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Time-Dependent and Microbiota-Dependent Effects of Interleukin-22 in a Colitis Mouse Model

Dissertation

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1. Objectives

This thesis assessed the effect of interleukin (IL)-22 on colitis and colitogenic microbiotas by testing the following two scientific hypotheses.

Hypothesis I: Germline knockout of *Il22* and *in vivo* neutralization of IL-22 has been shown to aggravate T cell-mediated colitis in mice. We hypothesized that also short-term neutralization of IL-22 exacerbates colitis. Here we use an adoptive T cell transfer colitis model, which has not been readily used with *in vivo* IL-22 neutralization in the past, to test this hypothesis.

Hypothesis II: IL-22 has been shown to directly impact the microbiota composition. Interestingly, the protective effect of IL-22 appears to be limited to certain microbiota settings. We hypothesized that IL-22 ameliorates colitis in mice by influencing a colitogenic microbiota. To this end, we analyzed changes in microbiota composition and colitis pathology in mice that underwent different regimens of fecal microbiota transplant (FMT), anti-IL-22 treatment, and colitis induction.

2. Introduction

2.1. The intestinal mucosa in health

Homeostasis regarding nutrient absorption, defense against pathogens, and immune balance of the gastrointestinal tract is achieved by an interplay between at least three components: intestinal epithelial cells, the mucosal immune system, and commensal microbiota (1).*

2.1.1. Intestinal epithelial cells

The human gastrointestinal tract is an interface of mucosal tissue with microbial colonization, potential pathogens, and food antigens. The paradigm of intestinal homeostasis is to prevent inflammation by containing microbial organisms in the lumen. The cell population of intestinal epithelial cells (IECs; singular IEC) creates a strong barrier but allows communication and nutrient absorption. IECs are derived from intestinal stem cells residing in the crypts, and they give rise to six different cell types: enterocytes, enteroendocrine cells, goblet cells, microfold (M) cells, Paneth cells, and tuft cells. (2) In order to stabilize the epithelium, all intestinal epithelial cells are connected by multiple cell-cell junctions: tight junctions, desmosomes, and adherens junctions. The basal site of the IECs is connected to the underlying basal membrane by hemidesmosomes. Underneath is the lamina propria, which contains blood vessels, lymphatic vessels, and mobile cells, *i.e.*, immune cells. The last layer of the mucosa is the lamina muscularis mucosae. Next is the loose submucosal connective tissue followed by the muscularis propria. On the outside of the intestinal tube lays either an adventitia (connective tissue of retroperitoneal organs) or a serosal layer (epithelium of intraperitoneal organs, such as the transverse colon).

Two key functions are taken care of by IECs to help maintain intestinal immune homeostasis: firstly, active segregation of microbiota and the lamina propria, secondly, communication between microbiota and immune cells. Concerning active segregation, the maintenance of an effective barrier is the principal function of IECs. This is achieved by physical (anatomical structures, mucins, and continuous flow of chyme), chemical (pH), and biochemical factors. Mucins are colloidal, highly glycosylated proteins produced mainly by goblet cells. IL-22 stimulates the release of mucins. IL-22 will be broadly introduced in the section on pathophysiology. The three main types of mucins are mucin 2 (MUC2), trefoil factor 3, and resistin-like molecule-β. (3–5) Another central part of segregating microbiota

* To make the citation more precise, if a reference number is placed before the full stop of a sentence, the cited literature refers to the respective sentence specifically, not to the sentences before.

from the lamina propria immune cells is antimicrobial peptides, which are also under the stimulatory control of IL-22. These are produced partly by enterocytes, *e.g.*, regenerating islet-derived protein 3 gamma (REGIIIγ), and additionally by the specialized Paneth cells (*e.g.*, cathelicidin, α-defensin, REGIIIγ). In the thick mucus layer directly on top of the epithelium, REGIIIγ creates a zone free of Gram-positive bacteria in the mucin adjacent to the IECs (6). This "demilitarized zone" thereby is an important barrier between microbes and human cells. Interestingly, outer mucus that is found further away from the IECs is a biologial niche, which supports microbial growth, and some bacteria might be able to degrade mucins for their nutrition (7, 8).

Next, some key mechanisms regarding communication between microbiota and immune cells, which also enhance barrier integrity, will be described. IECs control microbial growth in the vicinity of the epithelial layer by immunoglobulin (Ig) A transcytosis. IgA, which is generated by lamina propria plasma cells, is internalized by IECs via the polymeric Ig receptor, which after apical secretion becomes the secretory component stabilizing the IgA dimers. IgA neutralizes microbes, acts as a decoy, and does not induce inflammation. (9) In rodents, such as mice, which are relevant models for our experimental questions, another way of IgA secretion exists in form of the "hepatobiliary route". There, IgA is transported via the portal venous system to hepatocytes, which in turn secrete it into the bile ducts. In humans, however, this hepatobiliary route is negligible. (10) In order to react to environmental or endogenous stressors and to enhance the epithelial barrier, another important mechanism is the unfolded protein response. If environmental stressors exceed the IECs' abilities to adapt, unfolded proteins accumulate in the endoplasmic reticulum. This triggers an intracellular response which leads to both increased cytokine production, which targets the lamina propria immune cells, and increased exocytosis of mucins and antimicrobial peptides. This rise in exocytosis is dependent on the transcription factor X-box binding protein 1 (XBP1). (11, 12) The unfolded protein response is also essential for dendritic cell and plasma cell function (13, 14). Next, IECs are capable of directly lysing bacteria that have reached the IECs' cytosol, such as *Salmonella (S.) typhimurium*. Such bacteria can be sensed by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) which recognizes muramyl peptides found in both Gram-positive and Gram-negative bacterial cell walls. NOD2 binds the protein autophagy related 16 like 1 (ATG16L1), which thereby activates a digestive autophagosome (15). NOD ligands, *i.e.*, microbial structures, enter the IECs

autonomously and via active transport that is mediated by peptide transporters or transporters of the solute carrier family. In addition to forming a stable yet flexible barrier, IECs send active signals to immune cells and promote wound healing. Pattern recognition receptors, *i.e.*, NOD-like receptors and Toll-like receptors, signal IECs to release cytokines, such as inflammasome-derived IL-1β, and promote wound healing. Wound healing is achieved for example via Wnt signaling. Re-activation of this developmental pathway promotes proliferation but also imposes risks for cancer development (16). Another mechanism that promotes wound healing is the production of reactive oxygen species by IECs (17). Lastly, IECs send direct signals to immune cells, *e.g.* via juxtacrine signaling through E-cadherin (18). This cell adhesion molecule binds to cluster of differentiation (CD)103 (integrin αE:β7), which is expressed on intraepithelial lymphocytes (IEL) and tissue-resident memory T cells (T_{RM}) (18). In the colon and small bowel respectively, IEC-derived CC-chemokine ligand (CCL)28 and CCL25 attract T cells and B cells to the lamina propria (19, 20). Hence, IECs play a central role in maintaining an intact intestinal barrier and in coordinating the wound-healing response in case of injury. This is achieved by means of active segregation and, on the other hand, communication.

In line with their protective barrier function, IECs distinguish between commensal microorganisms and pathogens. They do so by specific pattern recognition receptor signaling that exclusively recognizes live organisms (*i.e.*, no harmless microbial debris) and virulence factors of potential pathogens (*e.g.*, bacterial flagellin) (21, 22). These pattern recognition receptors mediate either a proinflammatory response or a more tolerogenic response, depending on their localization within the cell. Downstream signaling molecules are distributed unequally between the apical and basolateral cytosol of IECs. Basolateral encounter of microbes (*e.g.*, potentially invasive bacteria) is transduced by more signaling molecules than apical encounter. Thus, it results in stronger downstream signaling, resulting in a more proinflammatory immune activation (22). Correspondingly, cytokine secretions by IECs are relatively low during homeostasis. In infection, however, they increase massively, as seen for example with pathogen-triggered CCL20 release (23). So, IECs directly recruit immune cells in case of intestinal infection.

