates its involvement in the development of the immune system (Smith, McCoy, & Macpherson, 2007) and in the modulation of gene expression (Hooper, 2001). For instance, a simple modification of this experimental model allowed the research on the dynamics and longevity of immunoglobulin A in reversible microbe colonized GF mice (Hapfelmeier et al., 2010). In this study, to overcome the disturbances created by a continuous presence of the gut flora, a triple mutant *Escherichia coli* strain was utilized to establish a transient intestinal colonization for up to 48 hours. This allowed the exact measurement of the threshold at which the production of immunoglobulin A is activated and to

elucidate the dynamic memory characteristics of immunoglobulin A (Hapfelmeier et al., 2010). Therefore, GF mice colonized with bacteria, unable to proliferate, represent an interesting tool to unravel time-dependent mechanisms and to simulate recurrent infections through repetitive applications. Nevertheless, this model still cannot mirror the complexity of the interaction between the microbiota and the host.

A further widely employed approach is the usage of human microbiota-associated (HMA) mice (Arrieta, Walter, & Finlay, 2016). A human gut microflora sample is inoculated in GF mice combining the advantages of a mouse model and a human-like system. This model provides the possibility to investigate human diet- and disease-associated dysbiosis, which is hypothesized to predispose or contribute to the pathogenesis of various chronic diseases. However, the mammalian microflora is an evolutionary result of a symbiotic coexistence between the host and the commensal bacteria influenced by multiple factors and therefore is unique for every species. Colonization of mice with human bacteria perturbs this delicate homeostasis and leads to inevitable alterations such as an impaired development of adaptive and innate immune cells in the intestine (Arrieta et al., 2016). The deficiency of CD4+ and CD8+ T cells resembles that in observed GF mice (Chung et al., 2012). In addition, the intestinal expression of antimicrobial peptides such as Reg3g and immunoglobulin A is downregulated resulting in a disability to cope with infections efficiently (Chung et al., 2012). Even though the majority of the human microbiota can be established in GF mice (Turnbaugh et al., 2009), the abundance pattern of several bacterial strains is altered and does not represent a beneficial host-specific microbiota anymore, which affects the maturation of the immune system as well as metabolic pathways (Marcobal et al., 2013). Despite these evolutionary and ecological considerations, HMA mice still provide a powerful tool to investigate associations, to assess causality of disease and diet-induced microbial differences. They are even considered to be the gold standard in the gut microbiota research (Nguyen et al., 2015). However, the limitations of this experimental model need to be acknowledged, and the efforts to improve this approach must not be neglected.

Another interesting approach is the treatment of conventional mice with antibiotics prior to fecal microbiota transplantation (FMT) to facilitate the following repopulation of the gastrointestinal tract with exogenous bacterial communities by diminishing the endogenous gut flora. In contrast to HMA mice, this experimental setup circumvents the lack of mature immune system and other alterations observed in GF mice. Second, this model can mimic the chronological recovery of the microbiota after antibiotic intake with and without subsequent FMT, which depicts an approach close to reality. On the contrary, the long-term effects of antibiotics could influence the results due to the selection and favoring of resistant bacteria. However, lowering the bacterial load with antibiotics prior to FMT leads to a combined reshaping effect of the antimicrobial treatment as well as the bacterial

inoculation and not to a complete transplantation of the donor's microbiota (Manichanh et al., 2010). It is hypothesized that the antibiotics are deleterious to both endogenous and implanted commensal bacteria. Therefore, the observed effects could be dependent on the chronological design of the drug administration, albeit this consideration remains to be ascertained. Additionally, this procedure is a suitable method to investigate the course of an antibiotic-altered intestinal ecosystem and the factors that modulate this process.

Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis has been linked to the microbiota and intestinal mucosal integrity. To investigate this complex disorder, over 66 different kinds of animal models have been established and can be classified into four categories: chemically induced, cell-transfer, congenial mutant and genetically engineered models (Mizoguchi, 2012). Each model varies in strength and limitations regarding time of onset, degree of inflammation severity and phenotype. Since many of them only affect the large intestine, thereby resembling symptoms of an ulcerative colitis, they are usually referred to as colitis models (Peloquin & Nguyen, 2013). One common way to mimic IBD is the usage of genetically altered mice, where a gene involved in the preservation of the mucosal barrier function is knocked out. However, these genetically modified models are not capable of representing the whole pathophysiology observed in humans and can only represent a subset of IBD patients, who carry a genetic defect (Nguyen et al., 2015). The anti-inflammatory IL-10 is one of the earliest identified cytokine involved in the maintenance of the epithelial immune system, since administration of anti-IL-10 receptor antibodies can lead to colonic inflammation (Kiesler, Fuss, & Strober, 2015). However, for the development of colitis the stimulating presence of microbial communities is crucial, as IL-10 deficient mice do not suffer from colitis in a germ-free environment (Kiesler et al., 2015). Furthermore, an attenuated progression of colitis can be observed after antibiotic treatment of IL-10 knock-out mice. This underlines the importance of the interactions between an altered epithelial immune system and the microbial milieu (Madsen et al., 2000). In addition, the correct choice of the laboratory mice strains is essential, as the genetic background determines the resilience or predisposition to intestinal inflammation. In comparison to the resilient C57BL/6 strain, the 129/SvEv strain confers susceptibility to IL-10 deletion, leading to an earlier occurrence of colitis (Berg et al., 1996).

Other methods to initiate colonic inflammation include the application of chemical agents due to their simple manageability and possibility to challenge a predisposed experimental model at any time. For instance, dextran sulfate sodium (DSS) added to the drinking water of laboratory animals causes a disruption of epithelial integrity and leads to subsequent entry of luminal bacteria into the host's tissue (Kiesler et al., 2015). This toxic effect on the colonic mucosa induces a reproducible acute inflammation allowing the investigation of mechanisms that regulate the restoration of the

mucosal barrier function during and after the chemical injury. Hence it was discovered that the TLR4/MyD88 signaling pathway is essential for neutrophil recruitment and limitation of bacterial translocation (Fukata et al., 2005). Furthermore, MyD88 signaling in macrophages facilitates epithelial cell renewal (Pull, Doherty, Mills, Gordon, & Stappenbeck, 2005), indicating a strong activation of the innate immune system. Strikingly, GF mice suffer more severe DSS-induced colitis compared to conventional mice (KITAJIMA, MORIMOTO, & SAGARA, 2002). Therefore, it is questionable whether the underlying mechanism of the increased bacterial translocation can solely explain this phenomenon. In contrast to DSS, 2,4,6-trinitrobenzenesulfonic acid (TNBS) needs to be intrarectally administered and renders colonic proteins immunogenic to the host immune system, inducing a transmural colitis driven by T_H1-mediated inflammatory cytokines. Because affected mice show similar characteristics as humans suffering from Crohn's disease, TNBS serves as an important source of information about factors that modulate the progression of this disease (Kiesler et al., 2015). TNBS-induced colitis is associated with an increased expression of INFγ (Kiesler et al., 2015). This finding paved the way for the development of a monoclonal anti-IL-12 antibody, which proved its efficacy in controlling active Crohn's disease in clinical trials (Mannon et al., 2004; Sandborn et al., 2008). Similar to TNBS, Oxazolone operates via haptenating mechanisms. Its induced phenotype differs from that caused by TNBS, since it resembles many aspects of ulcerative colitis (Kiesler et al., 2015). Oxozolone-induced colitis stimulates the production of IL-13 originating from CD4+ NKT cells (Kiesler et al., 2015). Remarkably, the administration of different agents to initiate intestinal inflammation activates various pathways of the immune system. Therefore, every experimental model has its own strengths and limitations and can only mirror one aspect of the human complexity.

5.6 Aim of the project

Antibiotic administration has been shown to cause long-lasting alterations in the gut flora and is associated with the development of irritable bowel syndrome (IBS). Even though the pathogenesis of this chronic illness is multifactorial, we aim to elucidate the underlying mechanisms by investigating the impact of antibiotic-induced dysbiosis on the intestinal microbiota and host response, especially on Reg3 expression.

Specific objectives:

I. Antibiotic effects on epithelial immune responses and mucus formation.

   The impact of different antibiotic treatments on Reg3 expression and the function of GCs is measured in correlation to the mucus layer thickness.

II. Effect of IL-22 and MyD88-pathways in intestinal Reg3b regulation.

   To confirm previous studies showing that IL-22 is involved in the regulation of Reg3, Reg3 expression is measured after intraperitoneal application of IL-22. The involvement of MyD88 in the MyD88 – Reg3b axis is evaluated by assessing Reg3b expression profile of MyD88 Knock-out mice.

III. Microbiota-dependency of Reg3b response.

   We established FMT experiments to assess the impacts on Reg3b expression by using AMT-pretreated mice or mice of C57BL/6 and BALB/c strain.

IV. Effects of Reg3b deficiency on experimental colitis.

   The impact of impaired Reg3b regulation after antibiotic intake on the development of gastrointestinal inflammation is evaluated by challenging antibiotic treated mice with DSS.

V. Regulation of Reg3b

   Oral administration of Reg3b aims to disclose whether Reg3b is regulated in a feed-forward loop. Furthermore, oral administration of butyrate as one of the most important SCFA aims to test to which extend the anti-inflammatory property of butyrate modulates the release of Reg3b.

6 Material and Methods

6.1 Materials

6.1.1 Animals

8-10 weeks old male C57BL/6JOlaHsd and BALB/cOlaHsd (Harlan Winkelmann, Borchen, Germany, body weights from 22 to 28 g) were used in the present work.

MyD88 knockout mice (C57BL/6JOlaHsd background, bred in-house, MIH) were kindly provided by Dr. Thorsten Buch.

All mice were housed in groups of 3 mice per cage under SPF-conditions with unlimited access to water and food. The animals were kept on a 12 h light-dark cycle at room temperature of 22 ± 5 °C.

6.1.2 Reagents for tissue fixation

Acetic acid (glacial)	Merck KGaA	Germany
Chloroform	Carl Roth GmbH + Co. KG	Germany
Formaldehyde	AppliChem GmbH	Germany
Methanol	Merck KGaA	Germany

6.1.3 Reagents for immunofluorescence and histology

Citric acid	Carl Roth GmbH + Co. KG	Germany
DAPI	Thermo Fischer Scientific	USA
Disodium hydrogen phosphate	Carl Roth GmbH + Co. KG	Germany
DPX mounting media	VWR International GmbH	USA
Eosin (1%, watery)	Morphisto GmbH	Germany
Ethanol absolute	Pharmacy of MRI of TU Munich	Germany
Fetal bovine serum (pH 5)	AppliChem GmbH	Germany
Hemalum solution acid according to Mayer	Carl Roth GmbH + Co. KG	Germany
PAP pen	Vector Laboratories Inc.	USA
PBS	137 mM NaCl; 2,7 mM KCL; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4 (pH 7,4)	

Parafilm M	Parafilm	USA
Potassium chloride	Merck KGaA	Germany
Potassium dihydrogen phosphate	Carl Roth GmbH + Co. KG	Germany
Roticlear	Carl Roth GmbH + Co. KG	Germany
Sodium chloride	Carl Roth GmbH + Co. KG	Germany
Vectashield mounting media for Fluorescence H-1000	Vector Laboratories Inc.	USA

6.1.3.1 Primary antibody for immunofluorescence

Target	Species	Dilution	Company
MUC-2	Rabbit	1:100	Santa Cruz Biotechnology

6.1.3.2 Secondary antibody for immunofluorescence

Species	Dilution	Company
Anti-rabbit (IgG)	1:100	abcam

6.1.4 Reagents for quantitative real-time PCR and Gut Low-Density Array

Bio-Rad C1000 Touch™ Thermal Cycler	Bio-Rad Laboratories GmbH	Germany
DNA-free™ DNA Removal Kit	Thermo Fischer Scientific	USA
DNAse I Buffer	Thermo Fischer Scientific	USA
DNase Inactivation Reagent	Thermo Fischer Scientific	USA
dNTP	Promega	USA
FrameStar® 384-well PCR plate	4titude Ltd	UK
GenElute™ Mammalian Total RNA Miniprep Kit	Sigma-Aldrich	USA
MicroAmp™ optical adhesive film	Applied Biosystem	USA
NanoDrop 1000	Thermo Fischer Scientific	USA
Nuclease-free water	Promega	USA

QIAamp DNA Stool Mini Kit	Quiagen	Netherlands
Random Primers	Promega	USA
rDNase I	Thermo Fischer Scientific	USA
Reverse transcriptase 5x buffer	Promega	USA
Reverse transcriptase RNase	Promega	USA
RNAeasy® Mini Kit	Quiagen	Netherlands
SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad Laboratories, Inc.	USA

6.1.5 Primers for quantitative real-time PCR

mCrypt-4	Forward	GCTGTGTCTATCTCCTTTGGAGG
	Reverse	CGTATTCCACAAGTCCCACGAAC
mCXCL1	Forward	TGCACCCAAACCGAAGTCAT
	Reverse	TTGTCAGAAGCCAGCGTTCAC
mGAPDH	Forward	GCCTTCTCCATGGTGGTGAA
	Reverse	GCACAGTCAAGGCCGAGAAT
mIFNγ	Forward	TCAAGTGGCATAGATGTGGAAGAA
	Reverse	TGGCTCTGCAGGATTTTCARG
mIL-10	Forward	CTAGAGCTGCGGACTGCCTTC
	Reverse	CCTGCTCCACTGCCTTGCTCTTAT
mIL-17	Forward	GCTCCAGAAGGCCCTCAGA
	Reverse	AGCTTTCCCTCCGCATTGA
mIL-1β	Forward	CAACCAACAAGTGATATTCTCCATG
	Reverse	GATCCACACTCTCCAGCTGCA
mIL-2	Forward	GACACTTGTGCTCCTTGTCA
	Reverse	TCAATTCTGTGGCCTGCTTG
mIL-22	Forward	CGCTGCCCGTCAACACCCGG