The immunological function of IECs consists mainly of transmitting microbial signals to the underlying immune compartment and delivering antigens for direct immune recognition. Enterocytes deliver these luminal antigens to immune cells by three different routes:

unspecific transcytosis, delivery of intracellular pathogens through apoptosis, and neonatal Fc receptor-mediated transport of IgA back to the lamina propria. More specialized mechanisms include the transport of soluble antigens by goblet cells and transcytosis via M cells (24). M cells are located above specialized lymphoid tissue (isolated lymphoid follicles, Peyer's patches). Both unspecific phagocytosis and glycoprotein 2-mediated recognition of bacterial pili deliver luminal antigens to immune cells in the absence of (or prior to) microbial tissue invasion (25). M cells release CCL20 to attract immune cells, such as chemokine receptor (CCR) type 6^+ dendritic cells, so that they immediately recognize the transported antigen. Interestingly, M cell development itself is dependent on their interaction with immune cells via receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) ligand (RANKL) (26). In 2019, Ladinsky *et al*. have described a novel mechanism of antigen uptake by which direct commensal-IEC contact triggers endocytosis of segmented filamentous bacteria (SFB). They termed this process microbial adhesion-triggered endocytosis (MATE). (27)

IEC-derived cytokines include thymic stromal lymphopoietin (TSLP), transforming growth factor-beta (TGFβ), a proliferation-inducing ligand (APRIL), B cell-activating factor (BAFF) (28, 29), and IL-25 (30). These cytokines promote a proinflammatory or tolerogenic response in nearby immune cells. For example, the release of TSLP and TGFβ primes lamina propria dendritic cells into a tolerogenic state (28). These commensal-primed dendritic cells migrate into secondary lymphoid tissue (Peyer's patches, isolated follicles, mesenterial lymph nodes) and prompt B cells to develop into protective IgA-producing plasma cells (31). Also, commensal-primed dendritic cells induce gut homing in those B cells via paracrine retinoic acid signaling (32). Finally, gut-homed plasma cells receive direct stimulatory signals from IECs (29). Similar communicatory circuits exist for T cells (notably, forkhead box P3 (FOXP3) induction) (33)), innate lymphoid cells (ILCs) (34), basophils (35), and macrophages (36). This demonstrates the central role of IECs in transmitting microbial stimulation in order to sustain an adaptable barrier control.

2.1.2. Mucosal immune system

The intestinal immune system and the gut-associated lymphoid tissue (GALT) is, based on cell numbers, the largest immune compartment of our body. This is described below concerning intraepithelial lymphocytes (IEL). It is assumed that the development of the immune system is initiated in the gut by microbiota-immune interaction. Correspondingly, GALT

structures develop only after birth, concomitant with colonization with gut microbiota. Additional evidence for this hypothesis is provided by observations in germ free mice, which lack healthy GALT and show features of a diminished systemic immune system, *e.g.,* smaller global lymph nodes (37). In mice, microbiota is also transferred from the mother to its offspring during birth, but it is additionally acquired by coprophagy within mouse colonies. Functionally, soluble proteins lead to oral tolerance, *i.e.*, a systemically diminished immune response to orally taken food antigens (38). GALT is organized into four different anatomic compartments, namely, the isolated lymphoid follicles, which are found in the lamina propria of the entire intestinal tract, Peyer's patches, which underlie a special dome-shaped epithelial layer in the ileum, cryptopatches (only described in mice), which lie in the colonic lamina propria, and a compartment of scattered immune cells. Within the follicles, B-2 B cells are found, which are located within a network of stromal cells, called follicular dendritic cells, and antigen presenting dendritic cells. In between the follicles is the T cell-dominated area. In the following, some relevant immune cells of the GALT are briefly characterized.

GALT **T cells** express CCR9, which directs them to the lamina propria. Together with integrin expression, this is essential for homing to and staying in the gut. These T cells are thought to be mostly commensal-specific, *i.e.*, they recognize microbial peptides. Their function, however, is not to create a typical adaptive immune response. No complete adaptive response is unleashed because of several mechanisms. First, the barrier integrity of the gut prevents full proinflammatory activation. This is based on the observation that gut T cells do cause inflammation in the situation of pathologically high barrier permeability. (39) Second, the co-stimulation by the previously discussed tolerogenic dendritic cells primes T cells for a less aggressive phenotype. Third, transfer studies have demonstrated that IL-10 producing T cells balance out proinflammatory T cell cytokines (38, 39). In the colon, these immunomodulatory cells are mostly $FOXP3^+$ regulatory T cells (T_{reg}), while $FOXP3^-$ cells, such as type 1 regulatory T cells (Tr1), secrete IL-10 in the small intestine. Based on their location, GALT T cells are subdivided into lamina propria T cells (found scattered and organized in between follicles) and IEL.

Lamina propria T cells include $CD4^+$ effector T helper cells: type 1 (T_H1), type 2 (T_H2), type 17 (T_H17). Interestingly, IL-22 can be produced by T_H17 cells and by an IL-17A negative T cell subset, also referred to as T_H22 cells. Other T helper cells include follicular helper

cells (T_{FH}) and T_{reg} (40). The integrin α4:β7 (α4 = CD49b), which is essential for homing to the lamina propria, and CD45RO, which indicates prior antigen contact, are well expressed on lamina propria T cells (41). Specific functions of the different T helper cell lineages can be related to distinct aspects of barrier integrity. T_H2-derived cytokines, notably IL-13, stimulate goblet cells to produce mucins. T_H17-derived cytokines, notably IL-22, stimulate Paneth cells to produce antimicrobial peptides and stimulate the production of mucins.

In addition to T helper cells, there are closely related cells such as ILC2 and ILC3 that release the same cytokines as their corresponding T cells (*i.e.*, ILC2 typically release the same cytokines as T_H2; ILC3 typically release the same cytokines as T_H17). ILC3 are largely dependent on the aryl hydrocarbon receptor, which senses food-derived ligands, notably from cruciferous vegetables. A subset of ILC3 express receptors which are typical for NK cells, and these are termed NCR^+. (42) Flexible regulatory circuits control ILC3 activity, making ILC3s the main producers of barrier enhancing IL-22 in response to environmental stressors (43). Furthermore, ILC3 aid lymphoid tissue development (34). An additional subset of lamina propria cells include natural killer T (NKT) cells and mucosal associated invariant T (MAIT) cells, whose functions are the recognition of alternative antigens derived from the microbiota. NKT cells are activated by lipid antigens via CD1d, while MAIT cells recognize byproducts of bacterial riboflavin (vitamin B2) and folic acid biosynthesis (vitamin B9) (44, 45). Lastly, lymphoid tissue inducer (LTi) cells (found for example in cryptopatches) are mediators of postnatal development of secondary lymphoid structures such as Peyer's patches (46). Hence, lamina propria T cells are a heterogenous and highly flexible group of $CD4^+$ T cells which are crucial for homeostasis and reaction to environmental influences.

In contrast to lamina propria T cells, IEL are dominantly $CD8^+$ effector T cells (80% of T cells in the epithelium). Ten percent of IEL are not T cells; however, these are often disregarded in the literature when referring to IEL. IEL are a substantial part of the immune system, there is one IEL for every ten enterocytes. Mathematical estimates for the total count of murine enterocytes are roughly 8×10^8, resulting in an estimated IEL count of 0.72×10^8 (T cells only) (47). Compared to the average T cell number in the murine spleen (up to 0.25×10^8) (48), IEL are approximately three times more abundant than splenic T cells. This indicates how relevant IEL are for mucosal immunity. Therefore, when speaking of T cells in the intestine, one should always consider both lamina propria cells and IEL.

IEL usually express αE:β7 (αE = CD103), which binds E-cadherin as discussed above. Different IEL subsets are recognized**.** While T cell receptor $(TCR)^-$ IEL are very similar to ILC3, TCR^+ can be further divided into two categories (49). The first subset, termed *inducible*, are essentially usual $CD8^+$ cytotoxic T cells seen in the expression of the typical CD8αβ dimer (a costimulatory signal transducer molecule). The other subset, termed *natural*, expresses CD8αα. CD8αα is required for development and maintenance of these cells by binding thymus leukemia antigen on IECs. (50) The second factor required for natural IEL development is IL-15, which is induced by the microbiota and presented on IECs. The two main functions of IEL are intensive cytokine production, even in steady state, and killing of any abnormal (infected or pre-malignant) IECs. Just as lamina propria cells are kept tolerant by IL-10 production, IEL also demand immunomodulatory balancing. One interesting mechanism for this is CD8αα signaling. While CD8αβ acts as a stimulatory signal, CD8αα acts rather as an inhibitory receptor, presumably preventing *natural* IEL from unleashing their full proinflammatory potential when stimulated by their environmental antigens. (51, 52)

Plasma cells, terminally differentiated B cells, reside in the lamina propria and produce three to four gram of IgA daily. The plasma cell response is classically induced by the microbiota, and secreted IgA is mostly commensal-specific. For example, 75% of commensals are coated by IgA. Secretory IgA is important for barrier control and segregation of microorganisms and human cells without causing inflammation (little potential for complement activation or opsonization). However, patients with selective IgA deficiency usually do not experience any symptoms. This is typically explained by compensatory secretion of IgM, which – however – is capable of complement activation. (53) Thus, the function of IgA remains partly elusive. In mice IgA is also produced by innate B-1 cells at the peritoneal site of the liver. Murine B-1 cells can carry out a T cell independent class switch to IgA^+ plasma cells. (54)

GALT **Macrophages** can have tissue resident properties. They are characterized as $CD11c^+$ (integrin αX), $CD64^+$, and $CD103^-$. Unlike in a situation of classical inflammation, they do not tend to act as antigen presenting cells. But C-X-C chemokine receptor type $(CXCR)1^+$ macrophages pass on antigen to dendritic cells that present it to T cells as discussed above. Their two main functions comprise scavenging microbial, food-derived, or cellular debris and producing immunomodulatory cytokines, mainly IL-10. (55, 56)

Dendritic cells can migrate and transport antigen from the lamina propria to follicles, Peyer's patches, and mesenteric lymph nodes. They are subdivided into $CD11b^+$ (preferentially inducing T_H17) and $CD11b^-$ (preferentially inducing T_H1). Dendritic cells must be exposed to certain cytokines to become tolerogenic (IEC-derived cytokines; stromal-derived prostaglandin E2 and IL-10 from macrophages and $CD4^+$ cells). These tolerogenic dendritic cells lack full costimulatory potential. Thus, relatively unaggressive T cells are created under steady state conditions.

Taken together, for intestinal homeostasis the intestinal immune cells have the same purpose as the IECs. They aim at discriminating between microbiota and potential pathogens. They achieve this by strengthening the intestinal barrier while remaining flexible towards environmental and microbial influences.

2.1.3. Microbiota

First, the **structure of the microbiota** will be outlined. The human body is physiologically colonized by large numbers of microbial organisms, termed microbiota, and they outnumber human cells by up to factor ten (57). The microbiota, defined as all commensal microorganisms, live in a symbiotic relationship with the host, *i.e.*, it contributes to human health, physiologic development, and function. The gut, especially the distal small intestine and the colon, is intensively colonized. In this thesis, we use the term microbiota to refer to the intestinal microbiota. Their total microbial content corresponds to approximately 10^{13} cells or 150 g of total microbial mass. Microbiota composition can be analyzed with regards to different taxonomic levels by 1) domain, 2) kingdom, 3) *phylum*, 4) class, 5) order, 6) family, 7) *genus*, and 8) species. For some bacteria, such as *Escherichia (E.) coli*, even subspecies analyses are necessary to distinguish typical pathogenic from typical commensal strains. However, nowadays, the distinct differentiation in pathogenic and commensal bacteria has become somewhat outdated (58). In addition to living organisms, also viruses and bacteriophages are present. The next useful systematic level below the domains are *phyla*, two of which are most relevant for the bacterial microbiota, *Firmicutes* and *Bacteroidetes*.

Firmicutes are Gram-positive bacteria, and they make up circa 50% of the average human microbiota (Figure 1). The classes of *Firmicutes* include *Clostridia*, obligate anaerobes, and *Bacilli*. *Clostridia* are subdivided into their main families *Eubacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae*. *Ruminococcaceae* are further differentiated into the key

genera Ruminococcus and *Faecalibacterium (F.)* with its signature species *F. prausnitzii*. Another dominant *phylum*, making up circa 45% of most human microbiotas, is *Bacteroidetes*. A relevant class of these Gram-negative bacteria are *Bacteroidia* with two relevant *genera*: *Bacteroides (B.)* with the very dominant *B. thetaiotaomicron* (59) and *Prevotella (P.)*, with *P. copri*. Other important commensal bacteria *phyla* are *Proteobacteria* (*e.g.*, *E. coli*), *Actinobacteria* (notably *Bifidobacterium* of breastfed neonates) and *Verrucomicrobia* (*e.g., Akkermansia muciniphila*). These bacteria are sometimes referred to as "core microbiome" because of their almost ubiquitous presence in human microbiota. Interestingly, the abundance of the core species *Bacteroides*, *Faecalibacterium*, and *Prevotella* are inversely correlated (60). Therefore, analyzing microbiota by relative abundance of these species leads to clustering of the microbiota into one of three enterotypes (61). This does not mean that in the *Prevotella* enterotype *Prevotella* is the most common species. However, *Prevotella* is relatively enriched compared to the other enterotypes. Correspondingly, other species seem to display certain relative abundances, according to the enterotype.

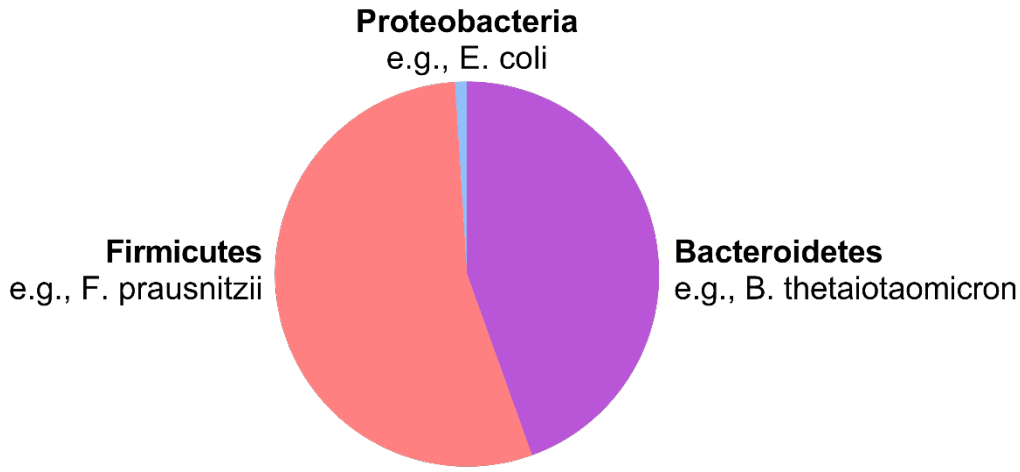


Figure 1: Average adult human gut microbiota under homeostatic conditions, showing the three main *phyla* and one example species each. Adapted from Dethlefsen *et. al.* (65).

The enterotype concept, however, has not been able to become widely accepted. One key criticism is that depending on the parameters and segmentation of clustering, more or less than three enterotypes could be found (62, 63). Also, metagenomic analysis provides evidence that, irrespective of the microbial composition, gut microbiota displays similar

functional pathways (64). Therefore, there is ongoing scientific debate if actual types of gut microbial composition exist or, likewise, if they mattered.

Microbiota compositions are highly variable. Major inter-individual differences exist both physiologically and in association with numerous diseases. Also, intra-individual differences are notable. These reflect different forms of symbioses in different regions of the gastrointestinal tract: Small intestinal microbiota mediates lipid absorption; for example, *Peptostreptococcaceae* (class: *Clostridia*) can be enriched in the duodenum but is normally absent in the distal small intestine (66). On the other hand, colonic microbiota, *e.g.*, *B. thetaiotaomicron*, degrades nondigestible polysaccharides into short-chain fatty acids (SCFA) as discussed below. These differences in microbial functions stem from different mucosal strategies to control microbial growth, which are suppression and separation. While in the small intestine immune-mediated suppression (such as antimicrobial peptides) subdues microbial growth, the colonic mucosa relies on spatial separation (via a thick mucus layer) allowing rigorous luminal growth of bacteria (58). In addition to different microbiota compositions along the gastrointestinal tract, there are also different ecological niches for microbial growth, more luminal or closer to the intestinal wall (7). This presents a challenge when sampling microbiota for analyses. Typically, fecal samples are collected. However, for sampling upper gastrointestinal microbiota or bacteria adherent to the intestinal wall, other methods are necessary. (67)

In the following, the **functions of the microbiota** will be discussed. The microbiota carries out numerous physiologic functions for the human host. Most importantly, the microbiota cleaves nutrients that are not digestible by human enzymes and makes them available to the host. Experiments in rats by Wostmann *et al*. showed that germ free animals had an 18% higher energy need because of fecal loss of calories (68). The main principles by which the microbiota provides their hosts with energy are fermentation, sulphate reduction, and hydrolysis of polysaccharides, such as resistant starch and fiber. These come notably from a carbohydrate diet, *i.e.*, whole grain products, fruits, or vegetables, and are often termed microbiota-accessible carbohydrates (69). The prototypical species for digesting dietary fiber is the in most microbiotas dominating *B. thetaiotaomicron*, which expresses numerous glycoside hydrolases and is able to break down starch, mannan, and glucan (70). Dietary fiber is broken down into 1) SCFA and 2) intermediaries. Key SCFA include acetate, butyrate, and propionate. Acetate is produced by all anaerobes, *e.g.*, *Bacteroides,*