	Reverse	CTGATCTTTAGCACTGACTCCTCG
mIL-23	Forward	AGCGGGACATATGAATCTACTAAGAGA
	Reverse	GTCCTAGTAGGGAGGTGTGAAGTT
mIL-6	Forward	AGTTGCCTTCTTGGGACTGA
	Reverse	CAGAATTGCCATTGCACAAC
mLysozyme	Forward	GATGGCTACCGTGGTGT
	Reverse	CACCCATGCTCGAATG
mMCP1	Forward	GCCTGCTGTTCACAGTTGC
	Reverse	TGTATGTCTGGACCCATTCCT
mReg3b	Forward	TCCCAGGCTTATGGCTCCTA
	Reverse	GCAGGCCAGTTCTGCATCA
mReg3g	Forward	TTCCTGTCCTCCATGATCAAAA
	Reverse	CATCCACCTCTGTTGGGTTCA
mTNFα	Forward	CGATGGGTTGTACCTTGTC
	Reverse	CGGACTCCGCAAAGTCTAAG

6.1.6 Primers for Gut Low-Density Array

Akkermansia	Forward	CAGCACGTGAAGGTGGGGAC
	Reverse	CCTTGCGGTTGGCTTCAGAT
Alistipes spp.	Forward	TTAGAGATGGGCATGCGTTGT
	Reverse	TGAATCCTCCGTATT
Bacteroidetes [universal]	Forward	GGARCATGTGGTTTAATTCGATGAT
	Reverse	AGCTGACGACAACCATGCAG
Bacteroidetes spp.	Forward	CGATGGATAGGGGTTCTGAGAGGA
	Reverse	GCTGGCACGGAGTTAGCCGA
Bifidobacterium spp.	Forward	GCGTGCTTAACACATGCAAGTC

	Reverse	CACCCGTTTCCAGGAGCTATT	
Clostridia cluster IV	Forward	GCACAAGCAGTGGAGT	
	Reverse	CTTCCTCCGTTTTGTCAA	
Clostridia cluster XIVa	Forward	AAATGACGGTACCTGACTAA	
	Reverse	CTTTGAGTTTCATTCTTGCGAA	
Desulfobrivio spp.	Forward	CCGTAGATATCTGGAGGAACATCAG	
	Reverse	ACATCTAGCATCCATCGTTTACAGC	
E. coli spp.	Forward	CATGCCGCGTGTATGAAGAA	
	Reverse	CGGGTAACGTCAATGAGCAAA	
Enterobacteriaceae	Forward	CATTGACGTTACCCGCAGAAGAAGC	
	Reverse	CTCTACGAGACTCAAGCTTGC	
Enterococcus spp.	Forward	CCCTTATTGTTAGTTGCCATCATT	
	Reverse	ACTCGTTGTACTTCCCATTGT	
Firmicutes [universal]	Forward	TGAAACTYAAAGGAATTGACG	
	Reverse	ACCATGCACCACCTGTC	
Lactobacillus spp.	Forward	AGCAGTAGGGAATCTTCCA	
	Reverse	CACCGCTACACATGGAG	
Methanobrevibacter	Forward	CCGGGTATCTAATCCGGTTC	
	Reverse	CTCCCAGGGTAGAGGTGAAA	
Prevotella spp.	Forward	CACCAAGGCGACGATCA	
	Reverse	GGATAACGCCYGGACCT	Y = C or T
Universal	Forward	ACTCCTACGGGAGGCAGCAGT	
	Reverse	GTATTACCGCGGCTGCTGGCAC	

6.1.7 Reagents for ELISA

Mouse Regenerating islet derived protein 3 beta ELISA Kit	abbexa	UK
NanoDrop 1000	Thermo Fischer Scientific	USA
Pierce® BCA Protein Assay Kit	Thermo Fischer Scientific	USA
RIPA buffer	50 mM Tris-HCL; 1% NP-40; 150 mM NaCl; 0,25% DOC; 1mM EGTA; ph 7,4; 1 dissolved cOmplete™ Protease Inhibitor Cocktail (Roche) per 10 ml Buffer	

6.1.8 Microscope and camera

AxiocamMRm	Zeiss	Germany
LEICA DMRBE immunofluorescence microscope	Leica	Germany

6.1.9 Software

Adobe Illustrator	Adobe Systems	USA
Axio vision imaging software 4.8	Zeiss	Germany
Bio-Rad CFX Manager software	Bio-Rad Laboratories, Inc.	USA
GraphPad Prism 5	Graphpad Software, Inc.	USA
NIH ImageJ Software	Public domain	

6.1.10 Reagents for experimental treatment

DSS	MP Biomedicals	USA
PBS	137 mM NaCl; 2,7 mM KCL; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4 (pH 7,4)	
Recombinant murine IL-22	R&D Systems	USA

6.1.11 Antibiotic treatment

AMT	Metronidazole (1 g/l) Ampicillin (1 g/l) Vancomycin (0,5 g/l) Neomycinsulfate (1 g/l)
RFX	Rifaximin (150 mg/l)

6.2 Methods

6.2.1 Tissue collection and processing

Mice were narcotized with isoflurane and subsequently sacrificed by cervical dislocation. Caecum and its stool content were harvested and immediately frozen at -80 °C for following RNA extraction. Colon, ileum and/or stomach were extracted and immersed in different tissue fixation solution. For MUC2-immunohistochemistry, a solution containing 60% methanol, 30% chloroform and 10% glacial acetic acid was used as fixation solution (Johansson et al., 2008). A 4% formalin solution was used for other stainings. The tissue was fixated for up to 96 h at 4 °C. Afterwards, a standard dehydration procedure was performed followed by paraffin embedding. Subsequently, each organ was sectioned into 4 µm thin sections.

6.2.2 Immunofluorescence und histology

6.2.2.1 MUC2 immunostaining

Immunostaining was performed as described by Johansson and Hansson (Johansson et al., 2008). Sections were incubated at 60 °C for 10 min and then incubated in a 60 °C pre-warmed Roticlear solution followed by incubation in a Roticlear solution at room temperature for 10 min each time. The following hydration was performed in decreasing concentration of ethanol (100%, 95%, 70%, 50%, 30%) with incubation time of 5 min each. In the next step, the samples were immersed in an antigen retrieval solution (10 mM citric acid, pH 6) and placed in a microwave oven (300 W, 5 min). This procedure was repeated two more times. After allowing the sections to cool down in the solution for 20 minutes, a brief washing step in PBS was performed. Blocking solution (5% fetal bovine serum in PBS) was applied and samples were incubated in a dark and humid chamber at room temperature for 30 min. For mucus visualization, a MUC2-specific antibody, diluted 1:100 in blocking solution was added to the samples and incubated at 4 °C overnight in dark. Samples were washed 3

times with PBS for 10 min (in dark). Secondary antibody (Alexa 488, anti-rabbit IgG, 1:100 dilution) was applied together with DAPI (1:100 dilution) to the sections and incubated in a dark and humid chamber for 2 h. After PBS washing (three times, 10 min each, in a dark environment), sections were cover-slipped with Vectashield mounting media (H-1000).

6.2.2.2 Immunofluorescence image acquisition and analysis

All images were acquired with a LEICA DMRBE immunofluorescence microscope using a 40-fold magnification lens, the AxioCamMRm and Axio vision imaging software 4.8.

To determine the width of the inner mucus layer, the arithmetic mean was calculated from 16 measurements taken of 4 fields per section. Thereby, the 4 thickest areas for mucus width evenly spread over the field were chosen and measured by means of the NIH ImageJ software.

The amount of goblet cells per crypt was determined by averaging the number of goblet cells in a crypt counted in 4 different areas of the slide in high power field at 40-fold magnification.

6.2.2.3 Hematoxylin and eosin staining

For dewaxing the slides, they were incubated at 60 °C for 20 min and then immersed in Roticlear solution three times for 10 min. Subsequently, samples were incubated in absolute ethanol two times for 10 min, followed by a decreasing concentration of ethanol (90%, 70%, 50%) for 5 min each. After a 5-min immersion in ddH_2O, sections were dyed with hematoxylin solution (Hemalum solution acid according to Mayer) for 6 min. Then, the slides were washed in tap water. In the next step, samples were immersed in eosin (1% watery) for 6 min. Afterwards, dehydrating in 80% ethanol for 1 min and twice in absolute ethanol for 5 min was performed. Thereafter, the samples were incubated in Roticlear solution three times for 5 min. In a final step, DPX mounting media was applied to the slides.

6.2.2.4 Hematoxylin and eosin image acquisition and analysis

All images were acquired with a LEICA DMRBE microscope using a 20-fold magnification lens, the AxioCamMRm and Axio vision imaging software 4.8.

Assessment of sections was performed after blinded randomization. The number of lymphocytes per section was determined by averaging the number of lymphocytes counted in 3 different areas per slide in high power field at 20-fold magnification.

Histology score was based on three factors as previously described by Knoop et al. (Knoop, McDonald, Kulkarni, & Newberry, 2016b):

1. Infiltration with lymphocytes
2. Amount of edema
3. Are of ulceration

Assessment of sections was performed after blinded randomization. First, 5 random pictures per slide were acquired and the extent of lymphocytes infiltration and edema were evaluated to calculate an average score. This score and the ulceration score considering the ulceration area of the whole slide were added to a cumulative score between 0 and 9.

6.2.3 Quantitative real-time PCR and Gut Low-Density Array

6.2.3.1 RNA extraction

For RNA extraction either GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) or RNeasy® Mini Kit (Quiagen) was used according to the kit's manual. 30-50 mg of caecal tissue were used for RNA extraction. To survey quality of extracted RNA, its concentration was measured with NanoDrop 1000.

6.2.3.2 DNA digestion

First, 5 µl 10X DNAse I buffer and 1 µl rDNAse inactivation reagent of the DNA-free™ DNA Removal Kit (Thermo Fischer Sientific) were applied to the RNA samples, mixed gently and then incubated at 37 °C for 20 min. Subsequently, 5 µl suspended DNase inactivation reagent (Thermo Fischer Scientific) was added followed by a 2-min incubation at room temperature. After a 90-sec centrifugation step at 10 000 x g, RNA was transferred to fresh tubes and stored at -80 °C. Before storage, the RNA concentration was again measured to determine quality with the NanoDrop 1000.

6.2.3.3 Reverse transcription

Extracted RNA was diluted with 1 µl of random primers (150 ng/µl) and nuclease-free water to reach a 15 µl probe with a total RNA content between 750 and 1000 ng depending on the extracted yield. Samples were incubated at 70 °C and then cooled down on ice for 5 min each. For retro transcription into cDNA, 11 µl solution containing 5 µl reverse transcriptase buffer (5x), 1,25 µl dNTP mix (10 mM), 1 µl reverse transcriptase and 3,75 µl nuclease-free water were prepared and applied to each sample. In the next step, probes were incubated at room temperature for 10 min, then at 50 °C for 50 min and finally at 70 °C for 15 min. The concentration of cDNA was also measured by the NanoDrop device. Samples were stored at -20 °C until further use.

6.2.3.4 Quantitative real-time PCR protocol

Samples were diluted with nuclease-free water to reach a cDNA concentration of 200 ng per µl. 6 µl of each cDNA sampe were taken and pipetted into a FrameStar® 384-well PCR plate together with 5 µl SsoAdvanced™ Universal SYBR® Green Supermix, 0,5 µl forward and 0,5 µl corresponding reverse primer (10µM) of the desired target. Subsequently, the PCR plate was sealed with MicroAmp™ optical adhesive film and centrifuged at 300 rpm for 1 min. The qPCR was done with a Bio-Rad C1000 Touch™ Thermal Cycler using an amplification program with the following steps:

Initial denaturation	95 °C	3 min	
Denaturation	95 °C	15 s	40x
Annealing	60 °C	45 s	
Melting curve	65-95 °C	0,5 °C/5 s	

6.2.3.5 Fecal DNA Extraction

Fecal DNA was obtained from stool samples by using QIAamp DNA Stool Mini Kit (Quiagen) according to the manufacturer's manual. DNA concentrations were measured with the NanoDrop device.

6.2.3.6 GULDA protocol

For characterization of microbial 16S rRNA, samples were diluted to a DNA concentration of 10 ng/µl and were analyzed according to targeted 16S rRNA qPCR sequencing according to a protocol of Bergström et al. (Bergstrom et al., 2012). Following steps were used as amplification program:

Step 1	50 °C	2 min	
Step 2	95 °C	10 min	
Step 3	95 °C	15 s	40x
Step 4	60 °C	1 min	
Step 5	95 °C	15 s	
Step 6	60 °C	15 s	
Step 7	60-95 °C	2% ramp rate	

6.2.3.7 Quantitative real-time PCR analysis

The qPCR raw data were analyzed with the Bio-Rad CFX Manager software. Subsequently, data were exported into excel and analyzed using the ΔΔCq method. The mRNA levels were normalized to GAPDH.

6.2.4 ELISA

6.2.4.1 Protein Extraction

Caecum´s content weighting between 30 and 80 mg were harvested and diluted with 10 µl RIPA buffer per 1 mg fecal matter. Subsequently, the solution was mixed thoroughly and centrifuged at 10000 rpm for 10 min at 4 °C. 10 µl of the supernatant were diluted with 90 µl ddH_2O and used for determination of protein concentration. Total protein concentrations in the extract were assessed using Pierce® BCA Protein Assay Kit according to the manufacturer's manual. Protein was stored at -80 °C overnight and served as samples for ELISA the next day.

6.2.4.2 ELISA protocol

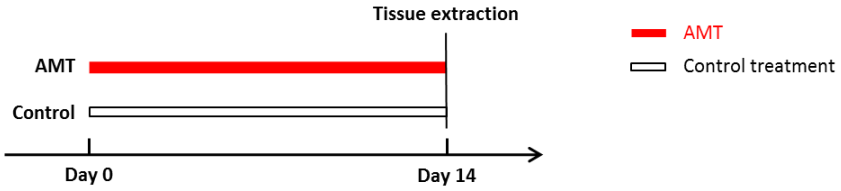
For determination of Reg3b concentration in caecal feces the mouse regenerating islet derived protein 3 beta ELISA Kit (abbexa) was used according to the manufacturer`s manual.