Faecalibacterium, Ruminococcus, Fusobacterium, and it reaches the highest systemic plasma levels. Butyrate is produced by *Firmicutes*, *i.e.*, *Lachnospiraceae* and *Clostridia* with the main producing species *F. prausnitzii*. The main role of butyrate is the provision of local energy supply for colonic enterocytes. Indeed, lack of SCFA in a surgically diverted colonic segment, for example following Hartmann's procedure, can lead to diversion colitis, which, stunningly, can be successfully treated with local SCFA (71). Butyrate production is highly dependent on dietary carbohydrates. In low-carb diet, Russell *et al*. observed a disproportionate decrease of butyrate levels. (72) Propionate, on the other hand, stems from both carbohydrate and protein breakdown (73). Approximately 13 g of peptides reach the colon every day that can be hydrolyzed by the bacterial catalytic machinery (74). Main producer of propionate is *Bacteroides* and a possible key function is energy supply for hepatic gluconeogenesis from portal vein propionate (75). Next to SCFA, bacterial metabolism yields numerous intermediary substances, notably organic acids, such as lactate and succinate. Succinate acts as a substrate for gluconeogenesis in enterocytes and thereby aids systemic glucose homeostasis and normal body weight development in mice (76).

Another important function of the microbiota is the synthesis of vitamins, such as vitamin K2 (menaquinone) (77). While functionally relevant vitamin K deficiencies have only rarely been reported from lack of dietary intake, chronically managed patients receiving long-term antibiotic treatment are relatively susceptible to vitamin K deficiency (78). This demonstrates the relevance of microbiota-derived menaquinones.

Another key function of the microbiota is protection against infection by pathogens. Both direct biological mechanisms and immune-related control circuits play a role (79). Direct biological mechanisms include secretion of bacteriocins and competition for space and nutrients. Some metabolites might be further used by other microbes, thereby some bacteria facilitate growth of other commensals. For example, the metabolite lactate of *Cutibacterium* can be further oxidized by *Enterobacteriaceae* to CO_2 and H_2. These can, finally, be metabolized to methane by other strains. Commensals also deplete sugars that could be otherwise utilized by pathogens. Also, commensal-derived SCFA might directly inhibit certain pathogenic microbial growth, such as Shiga-toxin 2 producing *E. coli* 0157:H7, which is the cause of hemolytic uremic syndrome (80). Conversion of bile acids to secondary bile acids by *Clostridium (C.) scindens* mediates resistance to the pathogenic *Clostridioides difficile* (81). Lastly, quorum sensing-mediated repression of *Vibrio cholerae* growth by *Ruminococcus*

seems to be important for recovery from diarrhea (82). Immune-related control circuits are discussed in the following.

Commensals, such as *B. thetaiotaomicron*, stimulate IECs via Toll-like receptor ligation to produce antimicrobial peptides, *e.g.*, LL-37, which inhibits colonization by the facultative pathogen *Candida albicans* (83, 84). Moreover, immune cell development depends on the microbiota: *Lactobacillus reuteri* metabolizes tryptophane (an essential amino acid and precursor of vitamin B3 (niacin) and serotonin) to aryl hydrocarbon receptor ligands, which leads to ILC3 expansion and IL-22 expression (85). SFB, part of the family *Clostridiaceae*, induce the development of ileal nonpathogenic T_H17 cells in mice (86). Contrarily, colonic T_H17 are induced by *Helicobacter (H.) hepaticus* and cause colitis. Interestingly, in mice that were not susceptible to the development of colitis these *Helicobacter*-induced T_H17 are contained by induced RAR-related orphan receptor gamma $(ROR\gamma)^+$ $FOXP3^+$ T_{reg} (87). Also, *Clostridium* species can induce T_{reg} in the colon (88). These inducing effects are largely mediated by microbiota-derived SCFA that bind G protein-coupled receptors on IECs, notably seen as T_{reg} induction via SCFA binding to GPR43 (89). Overall, the microbiota is essential for normal immune development as outlined by low systemic immune globulin levels, small lymphoid organs, low mucosal immune cell count, and underdeveloped Peyer's patches of germ free animals (37, 90, 91).

Next, **microbiota development** is outlined. The microbiota is largely inherited from the mother (92). There is a lack of studies on the initial development of the neonatal microbiota, but it is generally assumed that vaginal and skin microbiota of the mother play the most important role. Correspondingly, neonates delivered by cesarean section show structurally different microbiotas (93, 94). Microbiota development underlies constant change in structural composition, beginning in infancy. Compositional changes also occur in response to stimuli or in association with disease states throughout life. Change occurs via outgrowth of existing bacteria by new species and via genetic alterations of the existing microbiota by mutations and horizontal gene transfer (95, 96). Change during ageing is particularly prominent: During lactation, *Actinobacteria (e.g.*, *Bifidobacterium*) make lactate the most abundant bacterial metabolite (as opposed to acetate in later life). During this period, *Proteobacteria*, such as *E. coli*, dominate, while in adulthood they comprise usually only approximately one percent of the gut microbiota. These neonatal *phyla* are quickly outgrown by *Firmicutes* and *Bacteroidetes* (97). One key abiotic component is the partial pressure of

oxygen. While some bacteria rely on a high oxygen supply, others can only survive in the absence of oxygen (obligate anaerobic bacteria). Two factors allow for anaerobic colonization. First, aerobic bacteria closer to the mucosa (which has a high supply of oxygenated blood) use up all oxygen, so that luminal bacteria, such as *Bacteroides spp.*, *Clostridium*, and *Eubacterium*, can grow in their required anaerobic environment. Second, the partial pressure of oxygen gradually falls towards more distal parts of the gastrointestinal tract. These mechanisms lead to predominant (more than 95%) anaerobic bacterial species in the colonic microbiota (98).

Factors that affect microbiota composition are plentiful. Changes in the microbiota are driven by both host factors and the exposome. Thereby, the microbiota is viewed as a signaling hub that integrates environmental, genetic, and immune-related influences. Thus, numerous effects might be mediated via a change in the microbiota's composition or function. (99) For example, mice deficient in NOD-like receptor family pyrin domain containing 6 (NLRP6) are more susceptible to colitis than *wild type* (*wt)* controls. This effect is probably carried out by a change of the microbiota. (100) Examples for immune-related shaping of the microbiota include altered microbiota in IL-22 deficient ($Il22^{-/-}$) mice (101) and in situations of abnormal mucin utilization by the microbiota (102). Additionally, disturbance of bile acids has been implied in the etiology of dysbiosis (103). Since bile acid synthesis is dependent on the diet, *i.e.*, fat intake, this displays an example of a three-step mechanism from diet to host factors to change in microbiota. External factors also shape the microbiota, most importantly diet and the resulting availability of microbiota-accessible carbohydrates (104). For example, Western pattern diet is associated with less microbiota-accessible carbohydrate and consequently lower alpha diversity (105). Another example of diet-induced alteration of the microbiota is the observed difference in microbiota composition between breastfed and formula-fed infants (106). Furthermore, xenobiotics, most commonly drugs, have a high potential of influencing the microbiota; after broad-spectrum antibiotic treatment, the recovery time back to a similar microbiota compared to the status quo is six months. (107, 108) Lastly, living arrangements, habits, such as smoking, and geographic area of residency (*e.g.*, rural *vs.* urban) have been implied to shape microbiota development (109, 110). Ultimately, microbiota composition is also dependent on the microbiota itself as seen by the inversely correlated abundances of *Bacteroides, Faecalibacterium*, and *Prevotella*. Mechanistically, *Bacteroidetes* do not tolerate low pH values that are created

by butyrate producers, *i.e.*, *Firmicutes* (111). Also, concurrence for nutrients and, conversely, cooperation in nutrient supply (microbial cross feeding) enforce mutual dependencies of commensal species (112).

Integrating the mutual effects of microbiota, environment, and the host (both in the gastrointestinal tract and systemically), it does not surprise that certain microbiota alterations are associated with and even causally involved in numerous common disease states. Key examples include insulin resistance, circadian rhythm disturbances, and inflammatory bowel disease (IBD) (113–116). How disruption of the three components of homeostasis, microbiota, IECs, and immune system, leads to IBD will be addressed in the next section.