6.2.5 Statistics

All statistical calculations were done using GraphPad Prism 5 software. All data were analyzed by Kolmogorov-Smirnov test to check for a Gaussian distribution. Normally distributed data were analyzed by unpaired t-tests for 2-group comparisons or by one-way ANOVA with consecutive Bonferroni test for 3 or more group comparisons. Non-normally distributed data were analyzed by nonparametric Mann-Whitney test for 2-group comparison or by Kruskal-Wallis test with consecutive Dunns test for 3 or more group comparisons. Results with p-value < 0.05 were considered as significant.

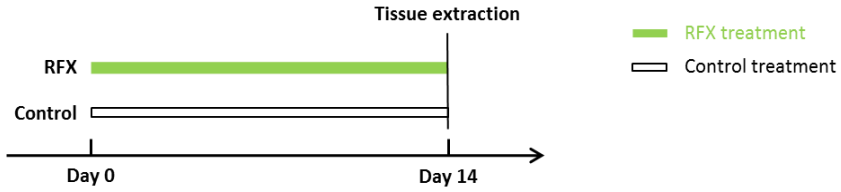
6.3 Animal experiments

6.3.1 Experiment 1: Effect of antimicrobial treatment (AMT)



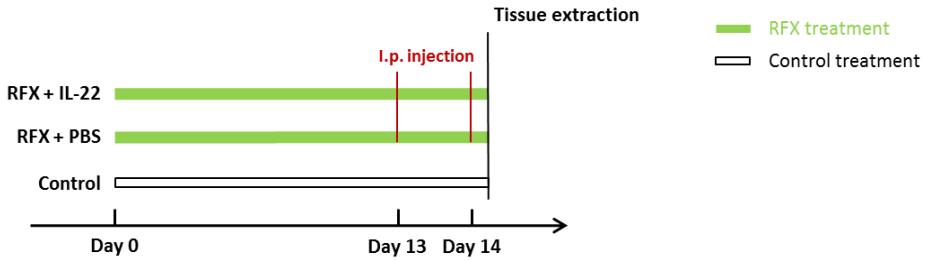
Mice were divided into two cohorts of 6 mice each and were treated from day 0 to 14 with a cocktail of different antibiotics (AMT: Metronidazole 1 g/l, ampicillin 1 g/l, vancomycin 0,5 g/l and neomycinsulfate 1 g/l) or tap water (control treatment). At day 14 rodents were sacrificed.

6.3.2 Experiment 2: Effect of Rifaximin treatment



Mice were divided into a control group receiving tap water and one rifaximin (RFX: Rifaximin 150 mg/l) treatment group (6 mice per group). Each group was treated orally for 14 days. At day 14 rodents were sacrificed.

6.3.3 Experiment 3: IL-22 treatment

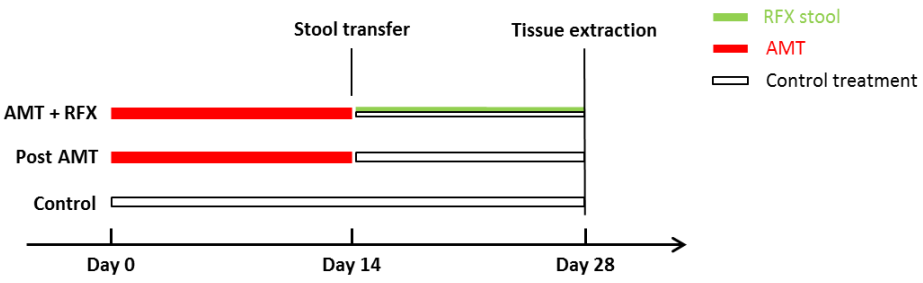


In an initial treatment phase, control mice received tap water, whereas two other groups of mice received oral RFX for 14 days. Afterwards, one RFX group received intraperitoneal (i.p.) injection of recombinant mIL-22, whereas the other group was i.p.-treated with PBS. Intraperitoneal injections were performed for administration of recombinant mouse IL-22 at a dose of 1 µg, diluted in 0,3 ml PBS, per mouse. Injections were done at the last two days at an interval of 24 h. At day 14 laboratory animals were sacrificed three hours after last intraperitoneal injection.

6.3.4 Experiment 4: MyD88 knockout

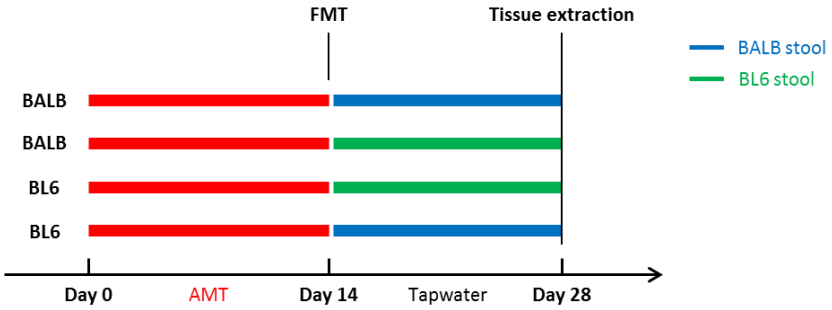
We compared Reg3 and IL-22 expression in the cecum of naïve wild-type mice (C57BL/6 background) with MyD88 knockout mice (C57BL/6JOlaHsd background, bred in-house, MIH, kindly provided by Dr. Thorsten Buch). Mice were between 6 and 10 weeks old when killed.

6.3.5 Experiment 5: Rifaximin-treated fecal microbial transplantation



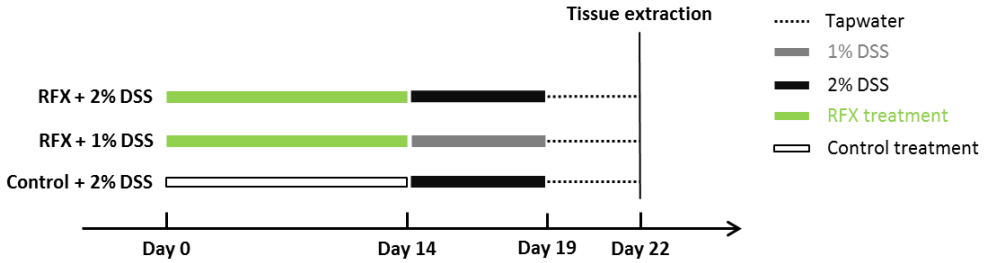
Mice were divided into three cohorts with 6 mice per group. For a period of 14 days, two groups received an antimicrobial treatment to reduce endogenous gastrointestinal microbiota. Meanwhile, the control group had only access to tap water and autoclaved food. At day 14, caecal contents of RFX-pretreated mice (3 mice; treated with RFX for 10 days, pooled caecal contents according to a protocol from Ridaura et al.(Ridaura et al., 2013a)) were transferred to one of the interventional group via a one-time oral gavage. From day 14 to 24, all groups received the same diet and tap water. At day 24 mice were sacrificed. Furthermore, stool within the caecum was utilized for ELISA procedure and 16S rRNA microbiome sequencing.

6.3.6 Experiment 6: Effect of stool transfer between BL6 and BALB mouse strains



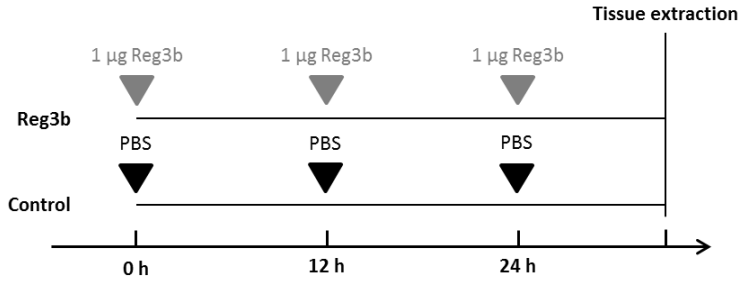
Two different mouse strains (C57BL6/JOlaHsd [BL6] and BALB/cOlaHsd [BALB]) were used. All rodents received an AMT treatment to diminish endogenous microbiota for 2 weeks. Subsequently, caecal contents of untreated BL6 and BALB mice were transferred to the animals via oral gavage (same transfer protocol as in experiment 5). Thereby, half of the mice of each strain (6 animals per group) received caecal material from the other strain (allogenic transfer), the other mice (5 animals per group) from the same strain (autologous transfer). To facilitate the colonization of the intestines rodents had free access to normal laboratory animal diet (autoclaved food) and tap water for further 14 days. Afterwards, animals were sacrificed.

6.3.7 Experiment 7: DSS colitis



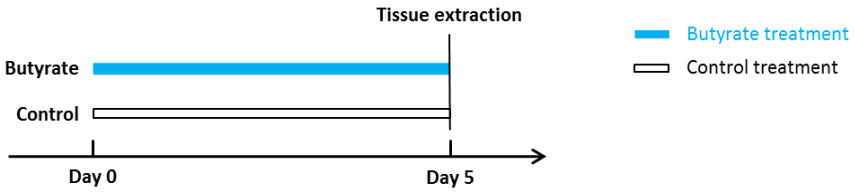
Mice were divided into three cohorts with 6 mice per group. Two groups were initially treated with RFX for 14 days, while controls received only tap water. Thereafter, 1% or 2% dextran sodium sulfate (DSS) was added to the accessible water to induce a chemical colitis. After a period of 5 days, DSS treatment was stopped and replaced with tap water. During the experiment animals' weights were assessed every day. At day 22, rodents were sacrificed.

6.3.8 Experiment 8: Effect of Reg3b treatment



This experiment was performed with one control group receiving PBS and one group receiving a rmReg3b treatment (6 mice per group). The substance was administered three times at an interval of 12 h by oral gavage. The interventional group received 1 µg Reg3b per mouse. 3 h after the last treatment mice were sacrificed.

6.3.9 Experiment 9: Effect of Butyrate treatment



Mice were divided into a control group receiving tap water and one butyrate treatment group (6 mice per group). One group was treated orally with 10 mg butyrate per kg body weight each day for 5 days. At day 5 rodents were sacrificed.

7 Results

7.1 Antimicrobial treatment reduces mucus layer thickness and Reg3 expression

MUC2 is the main component of the mucus layer separating colonic tissue from luminal bacteria. We investigated to which extend the mucus layer is affected by administration of antibiotics. Thus, mice received either AMT (metronidazole 1 g/l, ampicillin 1 g/l, vancomycin 0,5 g/l, neomycinsulfate 1 g/l) or RFX (150 mg/l). RFX was used, since it has been reported to have anti-inflammatory effects by inhibiting bacterial adherence to epithelial cells and bacterial translocation without effecting the bacterial count (Dupont, 2015). Since MUC2 is secreted by goblet cells, a possible correlation between mucus layer thickness and goblet cell count was determined. Furthermore, a possible link between the mucus layer and epithelial immune response in the caecum was analyzed. In contrast to other studies, the caecum was selected instead of the ileum, because the Reg3b secretion into the caecal feces was compared to the Reg3b expression in the caecum. In addition, 16S rRNA analysis was performed on caecal feces. Therefore, the expression profile in the caecum was analyzed in every experiment to ensure comparability. The mRNA levels of Reg3 defensins were measured in correlation with the expression of IL-22 and IL-23 to detect any modulations on the release of Reg3. To check for unimpaired functionality of the innate immune response, mRNA levels of cryptdin 4 (Crypt-4) were analyzed, since it is a member of the α-defensin family that has been reported to be expressed in the caecum (Karlsson et al., 2008). Additionally, the expression of lysozyme, an antimicrobial peptide, was measured, which is expressed in the colonic tract (Wang et al., 2011).

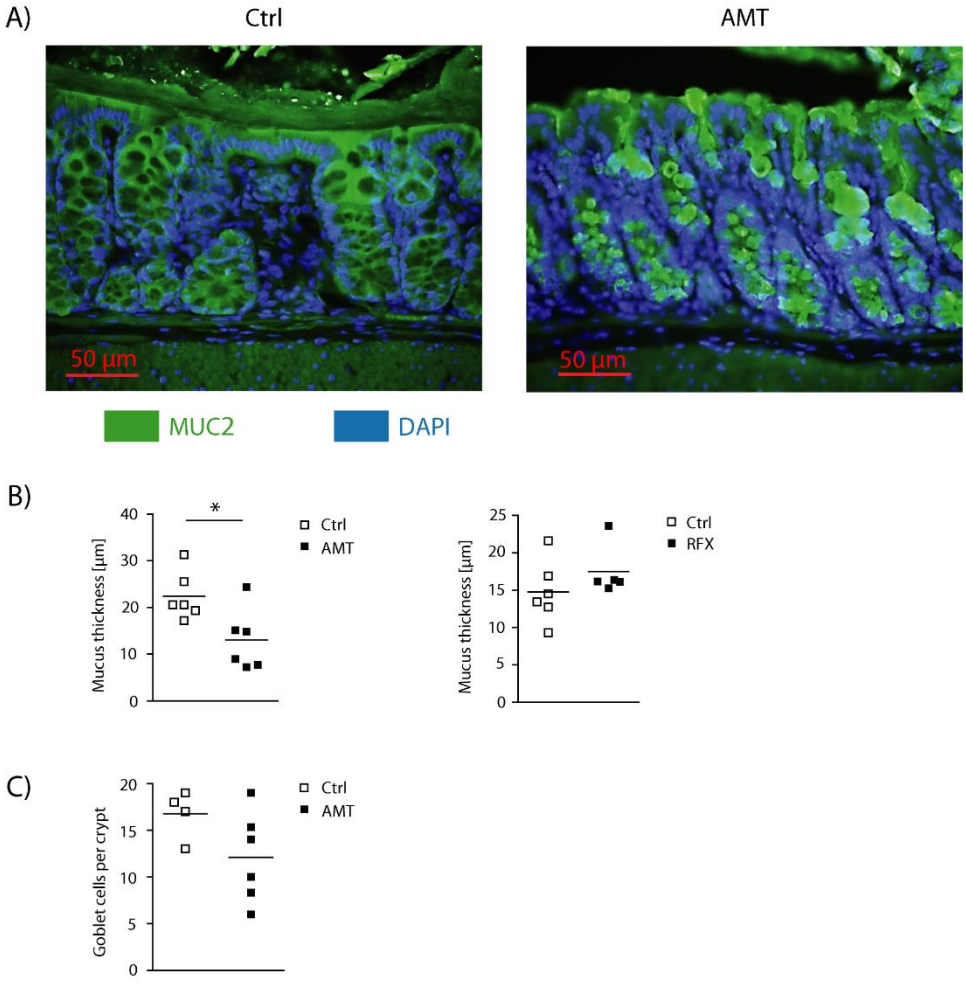


Figure 2 - Antibiotic therapy influences mucus formation and epithelial goblet cells.

A) Representative images of MUC2 immunostaining of colon sections. **B)** Quantification of colonic mucus layer thickness of AMT and RFX-treated mice. **C) G**oblet cell count per crypt following AMT. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test. Horizontal lines represent medians; *p<0.05; each dot represents one mouse.

Immunostaining for MUC2 in colonic sections revealed that the colon of AMT-treated mice showed a significantly thinner mucus layer compared to the control cohort (Figure 2A, 2B). Additionally, a trend towards a lower count of goblet cells in the colonic crypts could be observed (Figure 2C). Due to technical errors, the goblet cells of two control mice and the mucus thickness of one sample of the RFX group could not be determined. Treatment with a different antibiotic, RFX, did not affect thickness of the mucus layer (Figure 2B).

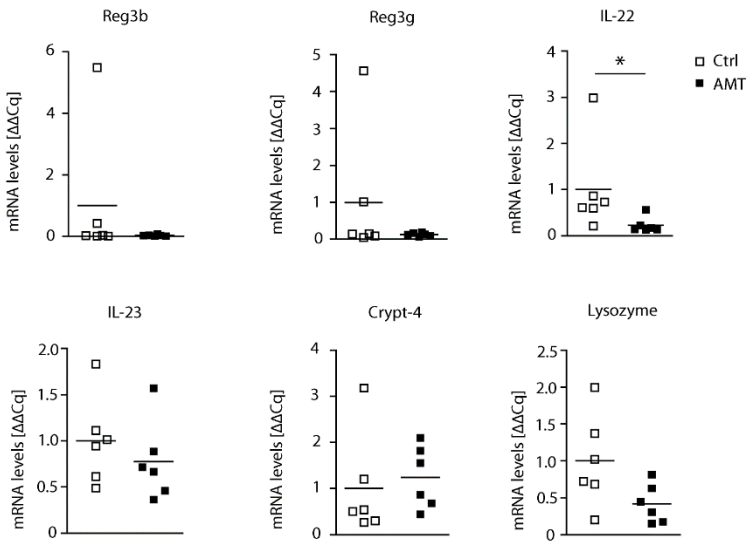


Figure 3 - Suppression of epithelial immune response is associated with antimicrobial treatment.

Quantitative PCR analysis of Reg3b, Reg3g, IL-22, IL-23, Crypt-4 and lysozyme mRNA levels in caecum of AMT-treated mice. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test. Horizontal lines represent medians; *p<0.05; each dot represents one mouse.

Concerning innate defensins, quantitative PCR analysis revealed reduced levels of Reg3b expression in the caecum after AMT (Figure 3). Furthermore, AMT treatment led to lower expression levels of Reg3g and reduced IL-22 mRNA levels. A tendency towards a downregulation of lysozyme was also observed in the AMT group. IL-23 and Crypt-4 expression levels were similar in both groups.

RFX did not appear to influence the mucus layer thickness (Figure 2B), therefore, we examined whether a similar trend could be observed in the defensin and cytokine expression profile. RFX treatment led to reduced Reg3b and Reg3g mRNA levels (Figure 4). In contrast, RFX treatment did not seem to affect Crypt-4 expression. No significant difference could be detected in IL-22, IL-23, Crypt-4 and lysozyme mRNA expression. In summary, although RFX did not affect the mucus layer thickness, Reg3b and Reg3g expression were downregulated.

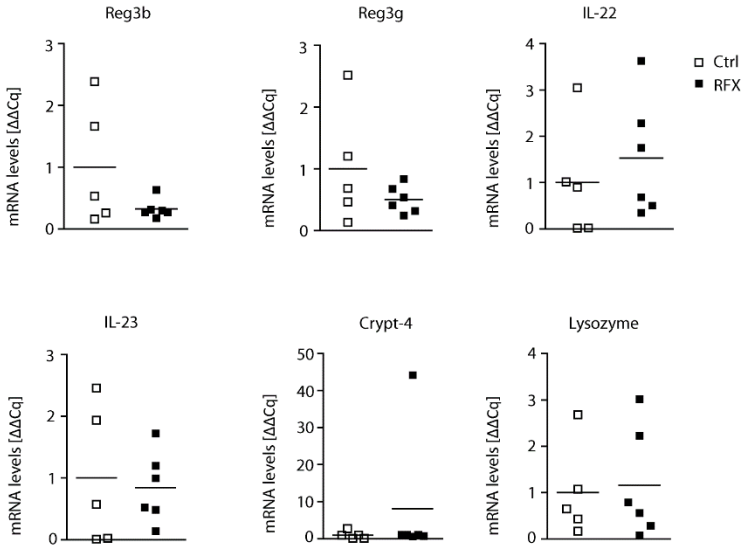


Figure 4 - RFX-treatment downregulates the expression of Reg3b and Reg3g in the caecum.

Quantitative PCR analysis of Reg3b, Reg3g, IL-22, IL-23, Crypt-4 and lysozyme mRNA levels in caecum of RFX-treated mice. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test. Horizontal lines represent medians; each dot represents one mouse.

7.2 IL-22 treatment restores Reg3 expression

Because RFX treatment downregulated Reg3 mRNA expression without affecting the mucus layer, we hypothesized that RFX could selectively impair the IL-22 – Reg3 axis in enterocytes, which regulates Reg3 expression. Therefore, mice were treated with IL-22 to check whether Reg3 expression can be restored after RFX treatment.

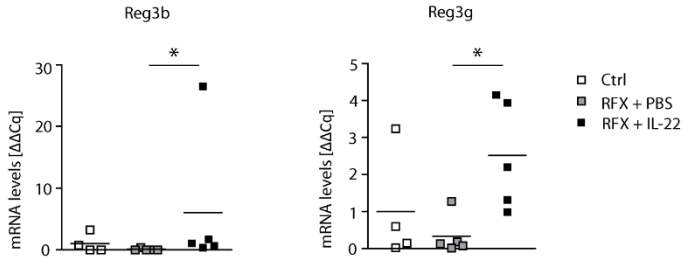


Figure 5 - Intraperitoneal application of IL-22 reverses suppressive effect of RFX on caecal Reg3 expression.

Reg3b and Reg3g mRNA levels in the caecum detected by qPCR. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed by Kruskal-Wallis test with consecutive Dunns test. Horizontal lines represent medians; *p<0.05; each dot represents one mouse.

After intraperitoneal IL-22 injection, Reg3b levels were significantly upregulated in the caecum in comparison to Reg3b expression in PBS-treated mice (Figure 5). Reg3g was also expressed at

significantly higher levels compared to PBS controls. However, in RFX-treated mice, which did not receive intraperitoneal IL-22 injections, Reg3b mRNA levels were the same as in the control cohort. Concerning Reg3g mRNA levels, they were expressed at lower levels in RFX with PBS-treated mice than in the control group.

To check whether Reg3 expression in the ileum could be also regulated through a IL-22 – Reg3 axis, defensin mRNA levels in the ileum were analyzed to examine whether the observed effects in caecal tissue could be reproduced in ileal tissue.

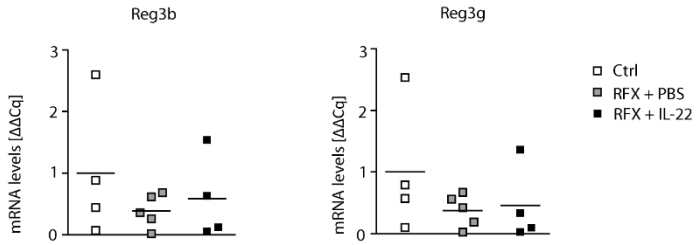


Figure 6 - Intraperitoneal injection of IL-22 leads to partial recovery of ileal Reg3 expression after RFX treatment.

Reg3b and Reg3g mRNA levels in the ileum determined by quantitative PCR analysis. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed by one-way ANOVA with consecutive Bonferroni test. Horizontal lines represent medians; each dot represents one mouse.

IL-22 upregulated Reg3b and Reg3g in the ileum of RFX-treated mice. Furthermore, expression levels of Reg3b and Reg3g were higher compared to the RFX-pretreated PBS control cohort. Thus, administration of IL-22 upregulates the expression of Reg3 in colonic tissue supporting the idea that IL-22 is involved in the signaling cascade of Reg3 expression. Due to the similar trend observed in ileal tissue, we assume a resembling signaling pathway in both colonic and ileal compartment.

7.3 Knocking out MyD88 seems not to affect IL-22 – Reg3 axis

Reg3 expression has been reported to be regulated through TLR-MyD88 signaling pathway, therefore, genetically deleting MyD88 would disrupt the TLR-MyD88 signaling cascade and hypothetically would lead to a decrease of Reg3 expression. In comparison to wild-type mice, MyD88 Knock-out mice showed a tendency towards higher Reg3b and Reg3g mRNA expression levels (Figure 7). In contrast, genetically deleting the MyD88 gene did not seem to influence IL-22, Crypt-4 and lysozyme mRNA expression levels.

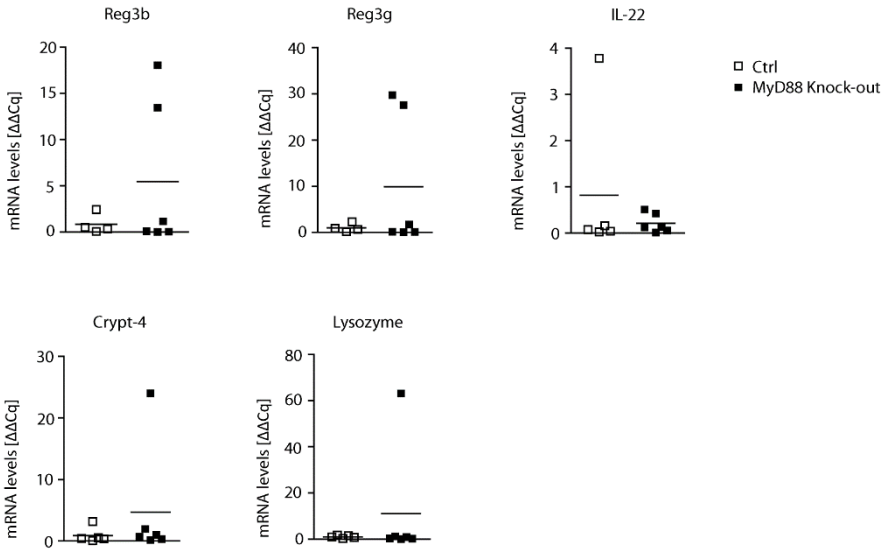


Figure 7 - Comparison of defensin and cytokine expression profiles in the caecum of wild-type and MyD88 Knock-out mice.

Quantitative PCR analysis of Reg3b, Reg3g, IL-22, Crypt-4 and lysozyme mRNA levels in caecum of MyD88 Knock-out mice. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed by Mann-Whitney test. Horizontal lines represent medians; each dot represents one mouse.

Since big differences within the groups were measured, a contamination or systemic error in the qPCR must be considered. Therefore, a conclusion cannot be drawn and further experiments are needed.

7.4 Fecal material transfer of RFX-treated stool leads to distinct microbial alterations

AMT decreased the mucus layer, Reg3 and IL-22 mRNA levels, whereas RFX did not affect the mucus layer and IL-22 expression. Therefore, it was tested whether AMT-induced alterations could be restored by FMT using stool of mice, which had been treated with RFX. Furthermore, a possible link between the amount of secreted Reg3b and the mucus layer was examined. The control group did not receive any kind of antibiotic treatment for 28 days. One interventional group (post AMT) was treated with AMT for 14 days and subsequently did not receive any antibiotics for 14 days. The second interventional cohort (RFX FMT) was also treated with AMT for 14 days. At the 14th day stool of RFX-treated mice was transferred to RFX FMT mice. Afterwards, RFX FMT mice did not receive any antibiotic treatment for 14 days.