2.2. Inflammatory bowel disease

2.2.1. Clinical features and epidemiology

IBD affects approximately half a percent of the Western world population (117). It features recurrent flares of gastrointestinal and, in some cases, systemic inflammation. The two typical entities of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Each of the two forms of IBD is an idiopathic inflammation of the gastrointestinal mucosa. In CD also the submucosal and muscular layer can be affected (termed transmural). Disease localization is grouped into ileal, colonic, ileocolonic, and isolated upper gastrointestinal by the Montreal classification (118). Transmural inflammation leads to the two most common local presentations of severe CD. First, transmural inflammation and subsequent wound healing can narrow the gastrointestinal tube, which can grow into mesenterial fat ("creeping fat"). This pattern of involvement is termed fibrostenotic CD. Second, transmural inflammation can lead to perforations and thereby fistulas to other organs or to abscesses, as seen in perianal CD. This pattern is called fistulizing. CD has four cardinal symptoms, which are abdominal pain, diarrhea (in some cases bloody), fatigue, and weight loss (119). The symptoms result from the underlying pathologies: Lower right quadrant pain can be a sign of terminal ileitis, which is the typical site for CD to develop. Cramping abdominal pain can be a sign of partial bowel obstruction that stems from strictures. Diarrhea in CD is probably inflammatory diarrhea, however, also malabsorption (for example of bile acids) might contribute to diarrhea in the sense of osmotically active compounds or steatorrhea. Fatigue is a systemic symptom of inflammation; hitherto its pathophysiology remains unclear (120). Weight loss is thought to be due to reduced oral intake in view of the mentioned symptoms. Extraintestinal symptoms

include arthritis, uveitis or episcleritis, erythema nodosum or pyoderma gangrenosum, and IBD-associated primary sclerosing cholangitis. Concerning their diagnosis, CD must be distinguished from ulcerative colitis to allow for optimal treatment. Main features that are indicative of CD rather than UC are: small bowel involvement, rectal sparing, and skip lesions (UC normally is continuous and almost always involves the rectum). If no clear distinction between the two types of IBD can be made, this is termed indeterminate colitis. Patients are managed based on risk stratification, assessment of disease activity, and treated with glucocorticoids, antibiotics, thiopurines, and biologicals (mainly, cytokine neutralizing antibodies). In many cases, also surgical interventions are necessary.

Ulcerative colitis has similar key symptoms, (bloody) diarrhea, which may be associated with incontinence, and colicky abdominal pain, *e.g.*, with tenesmus and fever (119). Local complications are bleeding, perforation, and fulminant colitis with toxic megacolon (which can, more rarely, also occur in CD) (121). Similar to CD, also patients diagnosed with ulcerative colitis have a high risk of circa 25% to develop extraintestinal complications. UC patients also have a relevant risk for developing inflammation-associated colorectal cancer. Therefore, a portion of patients, whose colitis cannot be controlled satisfactorily, require prophylactic colectomy, which generally heals the disease (122). Medical management of UC includes treatment according to risk stratification by clinical and endoscopic evaluation (*e.g.*, Mayo Clinic score) (123). Effective drugs are mesalamine, glucocorticoids, 5-aminosalicylic acid, biologic agents (including anti-integrin therapy with vedolizumab), and Janus kinase inhibitors.

A positive family history remains the only substantial risk factor with a relative risk of three to 20 for first-degree relatives and 20% (UC) to 50% (CD) risk for monozygotic twins (124, 125). Interestingly, this vast polygenetic risk component could hitherto not be mapped sufficiently onto specific genetic variants: Extensive genome-wide association studies (GWAS) found variants which explain only a small portion of the genetic risk that is known from twin or cohort studies. A lot of undetected variants carry genetic susceptibility, and, of the detected loci, only some could be traced down to specific genes and specific single-nucleotide polymorphisms (126–128). Next to disease susceptibility and risk for severe disease activity, also the phenotype of disease is partially genetically predisposed. Both location (*i.e.*, ileal, colonic, ileocolonic, *etc.*) and behavior (fibrostenotic *vs.* fistulizing) have a heritable component (129). IBD typically develops in younger adults, and both sexes are

affected. There is a weak tendency for women to develop CD and for men to develop UC. Thus, hormonal factors might play a role. The use of oral contraceptives increases the risk for UC. (117, 130, 131)

The incidence of IBD has been increasing over the last decades. Since genetic risk factors do not change such rapidly, this observation is being attributed to environmental and lifestyle factors. Smoking has been shown to increase susceptibility for CD (132). In ulcerative colitis, however, smoking cessation of active smokers leads to a higher risk of disease severity and complications (133). In addition, physical inactivity has been linked to susceptibility for CD (134). Interestingly, also geographic heterogeneity of IBD incidence points towards risk factors: Based on the observation that in northern latitudes IBD susceptibility is higher, low vitamin D levels have been established a risk factor (135, 136). Also, dietary factors play a role. While high-fiber diet seems to lower the risk for CD specifically, high-fat diet implies risk for both types of IBD (137). Furthermore, medical conditions that alter the mucosal immune system can increase IBD risk. This has been shown for acute infectious gastroenteritis as an IBD trigger (1, 138). Lastly, there are numerous studies on drugs and medical or surgical procedures as risk factors. Antibiotics might be a risk factor for CD whilst, at the same time, being utilized for treatment of CD in some cases (139, 140). Very frequent use of nonsteroidal anti-inflammatory drugs (>15 days per month) might increase the risk whilst also being sometimes used as a treatment for IBD (141). Appendectomy for a non IBD cause under the age of 20 years lowers the risk for the development of UC (142). Concerning psychological factors, so far, no clear association has been found. However, some data suggest that relapses of disease might depend on the patient's perceived stress levels (143).

Despite UC and CD being distinct entities, the substantial overlap concerning risk factors, clinical features, pathophysiology, and efficacious treatment options justifies discussing them unified as *IBD* as we will do for the rest of this manuscript.

2.2.2. Pathophysiology and relevance of cytokines

IBD pathophysiology is understood as interplay of host microbiota, immune system, genetic susceptibility, and all dietary/external/environmental factors (exposome). Owing to rising incidences over the last decades, emphasis has somewhat shifted away from genetics as key factor and towards the exposome and subsequent alterations in microbiota. Essentially, all introduced principles of homeostasis of the intestinal mucosal and its three components

are disrupted in IBD. Pathologic changes in IECs, immune cells, and the microbiota have been implied in the pathogenesis. However, the causes remain elusive. So, IBD is an idiopathic and multifactorial disease.

Concerning the intestinal epithelium, different human susceptibility loci and animal studies have revealed mechanisms, by which disruption of normal IEC function leads to intestinal inflammation. If the anatomic barrier fails, inducers of inflammation, such as microbial structures, lead to proinflammatory activation of lamina propria cells, culminating in tissue destruction. This is, for example, seen following disruption of barrier integrity in mice by administering dextran sulfate sodium (DSS) (144). Similarly, in humans increased epithelial permeability and tight junction disruption has been implied in IBD pathogenesis, which might be precipitated by an acute gastroenteritis (145). Also, the key function of pattern recognition receptor signaling, *i.e.*, detection of microbial products as already explained, plays an important role. Additionally, Paneth cell dysfunction predisposes to IBD (146). The most relevant risk locus for IBD (in particular, CD) is *NOD2*. NOD2 disruption leads to reduced synthesis of antimicrobial peptides and impaired barrier integrity. (146, 147) Linked risk loci are *ATG16L1* and *XBP1*. These are – as discussed – essential for autophagosome formation around ingested microbes and IEC-derived cytokine release, respectively. Lastly, mucin single-nucleotide polymorphisms (*e.g.*, MUC19) have also been related to IBD risk.

Other risk loci account for the host immune system. Cytokines and their receptors have been assigned a central role in IBD pathogenesis. Both efficacy of anti-cytokine treatments and results from GWAS point towards cytokines as critical drivers of IBD. The physiologic function of cytokines is induction of T cell differentiation and amplification of immune modules.

Concerning T cell differentiation, for example *IL12B* and *IL23R* play a role. The IL-23 receptor (gene: *IL23R*) is expressed on T cells and is specific of p19-dependent signaling of T cells towards T_H17 lineage commitment. *IL12B* encodes for p40, the common subunit of IL-12 and IL-23. Thus, also the *IL12B* risk locus belongs to the T_H17 lineage development axis. (128) The importance of this axis is also demonstrated by the integrative human microbiome project: IL-17 receptor signaling-related genes were the highest differentially expressed genes (DEGs) between UC and healthy ileal biopsies (148). Strikingly, blocking this axis with neutralizing antibodies against the common IL-12/IL-13 subunit p40, *e.g.*, with ustekinumab (Stelara®), is an effective therapy for IBD (149, 150).