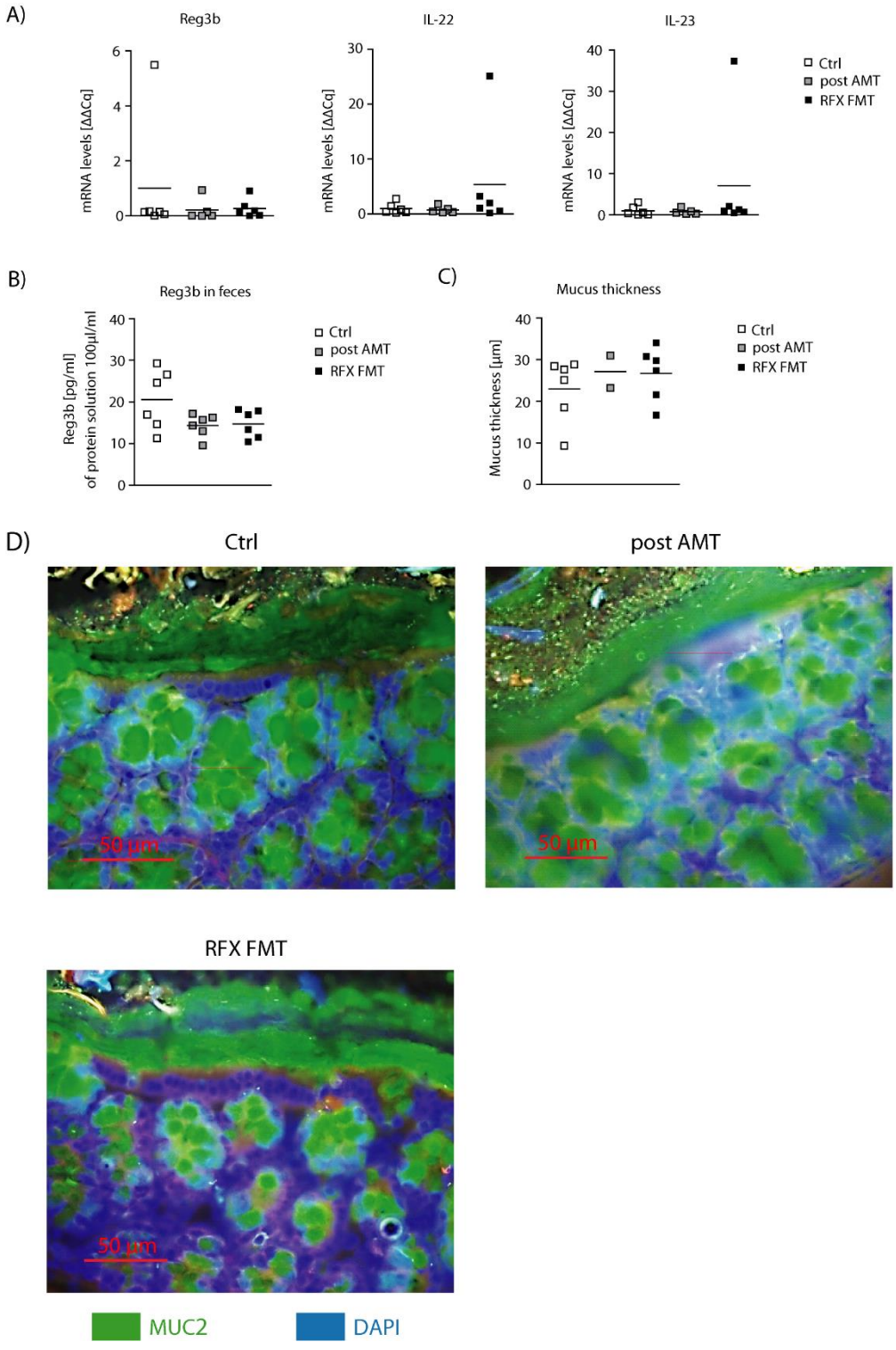


Figure 8 - Fecal microbial transplantation of RFX-treated stool reduces exclusively Reg3b expression without affecting the mucus layer.

A) Quantitative PCR analysis of Reg3b, IL-22 and IL-23 mRNA expression in the caecum. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by one-way ANOVA with consecutive Bonferroni test or by Kruskal-Wallis test with consecutive Dunns test. Horizontal lines represent medians; each dot represents one mouse. **B)** Reg3b secretion measured by ELISA in caecal content. **C)** Mucus layer thickness in the colon. **D)** Representative images of MUC2 immunostaining of colon sections.

Due to an outlier in the control group, mRNA levels of Reg3b were found to be reduced in the post AMT and RFX FMT cohort. The analysis of the IL-22 and IL-23 expression levels did not display any trend for changes after treatment, however, due to outliers this result should be interpreted carefully. An ELISA for Reg3b in the caecal contents revealed a trend towards lower amounts of secreted Reg3b in both interventional cohorts (Figure 8B). MUC2 visualization by immunofluorescence did not reveal any difference in the mucus layer thickness in the colon (Figure 8C and 8D).

A 16S rRNA GULDA analysis was performed to examine a possible link between the amount of secreted Reg3b and the relative abundance of various bacterial strains. Furthermore, we intended to determine whether FMT to antibiotic-treated mice leads to a combined reshaping effect of the gut flora as previously reported (Manichanh et al., 2010). As expected, the 16S rRNA GULDA analysis revealed a trend towards a reduction of the bacterial load in the antibiotic-treated cohorts compared to the controls (Figure 9), whereas the total bacterial load was slightly increased in RFX-treated stool donor animals. Strikingly, the bacterial abundance patterns of *Firmicutes*, *Clostridia cluster XIVa* and *IV*, *E. coli* and *Desulfobrivio spp.* were very similar. Highest abundance of bacterial strains could be detected in post AMT mice. The abundance in the control group was lower compared to the interventional groups. RFX-treated stool donor mice showed the lowest abundance of all groups. These microbial changes between the post AMT and RFX FMT cohort were even significant for the abundance of *Akkermansia* and *Prevotella spp.*, whereas the abundance pattern of *Enterococcus spp.*, *Desulfobrivio spp.*, *Clostridia cluster IV* and *XIVa* revealed a significant difference between the post AMT and RFX-treated cohort. Further, the bacterial strains of *Clostridia cluster IV*, *Enterobacteriaceae* and *E. coli spp.* were more abundant in post AMT mice than in the control cohort. Furthermore, *Bifidobacterium spp.* was detected more abundantly in post AMT mice. *Akkermansia* and *Enterobacteriacecae* were only found in the post AMT cohort. *Prevotella spp.* also appeared abundantly in post AMT mice. Abundance of *Prevotella spp.* was slightly elevated in RFX mice compared to control and RFX FMT animals. *Methanobrevibacter* was detected less abundantly in post AMT, RFX FMT and RFX animals, while post AMT mice showed the lowest abundance. No *Lactobacillus spp.* strain could be found in the RFX cohort. However, it could be detected more abundantly after RFX FMT treatment. Regarding the phylum of *Bacteroidetes*, lower abundances were detected in post AMT and RFX mice.

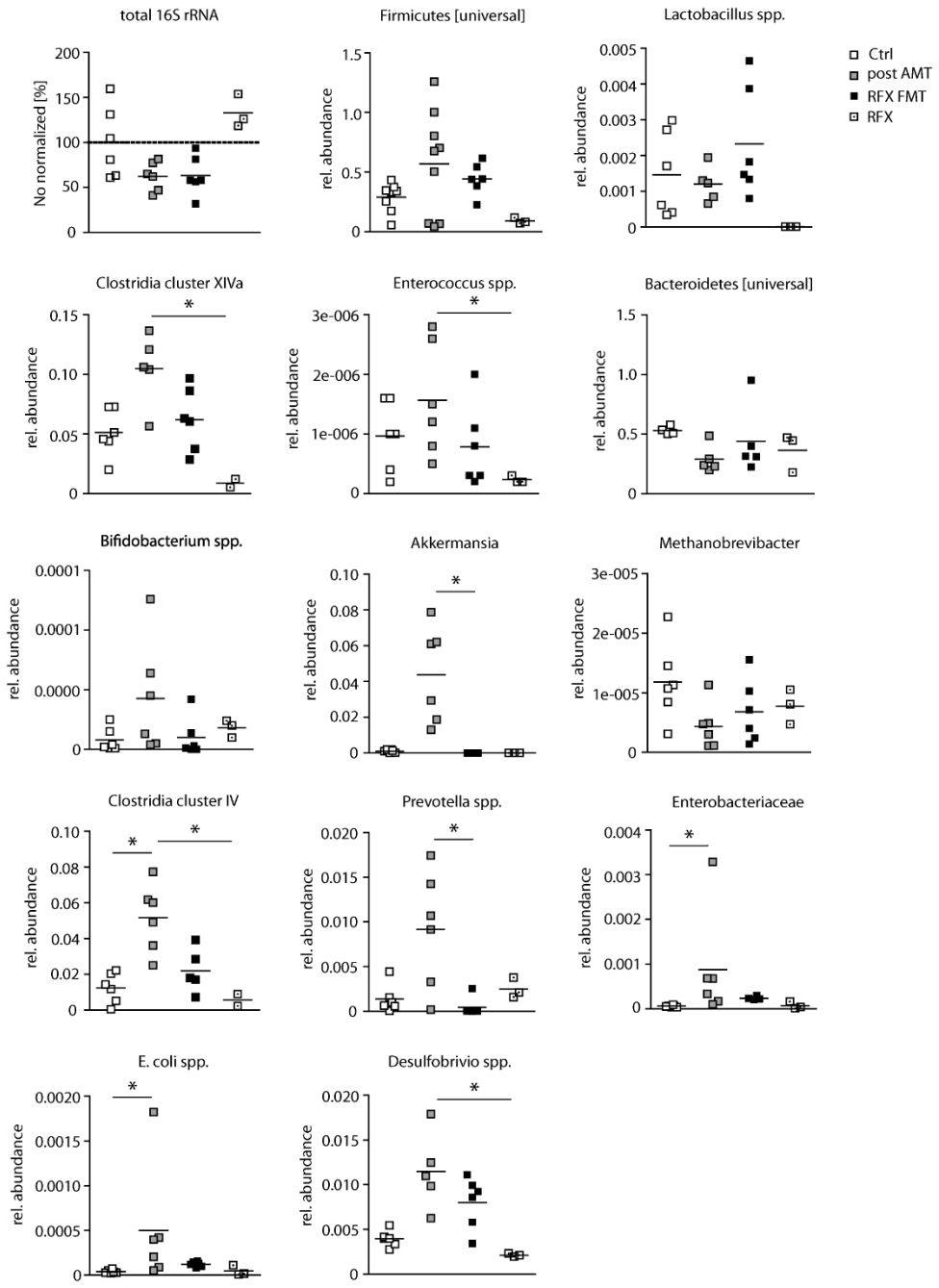


Figure 9 - Microbiota of RFX-treated mice is only partially transferable and leads to distinct shifts in the microbiota of recipients.

Relative abundance of selected 16S rRNA gene targets derived from analysis with GULDA. All values were normalized to universal 16S rRNA. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by one-way ANOVA with consecutive Bonferroni test or by Kruskal-Wallis test with consecutive Dunns test. Horizontal lines represent medians; *p<0.05; each dot represents one mouse.

In conclusion, no differences between the post AMT, RFX FMT and the control group could be detected regarding the Reg3b – IL-22 axis. Furthermore, the mucus layer of post AMT and RFX FMT mice seemed to be unaffected at the end of the experiment. Only the secreted amount of Reg3b was

slightly reduced in post AMT and RFX FMT cohorts. RFX FMT altered the microbiota of AMT-treated mice in a way that their microbiota partially resembled the microbiota of post AMT and RFX mice.

7.5 FMT reduces Reg3 expression

RFX treatment led to reduced Reg3 mRNA expression levels in the caecum via alterations of the gastrointestinal microbiota. The question arises whether microbial changes through transfer of untreated caecal content between different mouse strains could also induce a reduction of Reg3 expression. In a next step, it was assessed how microbiota, representing a dysbiosis-like status in comparison to the recipient's gut flora, would affect the IL-22 – Reg3 axis. To this end the caecal content of two different mouse strains, which are known to differ significantly in their gastrointestinal microbiota (Fransen et al., 2015), were used: C57BL/6 and BALB/c.

Mice receiving caecal matter from the same strain served as a control cohort, mice receiving the caecal microbiota of the other strain represented the interventional cohort. C57BL/6 rodents expressed IL-22, IL-23 and Crypt-4 at significant lower levels after microbial allogenic transfer, whereas BALB/c rodents treated with C57BL/6 caecal contents showed only a tendency towards a downregulation of IL-22, IL-23 and Crypt-4 (Figure 10). Furthermore, a similar pattern could be observed for Reg3 expression. In C57BL/6 animals receiving BALB/c microbial contents a trend towards suppression of Reg3b was detected. Reg3g was also expressed at lower levels in these animals in comparison to the controls. Furthermore, in C57BL/6 mice receiving BALB/c stool a reduction of mRNA levels of lysozyme were detected.

Similar to administration of antibiotics, transplantation of an exogenous gut flora reduces the expression of Reg3 and IL-22 indicating that changes in the composition of the microbial communities and consequently big interactions between luminal antigens and host tissue could be involved in the immune response.

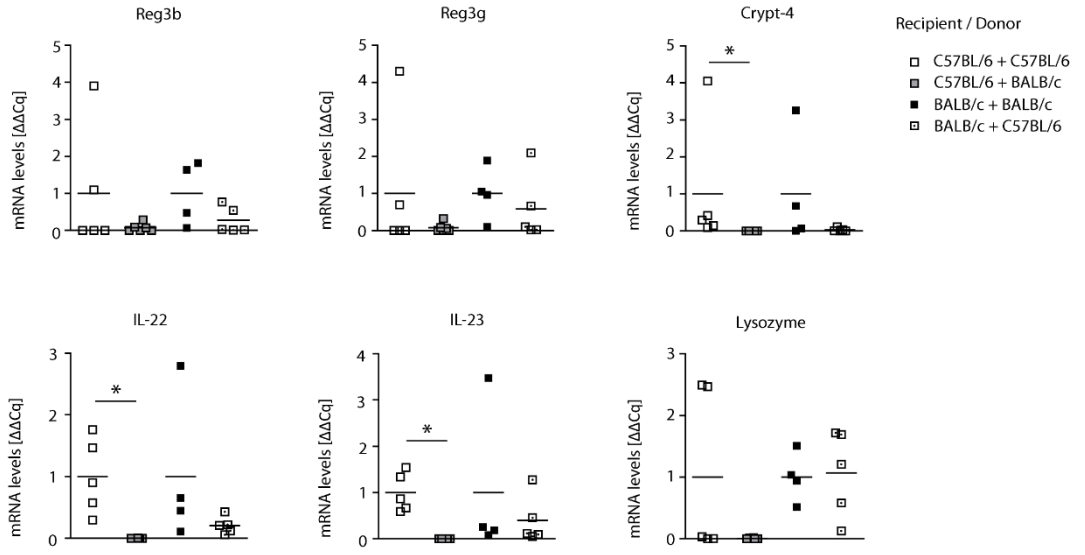


Figure 10 - Transfer of different microbiotas between inbred mouse strains leads to suppression of defensin and interleukin expression in the caecum.

Quantitative PCR analysis of Reg3b, Reg3g, IL-22, IL-23, Crypt-4 and lysozyme mRNA levels in caecum dependent on the donor-recipient relation. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Pairwise comparison was done. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test. Horizontal lines represent medians; *p<0,05; each dot represents one mouse.

7.6 RFX treatment predisposes towards colitis

Challenging RFX pre-treated mice with DSS represents an interesting approach to investigate whether the suppression of Reg3 after RFX treatment facilitates the development of chemically induced colitis. Colitis was induced with 2% DSS in control mice or mice after RFX treatment. As a third cohort, RFX-treated mice received 1% DSS. Then histological signs of inflammation and weight progression were evaluated.

On histological levels 2% DSS-treated control mice did not show any severe tissue damages in the colonic epithelium (Figure 11A). Also in RFX + 1% DSS-treated mice an almost intact histological structure of the colonic epithelium could be observed. Regarding the RFX + 2% DSS cohort, ulcerations reaching down to the lamina muscularis mucosae destroyed both the epithelial layer and crypt formation. Additionally, many lymphocytes were present in the edematous and damaged tissue. The weight progression under DSS exposure was documented over one week (Figure 11B). During the first four days, rodents of all three group did not lose any weight. The low DSS exposed animals even gained up to 1% weight, the controls even up to 3%. From day 5 on, the weight of the rodents treated with a high concentration of DSS dropped by up to 6% of baseline. The low DSS exposure cohort did not lose any weight during the observation period. By using a histology score

(considering three factors: infiltration with lymphocytes, amount of edema and area of ulceration), the severity of the chemical colitis was measured (Figure 11C). RFX + 2% DSS treatment led to a more severe inflammatory score compared to DSS only and RFX + 1% DSS.

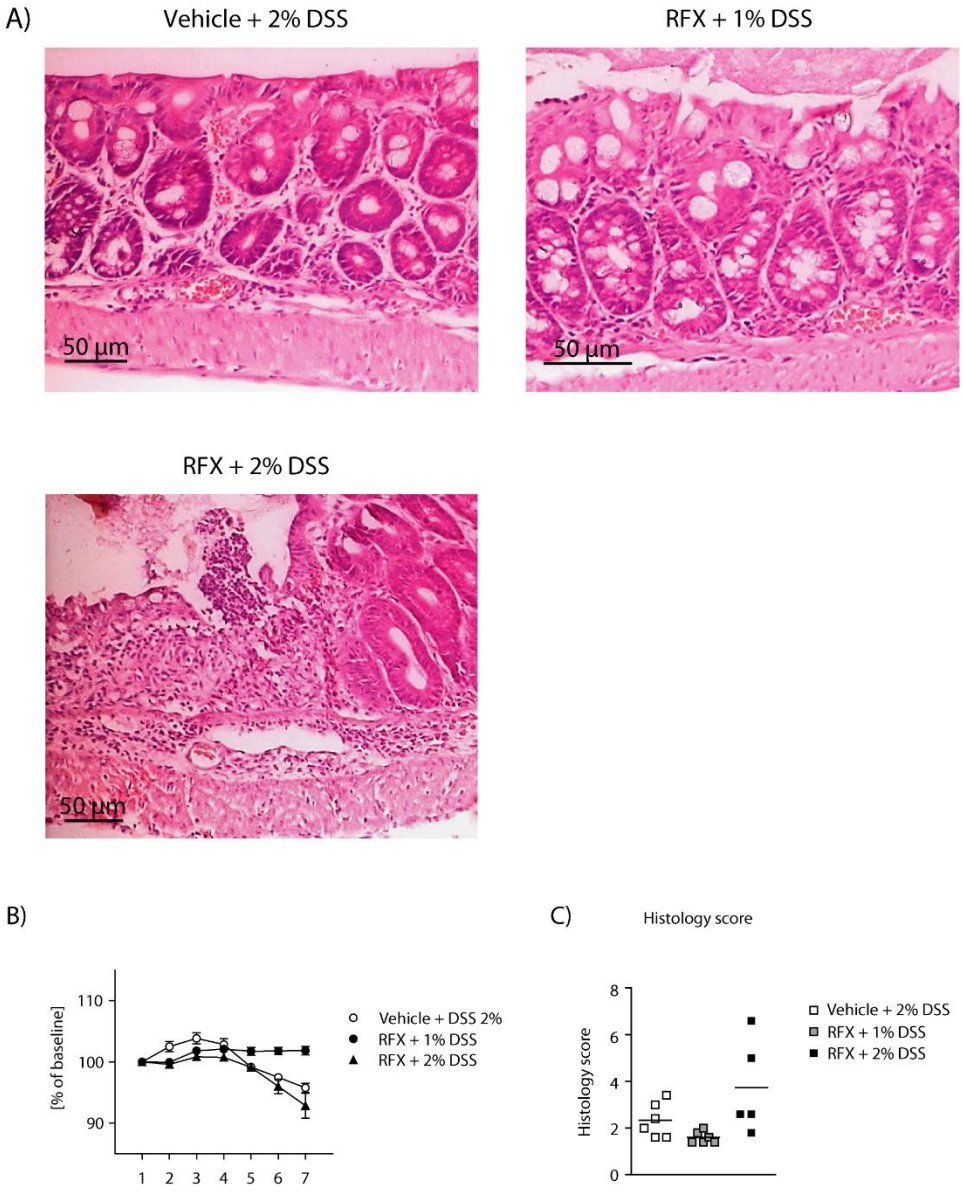


Figure 11 - RFX worsens colitis-associated weight loss and causes epithelial damages.

A) Representative images of hematoxylin and eosin stained colon sections demonstrating epithelial lesions after combined RFX treatment and DSS exposure. **B)** Weight progression of DSS-treated mice over one week. **C)** Histology scores indicating the severity of DSS-induced colitis. Kolmogorov-Smirnov test was followed by one-way ANOVA with consecutive Bonferroni test. Horizontal lines represent medians; n=6 mice per group.

Since 2% DSS treatment induced severe colitis in animals pretreated with RFX inflammation-associated parameters were analyzed. Besides Reg3b and Reg3g, IL-1β, IFNγ, CXCL1, MCP1 and TNFα

mRNA expression levels were examined as representatives of inflammatory cytokines involved in the neutrophil recruitment.

Quantitative PCR analysis revealed that RFX + 2% DSS mice expressed Reg3b and Reg3g at higher levels than 2% DSS-treated control mice (Figure 12). Additionally, 2% DSS + RFX treatment led to increased IL-1β and MCP1 mRNA expression levels. In contrast, no difference could be detected in TNFα and CXCL1 mRNA expression between RFX + 2% DSS and 2% DSS control cohort. Reduced IFNγ mRNA expression was observed in RFX + 2% DSS mice in comparison to 2% DSS control mice. Regarding the RFX + 1% DSS cohort, Reg3b, Reg3g and MCP1 mRNA expression levels were not increased compared to 2% DSS control cohort. Furthermore, RFX + 1% DSS-treated mice displayed lower IL-1β, IFNγ, TNFα and CXCL1 mRNA levels than 2% DSS-treated control mice.

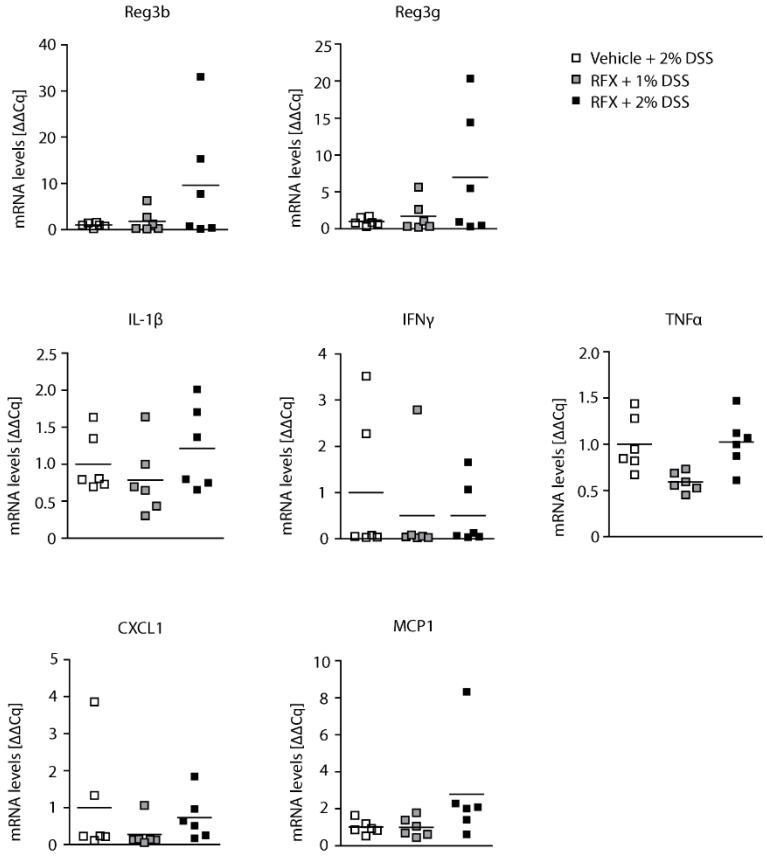


Figure 12 - Administration of RFX modulates inflammatory responses of the caecum upon DSS treatment.

Quantitative PCR analysis of inflammatory markers expressed by the caecal epithelium. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test for comparison between Vehicle + 2% DSS and RFX + 2% DSS cohort. Horizontal lines represent medians; each dot represents one mouse.

In conclusion, RFX treatment prior to a high DSS exposure induces a strong inflammatory response and consequently causes severe tissue damages in comparison to high DSS only exposure cohort. RFX

and high DSS treatment induced an upregulation of the Reg3 family, IL-1β and MCP1. However, no strong upregulation of IFNγ, CXCL1 and TNFα was detected. Since the combination of high DSS exposure and RFX treatment led to the most severe colitis and highest level of defensin markers, we assume that RFX induces tissue damages, thereby aggravating colitis.

7.7 Reg3b is subjected to a negative feedback loop and effective against both gram-negative and gram-positive bacterial strains

Since RFX treatment suppressed Reg3b expression and consequently predisposed towards colitis, the role of Reg3b was analyzed. Oral administration of Reg3b aimed to unravel the impact of Reg3b on the microbial composition and the defensin expression pattern. Therefore, Reg3b mRNA expression levels were measured. In order to assess to which extend Reg3b-induced modulations on the gut flora could influence the host immune response, various pro- and anti-inflammatory cytokine markers were screened. Furthermore, it was tested how the oral administration of Reg3b affects the Reg3b – IL-22 axis.

After three-times administration of rmReg3b, expression levels of Reg3b were downregulated in the murine caecum (Figure 13). In contrast, the RNA amount of TNFα was upregulated. Furthermore, IL-1β was expressed at lower levels compared to the controls. Simultaneously, a tendency towards suppressed levels of IL-2 was observed after Reg3b treatment. Both cohorts did not differ in IL-6 expression pattern. On the contrary, a trend towards higher IL-10 expression levels was detected in Reg3b-treated mice. Additionally, slightly increased amount of IL-17 RNA was found in the interventional cohort. In contrast, IL-22 was observed to be inhibited. However, Reg3b application induced an upregulation of MCP1 and a suppression of CXCL1. Higher amount of IFNγ RNA was found in the murine caecum of control animals.

As we observed a mixed pro- and anti-inflammatory pattern after Reg3b administration, we assessed the number of intraepithelial lymphocytes in hematoxylin and eosin stained sections (Figure 14). So far, we observed a tendency towards higher numbers of intraepithelial lymphocytes in the colonic tissue of the interventional cohort. S16 rRNA quantitative PCR analysis of the microbiome revealed, that Reg3b application only marginally reduced the total bacterial load (Figure 15).

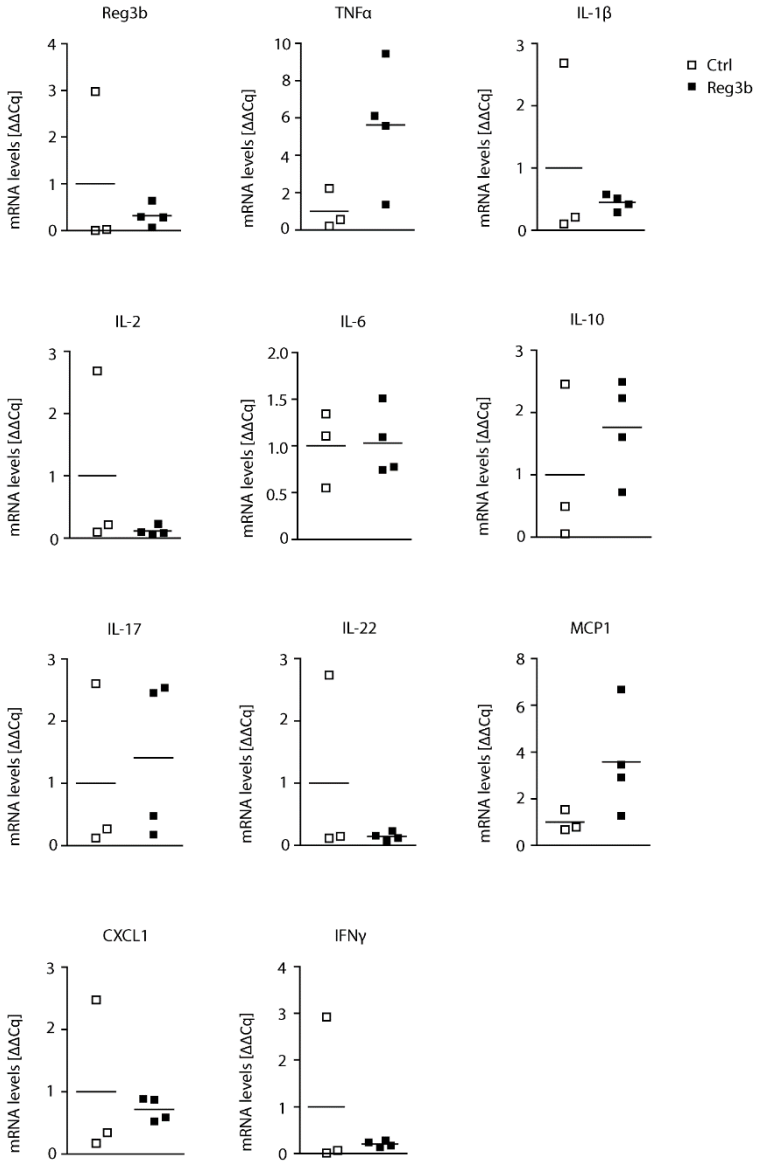


Figure 13 - Oral application of Reg3b modulates the defensin and cytokine expression pattern in the caecum.