Other cytokines are primarily involved in amplifying innate immune modules. Innate immunity can be viewed as the effector module of inflammation. Its relevance is for example demonstrated by the observation that genes of the complement cascade are the most significant DEGs of rectal samples of UC patients compared to non IBD controls (148). Among these cytokines, tumor necrosis factor alpha (TNFα or TNF) is viewed as the most important one for IBD pathophysiology. Anti-TNF therapy, *e.g.*, with infliximab (Remicade®), is a very effective therapy for moderate to severe IBD. In IBD, TNF is produced in aberrantly high amounts by multiple cell types, mostly by $CD14^+$ macrophages (but also, by dendritic cells, T cells, and fibrocytes or adipocytes) (151). TNF is synthesized as a membrane-bound homotrimer (26kD), which is cleaved by ADAM metallopeptidase domain 17. Both membrane-bound (mTNF) and the usual soluble forms are biologically active. Some data suggest that mTNF, in particular, is essential in IBD pathogenesis. (152–155) TNF acts by binding its receptors tumor necrosis factor receptor 1 (TNFR1) (ubiquitously expressed) and tumor necrosis factor receptor 2 (predominantly expressed on lymphocytes). All lamina propria immune cells and the IEC layer are potential targets of TNF. TNFR1 is associated with three distinct pathways, which indicate the destructive potential of TNF signaling. TNFR1 can interact with complex I, which activates receptor-interacting serine/threonine-protein kinase 1, leading to a) NF-κB translocation and b) mitogen-activated protein kinase-catalyzed phosphorylation of activator protein 1. NF-κB and activator protein 1 activity lead to inflammation. Via interaction with complexes IIa and IIb, TNFR1 signaling can directly lead to apoptosis via death-domain-dependent caspase 8 activation. Via complex IIc it can induce necroptosis. One specific example of these lethal pathways is TNF-induced IEC disruption and Paneth cell death. (156, 157)

Another key cytokine is **IL-22**, which is produced by T cells (mainly of those towards the T_H17 lineage or, similarly, T_H22), ILC3, NK cells, and others (158). Target cells are nonhematopoietic, including epithelial cells, such as IECs. At these target cells, IL-22 signals through a heterodimeric receptor, comprising the alpha 1 subunit (IL22RA1) and the IL-10 receptor beta subunit (IL10RB), which is a common receptor chain for all cytokines of the IL-10 superfamily (159). This signaling leads to transcription of antimicrobial peptides, mucin-related proteins (MUC1, MUC3), proliferative pathways (Ras, c-Raf, mitogen-activated protein kinases), and activation of the deoxyribonucleic acid (DNA) damage response (43, 160). So, the main function of IL-22 in the gut is enhancing barrier protection

via antimicrobial peptides and regeneration/renewal of damaged IECs (158). Gene knockout leads to increased susceptibility to infections and colitis (160–162). Indeed, also in IBD IL-22 plays a protective role (163, 164). However, there are also unfavorable effects of IL-22, which include increased inflammation (at skin or joints) or augmented intestinal tumor development, which seems to be the case for unconstrained IL-22 activity (43, 165–167). Therefore, IL-22 can be assumed to display different functions depending on the situation and/or the surrounding circumstances, such as microbiota. In the setting of intestinal inflammation and mucosal injury, IL-22 would physiologically confine inflammation and induce wound healing. In IBD, however, this response to inflammation and injury is altered. Samples from IBD patients and murine studies revealed that the natural inhibitor of IL-22, IL-22 binding protein (IL22BP), is overproduced, such that the protective effect of IL-22 cannot ensue (168). Strikingly, IL-22 also acts as a key mediator of the careful balance between absorptive and barrier functions of IEC. Recently, different diet-regulated circuits of IL-22 release have been discovered. In the presence of genotoxic stress from the diet, IL-22 can be upregulated, strengthening the barrier and inducing apoptosis in damaged cells (43). In 2020, Talbot *et al*. showed an opposite circuit: Following nutrient-rich food intake, IL-22 can be downregulated via a neuro-immune circuit leading to enhanced lipid absorption (169).

Some human monogenetic syndromes convey an exceptionally high risk for developing IBD at young age (very-early-onset IBD). Notable is the immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. Essentially, a loss of function of FOXP3 leads to an imbalance in T cell polarization. This T cell imbalance is characterized by lack of T_{reg} that could outweigh proinflammatory T cell effects. This shows that systemic immune dysregulation can result in intestinal inflammation. In this sense, colitis can be viewed as a nonspecific symptom of immune disbalance. Correspondingly, IL-10 receptor loss-of-function mutations also lead to very-early-onset IBD. Interestingly, both principles can be utilized for murine models of IBD (see section of IBD models below).

In addition to the host immune system, the host **microbiota** also appears to contribute causally to pathology. Five main pieces of evidence point towards this. First, microbiota in IBD shows distinct changes from normal. Second, microbiota reacts vigorously to environmental factors (as discussed above) and could thereby integrate different risk factors. Thirdly, murine models of colitis require presence of microbiota in that most models do not lead to substantial inflammation in germ free mice. Fourthly, while healthy human intestinal

surfaces are not colonized (similar to the Gram-positive "demilitarized zone"), colonic IBD biopsies show disruption of the mucus barrier with bacterial overgrowth (170). And, most importantly, probiotics, such as *E. coli* Nissle 1917, and FMT can be effective treatments for IBD patients, particularly in UC (116, 171). The distinct changes in IBD are profound and have diagnostic value, however, the integrative human microbiome project found that they are far less pronounced than normal inter-individual variations. In a profound machine learning analysis of 132 subjects, below 10% of the variation was explained by presence or absence of IBD, whereas circa 70% of the variation was explained by the individual subject. (148) However, some trends are quite consistent for most IBD patients and underline the principle of disrupted homeostasis: Biodiversity, *i.e.*, alpha diversity, is reduced, while general instability of microbiota is increased (148, 172). There is a shift between the two main *phyla*, from *Firmicutes* (normally dominating the microbiota) to *Bacteroidetes* (normally making up circa 45% of the gut microbiota). Since alpha diversity can be estimated by the F/B ratio, this structural shift from *Firmicutes* to *Bacteroidetes* reflects a lower biodiversity. This F to B shift can be translated to specific classes and species, which due to lack of more profound data might seem somewhat arbitrary: Reduced *Firmicutes* of the *Clostridia* class include *F. prausnitzii, Phascolarctobacterium*, *Roseburia*, and *Subdoligranulum*. Also, some of the *Bacilli* are reduced: *Leuconostocaceae* (family level) and *Odoribacter* (173). Most of these negatively correlate with disease intensity (174). Of all these, *F. prausnitzii* seems to be the most relevant. This is because it is (as discussed above) the main producer of butyrate, which is pre-eminent for intestinal homeostasis. Interestingly, substitution of *F. prausnitzii* alleviates murine colitis (175). Enrichment of *Bacteroidetes* includes the family *Prevotellaceae*. The F/B paradigm is a simplification since there also pronounced enrichments of some *Firmicutes* species (*i.e.*, *Ruminococcus gnavus*) and depletion of *Bacteroidetes genera (i.e.*, *Alistipes*) are observed. Also, members of the third relevant *phylum*, *Proteobacteria*, especially *E. coli* and *H. hepaticus*, are enriched. One example is the pathobiont enteroadhesive *E. coli* (176). Also, *Actinobacteria* (making up below one percent) are enriched, *e.g.*, *Rhodococcus*. Lasty, several *Enterobacteriaceae* are enriched and also correlate with disease severity (174). All these changes are in summary called *dysbiosis*. Interestingly, dysbiosis correlates with other disease markers, such as serologic titers of perinuclear antineutrophil cytoplasmic antibodies (p-ANCA – in UC), anti-saccharomyces cerevisiae antibodies (ASCA – in CD), anti-CBir1 flagellin antibody (CBir1), and anti-E. coli Outer membrane porin C precursor (OmpC), which usually is part of the p-ANCA group.

Structural changes in microbiota also affect viruses. For example, enrichment of bacteriophages has been observed at the beginning of developing dysbiosis in the integrative human microbiome project. (148)

In addition to structural changes in the microbiota, substantial functional alterations have been implied in metabolomic experiments. Vitamins B3 (niacin) and B5 (pantothenate) are reduced. At the same time, niacin metabolites are enriched, suggesting microbial decay of vitamin B3. Intestinal bile acids are also disrupted. While primary bile acids, such as cholate and related conjugates, are enriched, secondary (*i.e.*, microbially modified) bile acids are diminished. This implies that either bile acid-modifying *taxa* are reduced or that due to reduced transit time (diarrhea) secondary bile acids cannot be synthesized (148). Fatty acids are also markedly affected by microbiota and altered in IBD-related dysbiosis. In consistency with reduction of SCFA-producing *Firmicutes*, SCFA are also reduced. This implies loss of their immunoregulatory function during homeostasis. On the other hand, acylcarnitines, rather proinflammatory fatty acids that get heavily modified by microbiota, are enriched. Taken together, all components of the intestinal mucosa, IECs, immune system, and microbiota, play an important role in IBD. Key risk factors are affected first degree relatives and multifactorial aspects of the exposome.