Quantitative PCR analysis of caecal tissue analyzing defensins, pro- and anti-inflammatory cytokines in control and mice receiving Reg3b by oral gavage. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed by Mann-Whitney test. Horizontal lines represent medians; each dot represents one mouse.

A significantly reduced abundance could be observed regarding different bacterial phyla such as *Clostridia cluster XIVa*, *Enteroccus spp.* and *Prevotella spp.*. Furthermore, application of Reg3b led to a trend towards lower abundance of universal *Firmicutes*. However, the abundance of *Clostridia cluster IV* was similar in both groups. In addition, *Lactobacillus spp.* was found less abundantly after Reg3b treatment. The abundance of universal *Bacteroidetes* was at lower levels compared to the controls. This observation also applied to regarding the abundance of *Alistipes spp.*. In contrast, *Bifidobacterium spp.* was found in Reg3b-treated mice more present.

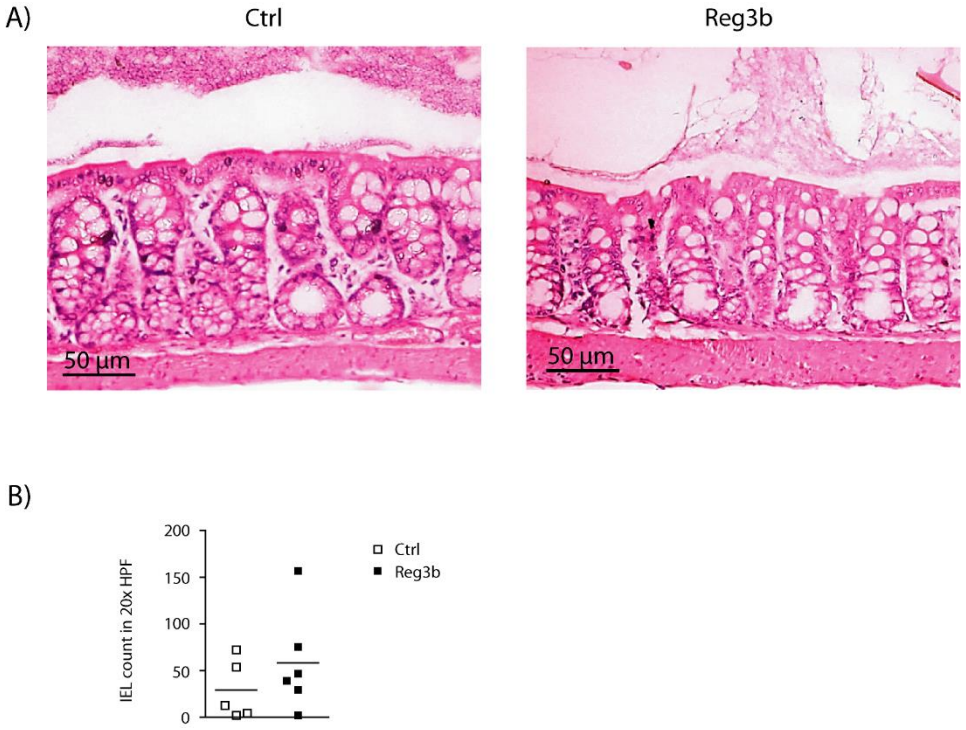


Figure 14 - Recruitment of intraepithelial lymphocytes after Reg3b application.

A) Representative images of hematoxylin and eosin stained colon sections after administration of Reg3b by oral gavage. **B)** Intraepithelial lymphocytes detected in hematoxylin and eosin stained sections of the colon in high power field at 20-fold magnification. Kolmogorov-Smirnov test was followed by unpaired t-test. Horizontal lines represent medians; each dot represents one mouse.

While the abundance of *Akkermansia* showed a slight tendency towards reduced levels in the interventional cohort, both groups did not differ in the abundance of *Enterobacteriaceae* and *Desulfobrivio spp*.. After application of Reg3b, *Bacteroides spp.* appeared less abundantly in comparison to the control animals. *Methanobrevibacter* was also observed to be less abundant in Reg3b-treated mice.

In conclusion, oral administration of Reg3b downregulates the expression of Reg3b and IL-22 indicating that the Reg3b – IL-22 axis could be subject to a negative feedback loop. The question, whether Reg3b directly inhibits the expression of Reg3b or changes in the microbial composition are responsible for the suppression of Reg3b still needs to be ascertained. Regarding the bacterial abundance, Reg3b targets both gram-negative bacterial strains (e.g. *Prevotella spp.*) and gram-positive strains (e.g. *Clostridia cluster XIVa*).

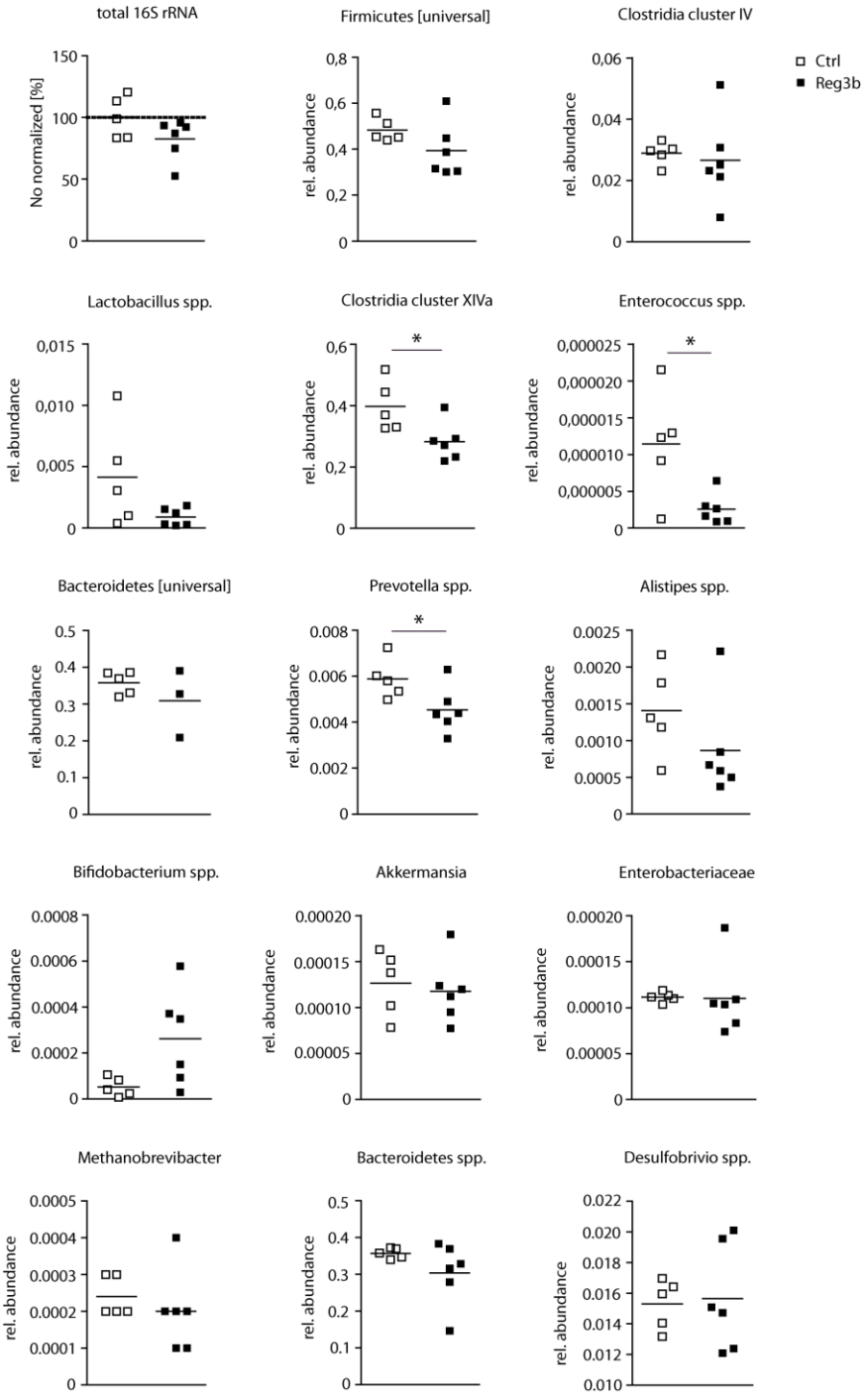


Figure 15 - Reg3b induces changes in the caecal microbial composition.

Gut Low-Density Array of fecal content extracted from caecum showing relative abundance of selected 16S rRNA gene targets. All values were normalized to universal 16S rRNA. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test. Horizontal lines represent medians; *p<0.05. Each dot represents one mouse.

7.8 Butyrate treatment suppresses Reg3b and IL-22 expression

Butyrate is an important SCFA strengthening intestinal barrier function during inflammation (Vieira et al., 2012). Therefore, a possible connection between its protective properties and the Reg3b – IL-22 axis was investigated.

Butyrate treatment reduced Reg3b expression in the caecum (Figure 16). Additionally, lower levels of IL-22 could be also observed. These results indicate that butyrate might be augmenting the barrier integrity, hence reducing the need of Reg3b and consequently downregulating the Reg3b – IL-22 axis.

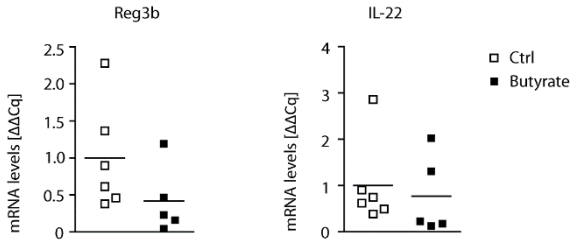


Figure 16 - Butyrate induces a decrease in the caecal expression of Reg3b

Quantitative PCR analysis of caecal tissue Reg3b and IL-22 in control and mice receiving 10 mg butyrate per kg body weight by oral gavage. All mRNA values were normalized to GAPDH. All samples analyzed were quantified in duplicate. Kolmogorov-Smirnov test was followed either by unpaired t-test or Mann-Whitney test. Horizontal lines represent medians; each dot represents one mouse.

8 Discussion

8.1 Microbiota modulation and mucus formation after antibiotic treatment

To investigate the impact of antibiotic administration on the epithelial immune response, laboratory mice received either an AMT (metronidazole, ampicillin, vancomycin and neomycinsulfate) or RFX treatment for two weeks. Subsequently, defensin and cytokine expression profile of caecal tissue was determined and mucus layer thickness was evaluated. Brandl et al. (Brandl et al., 2008) already published data proving that combined administration of metronidazole, vancomycin and neomycin induces a downregulation of Reg3g in the small intestine and linked this finding to decrease in commensal-derived metabolites and signals capable of promoting the release of antimicrobial peptides. We observed a tendency towards a suppression of Reg3g expression after AMT in the caecum and noticed, in addition, that Reg3b and IL-23 were also downregulated. IL-22 was expressed at significantly lower levels. IL-23 being involved in the release of IL-22 and consecutively Reg3b (Aden et al., 2016) represents a possible target of commensal-derived molecules as its reduction could subsequently lead to impaired release of Reg3b and even Reg3g. Strikingly, Reg3b expression was found to be significantly suppressed after RFX treatment. IL-22 and Reg3g expression levels were also reduced and, therefore, could support the theory of IL-22 being part of the interaction between microbiota and host immune system and its impairment due to alterations in the microbial composition. Lysozyme is a potent antimicrobial peptide of the innate immune system (Shimada et al., 2008), and its release likely depends on the presence of bacteria in the gastrointestinal tract. Since antibiotic treatment is known to diminish the endogenous gut flora (Ubeda et al., 2010), the reduced bacterial load in the gut could explain the impaired expression of lysozyme. However, RFX treatment did not influence the RNA amount of lysozyme in our experiments. This finding could indicate that RFX may not alter the number of bacteria in the intestinal lumen but instead reorganize some microbial populations as proposed by Soldi et al. (Soldi et al., 2015). Therefore, RFX creates dysbiosis-like alterations in the gut flora leading to downregulation of Reg3 without reducing the bacterial load. However, the expression of Crypt-4, another antimicrobial peptide of the innate immune system (Karlsson et al., 2008) belonging to alpha defensin family, is unchanged after both AMT and RFX treatment. This leads to the assumption that the release of Crypt-4 is not solely dependent on the bacterial presence in the gastrointestinal tract. Reduction of the inner mucus layer thickness of the colon observed after AMT treatment was reported to be caused by both ampicillin (Caballero et al., 2015) and metronidazole (Wlodarska et al., 2011). Metronidazole is assumed to impair the functionality of GCs (Wlodarska et al., 2011), which could explain the slightly decreased number of GCs we observed in AMT mice. However, metronidazole treatment induces an

upregulation of Reg3g (Wlodarska et al., 2011), whereas the combination of metronidazole, vancomycin and neomycin downregulates the expression of Reg3g (Brandl et al., 2008). This finding strongly emphasizes the interferences of simultaneous drug administration. Additionally, a study has shown that mono-colonization of *K. pneumoniae* restores the impaired mucus layer to pre-antibiotic treatment levels (Caballero et al., 2015). Thereby it is questionable whether the reduction of the mucus layer thickness we observed after AMT is solely attributed to killing bacteria or depends on microbial alterations. On the contrary, RFX does not seem to influence the mucus layer, but does disturb the commensal communities.