2.2.3. Murine models for IBD

Genetic models are based on the principles of homeostasis outlined above and on human very-early-onset IBD risk alleles. These models include mice deficient in IL-10 signaling, leading to disruption of the pro/anti-inflammatory balance, XBP1 deficient mice (disrupting the epithelial response to stress), and multidrug resistance protein 1 deficient mice, which fail to export xenobiotics into the intestinal lumen (144). **Chemically induced models** rely on disrupting the epithelial barrier so that inflammatory inducers (microbiota or microbial byproducts) can translocate to the intestinal surface and into the lamina propria. Two commonly used substances are oral DSS and rectal 2,4,6-trinitrobenzenesulfonic acid (TNBS) (144). Interestingly, most models require presence of intestinal microbiota. DSS leads to merely mild colitis in germ free mice (177).

An **adoptive transfer model** is the T cell transfer model of colitis. A lymphopenic host, typically recombinase activating gene 1 deficient (*Rag1*$^{-/-}$), is reconstituted with a specifically selected T cell population from a *wt* or, depending on the experimental question,

a specific knockout donor. The selected population is defined by cell surface proteins as $CD4^+$ $CD25^-$ $CD45RB^{hi}$ (in short, $CD45RB^{hi}$). $CD45RB^{hi}$ T cells are mostly naïve, nonregulatory, $CD4^+$ T cells. T_{reg} are absent both in the transferred cell population and in the lymphopenic recipient. Therefore, the T cell model of colitis nicely displays the effects of immune disbalance and its significance for IBD pathogenesis. T cell transfer colitis is based on a successful selection of the $CD45RB^{hi}$ population. The selection can be made solely based on surface markers because of the distinct biological functions of these proteins. $CD45RB^{hi}$ T cells are mostly naïve, nonregulatory, and induce colitis upon transfer into *Rag*$^{-/-}$ mice (178).

After peritoneal injection, $CD45RB^{hi}$ T cells need to expand and differentiate before they become relevant to the host. They migrate systemically, undergo *in vivo* expansion, and differentiation into effector T cell subsets and subsequently inflame the colonic mucosa (179). Excitingly, both T cell expansion and inflammation rely on major histocompatibility complex class II-dependent presentation of antigen. This implies a role for antigen presenting cells, such as dendritic cells, to ingest, process, and present foreign antigens to the expanding pool of $CD45RB^{hi}$ T cells. Considerable evidence indicates that these antigens could be derived from the microbiota. (180) The naïve T cells migrate after transfer. After migration to the colon, these T cell can be isolated from the lamina propria. Interestingly, they then express markers of effector or memory T cells. Also, their TCR repertoire is limited, suggesting that not all $CD45RB^{hi}$ T cells expand – but only those activated by microbiota. Moreover, in germ free mice transferred $CD45RB^{hi}$ T cells do not expand properly, *i.e.*, no T cells could be isolated in substantial numbers from the recipients. These germ free mice consequently did not develop colitis (179). Interestingly, more specific studies found that commensals, such as *B. fragilis*, were protective via T_{reg} induction. Pathobionts, such as *H. hepaticus*, increased colitis (181). These findings support the concept of human IBD being driven by an immune response to microbiota and the significant role for dysbiosis for this T cell development. In T cell transfer colitis the recipient host has no regulatory T cells to outbalance the highly active expanded naïve T cell population (144). Owing to this global immune disbalance, T cell transfer colitis has a broader phenotype than other colitis models: Next to colitis, also liver and small intestinal inflammation develop within the weeks after transfer (178, 182). Colitis is particularly pronounced because of the vast presence of microbial inflammatory inducers. T cell-derived cytokines are indispensable for T cell colitis and can

be functionally divided into two groups, those for further T cell development and those acting on innate immune modules. Generally, the essential cytokines can be linked to the T_H1 and T_H17 axis (183). IL-23, which is needed for T_H17 differentiation, is indispensable (184). Regarding effector cytokines, however, T_H1 cytokines dominate (185). Accordingly, IL-12, interferon (IFN)-γ, and TNFα are required and their neutralization is an effective treatment (186). The requirement of T_H17 cytokines despite the dominance of T_H1 cytokines in this model led to the question which of these two T helper subsets played the more important role (183). Evidence from reporter mice has made clear that this seemingly discrepant observation comes from $CD4^+$ T cell plasticity (187). There is one key transdifferentiation implied in $CD45RB^{hi}$ colitis and in CD. First, T_H17 develop which then later differentiate into T_H1. So, nowadays, the formerly distinct T helper subpopulations are viewed as plastic and less lineage committed (40). A main cytokine that ameliorates $CD45RB^{hi}$ T cell transfer colitis is IL-22, such that mice develop more severe disease in the absence of IL-22 (101, 162). This effect seems to be in part carried out by IL-22-dependent control of the microbiota since IL-22 deficient mice had an altered microbiota. When transferred to *wt* mice, this microbiota carried out a colitogenic effect even in these IL-22 competent mice (101). In summary, oligoclonally expanded T cells produce numerous cytokines and ultimately cause inflammation. The histologic hallmark of inflammation is an infiltrate of immune cells accompanied by edema. In the following, the pathological characteristics of the inflammation in $CD45RB^{hi}$ colitis are briefly summarized. Due to infiltrate and edema the colonic wall is thickened, which can also be observed macroscopically. Along with this, colon shortening can be observed. Histologically, the wall thickening is due to IEC hyperplasia and a transmural cellular infiltrate (186). Predominant immune cells of this infiltrate include macrophages, neutrophils, and expanded T cells, which account for a chronic inflammatory process. The infiltrating cells can form cryptitis, crypt abscesses, and cause epithelial erosion (188). Clearly, the above introduced barrier integrity is lost, which further emphasizes the relevance of microbial translocation to the lamina propria as an inflammatory driver. Practical details of this model are described later and in numerous publications which will be referred to in the methods section.

2.3. Current knowledge of microbiota-dependent cytokine effects

The paradigm that microbiota is altered by different external and host factors and thereby transmits the effect of such factors on the organism is founded on different experiments.

IL-22 shapes the microbiota in that IL-22 deficient mice develop dysbiosis and because of this higher colitis susceptibility (101). Another example is the NLRP6 inflammasome and its IL-1 and IL-18 cytokines. While *Nlrp6*$^{-/-}$ mice are highly susceptible to colitis, this effect does not come from NLRP6 deficiency itself but from subsequent change of the microbiota. This concept could be proven by transmitting the altered microbiota to *wt* mice which thereby where rendered equally susceptible (100). Also, human studies have revealed causal roles of the microbiota in numerous diseases. While shift work conveys a higher cardiovascular risk, this risk is transmitted by a change of the microbiota. Transferring microbiota from shift workers to germ free mice led to glycemic intolerance compared to healthy controls (114). Next, one 2019 study led the way for pharmacodynamic responses being dependent on microbiota: While immune checkpoint therapy induces remission in some melanoma patients, others do not respond. This response to therapy relies on the individual microbiota and could be transferred onto germ free animals (189). Also, pharmacokinetics depend drastically on the individual microbiota. Since many xenobiotics and drugs are metabolized by the microbiota, differences in microbiota can lead to substantial fluctuations in active metabolite concentrations and possibly adverse drug reactions. One study found that up to 70% of oral delivered brivudine, an antiviral agent, is metabolized by *B. thetaiotaomicron* to bromovinyluracil, an hepatotoxic metabolite (59). Different research groups have assessed that IL-22 protects mice from colitis in several, but not all, colitis models. Prior work from our laboratory found, intriguingly, that IL-22 did not protect mice in our facility. Strikingly, after transmission of microbiota from one of the research facilities that had shown a protective effect of IL-22, also our mouse lines showed the protective effect of IL-22. This led to the conclusion that IL-22 protects from colitis depending on the harboring microbiota (190).