8.2 Regulation of Reg3 release

To further investigate the IL-22 dependency of Reg3 release, RFX-treated mice received intraperitoneal application of IL-22. The caecal expression of Reg3b and Reg3g was indeed restored in RFX-treated mice and even surpassed the expression levels in untreated controls. Since we could reproduce the downregulated RNA amounts of Reg3 after RFX treatment, it strongly supports the theory of the IL-22 – Reg3 axis. Our findings are concordant with the study of Rendon et al., who showed that the impaired Reg3 expression in the ileum following acute alcohol exposure and burn injuries can be rescued by IL-22 administration (Rendon, Li, Akhtar, & Choudhry, 2013). In order to prove comparability of the immune response in both ileum and caecum, we also investigated the ileal expression of Reg3. Regarding the expression profile of Reg3b, we observed that IL-22 restored partially the expression of Reg3b after RFX treatment, but did not reach the level of the untreated control cohort. In contrast, Reg3g expression was not restored, however, we could not replicate RFX's suppressive effect, thereby indicating that both segments of the gastrointestinal tract display patterns in the regulation of the IL-22 – Reg3 axis.

In order to assess whether RFX-induced effects on the immune response are caused by alterations in the intestinal microflora or are mediated by killing bacteria, we conducted a fecal microbial transfer (FMT) experiment. Bacterial load was reduced after AMT treatment, whereas RFX seemed not to reduce bacterial count. Phylogenetic analysis revealed that RFX diminished *Clostridia cluster IV* and *XIVa* bacteria, two important strains for fermenting cellulose into butyrate (Brestoff & Artis, 2013), which is known to inhibit colitis-associated mucosa damages (Keely et al., 2014), thereby suggesting colitis aggravating properties of RFX. Strikingly, transplantation of RFX-treated fecal material into AMT preconditioned mice did not establish the transplanted microbiota, but the microbial composition rather resembled both donor's and recipient's gut flora as previously reported by

Manichanh et al. (Manichanh et al., 2010). Even though rats were used as laboratory animals and vancomycin and imipenem as antibiotic pretreatment for diminution of the endogenous gut flora, they also observed that the establishment of an exogenous microflora cannot be facilitated by an antibiotic administration prior transplantation (Manichanh et al., 2010). They also postulated that the antibiotic treatment may be deleterious to both bacterial communities, indicating that a combination of both treatments also combines their altering effects on the microbiota.

Genetically deleting MyD88 represents an interesting approach to uncover the relationship between MyD88 and IL-22 and has led to the discovery that, for instance, the gastric expression of IL-22 is indeed dependent on MyD88-mediated signaling pathways (Obonyo, Rickman, & Guiney, 2011). In addition, both IL-22 and MyD88 are essential components regulating the Reg3g expression in the ileum (Kinnebrew et al., 2012b). However, in our experiment we measured elevated levels of Reg3 (15 to 30-fold elevated) probably due to a contamination or systemic error. On the assumption of a MyD88 – Reg3 axis, we would have expected a downregulation of IL-22 and Reg3 in the caecum of MyD88 knock-out mice. This finding would have confirmed previous studies considering an impaired recognition of microbial products via TLR-MyD88 signaling cascades to result in a reduced homeostatic Reg3g expression in the small intestine under basal condition (Brandl, Plitas, Schnabl, DeMatteo, & Pamer, 2007; Vaishnava et al., 2008). Hypothetically, AMT and RFX treatment could be disturbing the physiological composition of bacteria. Subsequently, the TLR-MyD88 pathway would be impaired and not be able to release both Reg3g and Reg3b. However, in our project we could not confirm this theory, thus needing further evidence. TLR might be able to directly regulate Reg3b and Reg3g release without using MyD88 signaling cascade.

A fecal material transfer experiment between two different mouse strains aimed to investigate, whether an exogenous microbiota, mimicking a dysbiosis-like status in comparison to the recipient's gut flora, would affect the IL-22 – Reg3 axis. Regarding the Reg3 defensin expression profile, we observed a trend towards a downregulation of Reg3b and Reg3g. In addition, mRNA levels of Crypt-4 and both IL-22 and IL-23 were reduced. We, therefore, may speculate that any alteration of the microbial composition in relation to the original intestinal milieu disturbs the immune response. Furthermore, C57BL/6 mice receiving BALB/c feces expressed Reg3 at lower levels, and IL-22 and IL-23 expression levels were significantly downregulated in comparison to BALB/c mice transplanted with C57BL/6 caecal contents. This finding might indicate that the microbiota of BALB/c feces is less capable of inducing defensin expression in comparison to C57BL/6 feces. The C57BL/6 strain is known to be very resilient against *Listeria monocytogenes* infection (Czuprynski, Faith, & Steinberg, 2003), and its resistance properties could be conferred to BALB/c mice via the fecal material transfer in form of bacteria-derived metabolites. In contrast, the microbiota of BALB/c mice is more susceptible to

food borne listeriosis (Myers-Morales, Bussell, & D'Orazio, 2013). However, a previous study may challenge this hypothesis, as neither resistance nor susceptibility to listeriosis were transferred through transplantation of the gut flora between C57BL/6 and BALB/c inbred mice (Myers-Morales et al., 2013). Regarding the expression pattern of lysozyme, we noticed that only C57BL/6 mice receiving the microbiota of the BALB/c strain displayed lower levels of lysozyme RNA. This observation may indicate a lack of microbiota-derived products in the BALB/c metabolome, usually needed for the modulation of the expression of lysozyme in C57BL/6 mice. In contrast, the substitution of these metabolites by the gut flora of the C57BL/6 strain can facilitate lysozyme expression in BALB/c mice. Therefore, we assume lysozyme expression to be modulated by microbiota-derived metabolites similar to the release of Reg3.

Butyrate is an important SCFA strengthening intestinal barrier function during inflammation (Vieira et al., 2012). Therefore, we aimed to investigate a possible connection between its protective properties and the Reg3b – IL-22 axis. Strikingly, expression of Reg3b and IL-22 was lower after butyrate administration. This might indicate that butyrate alters the microbial composition of the gastrointestinal tract or augment the epithelial integrity, thereby reducing the need of defensins.

In this study, we focused on the effect of antibiotic-induced alterations on the expression of Reg3b, but its regulation and entire physiological function remains yet to be elucidated. To unravel further characteristics of this C-type lectin, we administered Reg3b orally and observed distinct microbial shifts in the gut flora without lowering the overall bacterial load. Gram-positive vancomycin-resistant enterococci (VRE) pose a serious threat to health care institutions due to their multi-drug resistance (MCGEER, 2000), and overabundance of gram-negative *Prevotella spp.* is associated with new-onset rheumatoid arthritis (NORA) (Hofer, 2014). Reg3b can limit the proliferation of these pro-inflammatory bacterial strains. This finding may contradict previous assumptions that Reg3b only targets gram-negative bacteria (Miki et al., 2012), and thereby represents a potent antimicrobial peptide to sustain intestinal homeostasis. However, Reg3b simultaneously reduces the abundance of gram-positive *Clostridial cluster XIVa*, a commensal bacterium with multiple benefits for the host due to its ability to produce butyrate (van den Abbeele et al., 2013). Other commensal bacterium *Lactobacillus spp.* was found to be reduced upon Reg3b treatment, while *Bifidobacterium spp.* was found to be more abundant. Even though both strains support the host immune system (Turroni et al., 2014), it remains to be ascertained, why Reg3b treatment diminishes one of them. Regarding chemokine expression profile in the caecum, various pro-inflammatory cytokines such as TNFα, MCP1 or IL-17 were increased, whereas the expression of other pro-inflammatory markers such as IL-1β and IFNγ was decreased. Additionally, the expression of the anti-inflammatory cytokine IL-10 was upregulated. In hematoxylin and eosin stained colon sections we noticed a trend towards a higher

count of intraepithelial lymphocytes, which could possibly indicate an enhanced recruitment of immune cells in consequence of the Reg3b administration. Furthermore, oral administration of Reg3b downregulated the mRNA expression of Reg3b and IL-22. Therefore, we speculate that Reg3b acts protectively through diverse modulation of the microbiota by targeting mainly pathogenic strains. This altered intestinal microflora could be responsible for the downregulation of the TLR-MyD88 mediated signaling cascade, which in turn would suppress the IL-22 – Reg3b axis. Thus, the subsequent alterations of the immune response could represent a negative feedback loop Reg3b is subjected to.

8.3 Relevance of Reg3 in translational clinical science

Inflammatory bowel disease is a chronic inflammatory disease affecting the gastrointestinal tract as Crohn's disease or ulcerative colitis. So far, its pathogenesis has not been completely elucidated. Accepted theories include aberrant immune responses against the gut flora triggered by environmental factors in genetically predisposed individuals (Shaw, Blanchard, & Bernstein, 2011). Additionally, various alterations of the intestinal microbiota have been reported in IBD patients, and it is controversially discussed whether dysbiosis is the cause or the consequence of relapsing intestinal inflammation (Matsuoka & Kanai, 2015). Antibiotics are known to alter the microbial composition and epidemiological studies revealed that antibiotic therapy is associated with the development of IBD (Shaw et al., 2011). Therefore, it is hypothesized that antibiotic-induced alterations of the microflora increase the susceptibility and likelihood for intestinal damage, which may finally trigger the onset of IBD. In the present study, we demonstrated the suppressive effects of RFX on the expression of Reg3b and, in addition, its microbiota modulating properties resulting in dysbiosis-like alterations. By challenging RFX-pretreated mice with DSS we aimed to test the hypothesis that RFX-induced dysbiosis and defensin downregulation would predispose the intestinal epithelium of the host to increased inflammation. In line with results published by Knoop et al. (Knoop, McDonald, Kulkarni, & Newberry, 2016c), we could demonstrate that antibiotic treatment alone cannot induce epithelial lesions. However, co-administration of a chemical agent as DSS significantly disturbed epithelial integrity (Knoop et al., 2016c). In our study, RFX pretreatment combined with a standard DSS dose (2%) induced a significant damage of the intestinal mucosa in comparison with vehicle pretreatment, indicating that RFX-induced microbial dysbiosis renders the epithelium more susceptible to pathogenic processes. However, RFX itself cannot trigger inflammation. Low dose DSS (1%) conditions with RFX priming did not induce considerable colitis in mice. The analysis of the animals' weights in the two 2% DSS treatment groups corresponded with

the histology score, although it did not reach statistical significance. The defensin expression profile in the caecum supported these considerations as Reg3 was expressed at higher levels after combined RFX treatment and high DSS exposure. This indicates an upregulation of Reg3 as an acute-phase protein in response to an aggravated colitis. Due to the suppressive nature of RFX on the protective Reg3 expression, RFX-induced microbial alterations could facilitate subsequent intestinal damages caused by DSS treatment. Therefore, DSS exposure after RFX administration induces an upregulation of pro-inflammatory markers such as TNFα, IL-1b and MCP1. In contrast, RFX administration per se did not significantly activate the immune system. Knoop et al. observed an increased bacterial translocation to mesenteric lymph nodes after antibiotic usage and proposed this phenomenon as an explanation for the DSS-colitis aggravating effect of antibiotics (Knoop et al., 2016c). Although not proven experimentally, bacterial translocation could – at least in part – explain RFX effects. Since bacterial translocation via GAPs is dependent on the TLR – MyD88 signaling cascade, RFX-induced microbial alterations could interrupt this pathway and modulate the predisposition to subsequent inflammation. The impact of microbial changes on the IL-22 – Reg3 axis could be dependent on the antibiotic. This consideration could explain why administration of some antibiotics did not aggravate the DSS-induced colitis in the study of Knoop et al. (Knoop et al., 2016c).

9 Conclusion

In this study, we could demonstrate that primarily RFX as a non-absorbable gram-positive and gram-negative bacteria targeting antibiotic suppresses the expression of Reg3 and that this effect is mediated partly through the TLR-MyD88 signaling pathway, which senses the presence of bacteria in the gastrointestinal tract. Antibiotic treatment probably disturbs the microbial composition leading to insufficient stimulation of the TLR-MyD88 signaling cascade, which in turns fails to upregulate the expression of IL-22 and IL-23. We found evidence indicating a possible dependency of Reg3 expression on IL-22 and/or IL-23 and supporting the theory of a IL-22 – Reg3b axis. Furthermore, we assume the expression of lysozyme in the caecum to be co-modulated by the TLR-MyD88 signaling pathway. Regarding Reg3b, we discovered that its effects are not limited to gram-negative strains and that it presents very potent antimicrobial properties to restore intestinal homeostasis and gut protection. Additionally, Reg3b-induced alterations of the gut flora impair the microbial sensing via the TLR-MyD88 signaling cascade and suppress the expression of Reg3b, which could represent a possible negative feedback loop. Reg3 is an essential part of the intestinal immune system as its diminution after RFX treatment and consequent microbial dysbiosis exposes the epithelium to aggravated inflammatory tissue damages. This indicates that antibiotic-induced alterations of the microbiota may be the cause for relapsing intestinal inflammations.

From a clinical perspective, one may therefore critically question whether the administration of antibiotics offers (long-term) benefits for IBD patients or impose risks for relapsing inflammatory conditions in the gastrointestinal tract.

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11 Presentation

Effects of microbiota modulation on gastrointestinal mucus formation and host defense responses

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