3. Materials and Methods

3.1. Tables of used materials and PCR reactions

3.1.1. Table 1: Chemicals and reagents used during the experiments

Chemical / reagent	Manufacturer
3M Sodium Acetate	AppliChem GmbH, Darmstadt, Germany
anti-IL-22 antibody (clone: AF582)	R&D Systems, Minneapolis, Minnesota, United States
Agarose Ultra Pure	Life Technologies, Carlsbad, California, United States
Ammonium chloride (NH4Cl)	Th. Geyer GmbH & Co. KG, Renningen, Germany
BBL Fluid Thioglycollate Medium	BD, Franklin Lakes, New Jersey, United States
BD FACS Clean Solution	BD, Franklin Lakes, New Jersey, United States
BD FACS Rinse Solution	BD, Franklin Lakes, New Jersey, United States
BD FACS Sheath Fluid Solution	BD, Franklin Lakes, New Jersey, United States
Brain Heart Infusion Broth	Sigma-Aldrich, St. Louis, Missouri, United States
Chloroform, J.T.Baker™	ThermoFischer Scientific, Waltham, Massachusetts, United States
Click's Medium	Sigma-Aldrich, St. Louis, Missouri, United States
Collagenase IV (100 U), from *Clostridium histolyticum*	Sigma-Aldrich, St. Louis, Missouri, United States
Diethyl Pyrocarbonate-treated (DEPC) water, Rnase-free	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Distilled water	B. Braun Melsungen AG, Melsungen, Germany
Dithiothreitol (DTT)	AppliChem GmbH, Darmstadt, Germany
DNase I	ThermoFischer Scientific, Waltham, Massachusetts, United States
dNTP Mix	Fermentas, Waltham, Massachusetts, United States

dNTP Mix (100mM)**	ThermoFischer Scientific, Waltham, Massachusetts, United States
Dream Taq 10x Green buffer	ThermoFischer Scientific, Waltham, Massachusetts, United States
Dream Taq DNA Polymerase	ThermoFischer Scientific, Waltham, Massachusetts, United States
Dulbecco's Phosphate buffered Saline (PBS)	ThermoFischer Scientific, Waltham, Massachusetts, United States
Eosin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Th. Geyer GmbH & Co. KG, Renningen, Germany
Ethanol (absolut)	Th. Geyer GmbH & Co. KG, Renningen, Germany
Ethanol 96%, vergällt	Walter CMP GmbH & Co. KG, Kiel
Ethidium Bromide (0.07%)	AppliChem GmbH, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA) [for microbial DNA isolation]	Th. Geyer GmbH & Co. KG, Renningen, Germany
Fetal Bovine Serum (FBS)	Sigma-Aldrich, St. Louis, Missouri, United States
Formaldehyde solution 36.5-38%	Sigma-Aldrich, St. Louis, Missouri, United States
GeneRuler 1kb Plus DNA Ladder	Fermentas, Waltham, Massachusetts, United States
Hank's Balanced Salt Solution (HBSS)	ThermoFischer Scientific, Waltham, Massachusetts, United States
Hydrochloric acid	Th. Geyer GmbH & Co. KG, Renningen, Germany
Ionomycin, Calcium Salt	Sigma-Aldrich, St. Louis, Missouri, United States
Isoflurane	Abbvie Inc., North Chicago, Illinois, United States
CD4 (L3T4) MicroBeads mouse (MACS antibody)	Miltenyi Biotec, Bergisch Gladbach, Germany
Mayer's hemalum solution (Hematoxylin)	Sigma-Aldrich, St. Louis, Missouri, United States
Monensin 1000x Solution	BioLegend, San Diego, California, United States

MultiScribe® Reverse Transcriptase (50 U/µL)**	ThermoFischer Scientific, Waltham, Massachusetts, United States
4-Nonylphenyl-polyethylene glycol (NP 40)	Sigma-Aldrich, St. Louis, Missouri, United States
Normal Goat IgG Control (polyclonal)	R&D Systems, Minneapolis, Minnesota, United States
Paraformaldehyd 4%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Pen Strep (penicillin G and streptomycin)	Gibco, Life Technologies GmbH, Darmstadt, Germany
peqGOLD TriFast™ and TriFast™ FL	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Percoll®	GE Healthcare, Chicago, Illinois, United States
Phenol/Chloroform/Isoamyl alcohol 24:24:1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, Missouri, United States
Phosphate-buffered saline (PBS)	Sigma-Aldrich, St. Louis, Missouri, United States
Potassium bicarbonate (KHCO3)	Th. Geyer GmbH & Co. KG, Renningen, Germany
Propan-2-ol (isopropanol)	Th. Geyer GmbH & Co. KG, Renningen, Germany
Proteinase K enzyme	Roche AG, Basel, Switzerland
RPMI Media 1640	ThermoFischer Scientific, Waltham, Massachusetts, United States
RT buffer**	ThermoFischer Scientific, Waltham, Massachusetts, United States
RT Random Primers**	ThermoFischer Scientific, Waltham, Massachusetts, United States
Sodium chloride	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS) 20%	AppliChem GmbH, Darmstadt, Germany
Sodium hydroxide	Th. Geyer GmbH & Co. KG, Renningen, Germany
TaqMan Fast Advanced Master Mix	ThermoFischer Scientific, Waltham, Massachusetts, United States
TE buffer	AppliChem GmbH, Darmstadt, Germany

Tris(hydroxyethyl)aminomethane (tris)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trypan blue 0.4% solution	Sigma-Aldrich, St. Louis, Missouri, United States
Xylene substitute	Sigma-Aldrich, St. Louis, Missouri, United States
Zombie Aqua™ Fixable Viability Kit (fluoro-chrome UV 379_28)	BioLegend, San Diego, California, United States

** part of the High-Capacity cDNA Reverse Transcription Kit, ThermoFischer Scientific, Waltham, Massachusetts, United States

3.1.2. Table 2: Antibodies used for flow cytometry

Antibody target	Clone	Fluorochrome	Dilution	Manufacturer
CD3	17A2	BV 650	1:400	all: BioLegend, San Diego, California, United States
CD4	RM4-5	Alexa Fluor 700	1:400	
CD11b	M1/70	PE Cy7	1:400	
CD11c	N418	FITC	1:200	
CD45	30-F11	BV 786	1:400	
FOXP3	NRRF-30	PE	1:100	
IL-17	9D3.1C8	Alexa Fluor 488	1:100	
Interferon-γ	XMG1.2	Alexa Fluor 647	1:100	
Ly6C	HK1.4	PE	1:400	
Ly6G	1A8	APC	1:400	
NK1.1	PK136	BV 421	1:200	

3.1.3. Table 3: Buffers used during the experiments

Buffer	Composition
10x Ammonium-Chloride-Potassium (ACK) buffer	NH4Cl 150 mM, KHCO3 10 mM, EDTA 1 µM, in DEPC-H2O
MACS buffer	10% FBS, 2mM EDTA in 1x PBS
Brain Heart Infusion (BHI) media	37 g BHI in 1 L H2O
4% formaldehyde solution	40 g PFA, in 1 l ddH2O, adjusted to pH = 7,4 with HCl
TE buffer	10 mM tris, 1mM EDTA, adjusted to pH 8.0 with HCl and NaOH
2x buffer A for fecal DNA Isolation	200 mM NaCl, 200 mM tris, 20 mM EDTA, adjusted to pH 8.0 with HCl and NaOH

All buffers are composed of chemicals from Table 1.

3.1.4. Table 4: Oligonucleotide primers used for microbiota analyses

Specificity	16S rRNA primer sequences	Amplicon	Ref.
All bacteria	7F: 5'-AGA GTT TGA TCC TGG CTC AG-3' 1492R: 5'-TAC GGT TAC CTT GTT ACG ACT T-3'	1200 bp	(191)
Prevotella spp.	181F: 5'-CGT CCC TTG ACG GCA TCC GAC A-3' 1032R: 5'-CAG CCC CGA AGG GAA GGG GTG-3'	849 bp	(192)
H. hepaticus	B38: 5'-GCA TTT GAA ACT GTT ACT CTG-3' B39: 5'-CTG TTT TCA AGC TCC CC-3'	417 bp	(193)
H. typhlonius	5'-AGG GAC TCT TAA ATA TGC TCC TAG AG-3' 5'-ATT CAT CGT GTT TGA ATG CGT CAA-3'	122 bp	

Primers are specific for the 16S rRNA gene of specific groups of bacteria. Amplicon size in base pairs (bp). Primers are ordered (forward primer, reverse primer). Ref.: Reference.

3.1.5. Table 5: PCR program

Step	Temperature	Duration
1	94 °C	2:00 min
2	94 °C	0:20 min
3	55 °C	0:30 min
4	72 °C	1:00 min
5	Go to step 2	30 times
6	72 °C	5:00 min
7	4 °C	0:00 min

3.1.6. Table 6: PCR reaction mix

Reagent	Volume per reaction
Dream Taq 10x Green buffer	3.0 µl
dNTP Mix	8.0 µl
Dream Taq DNA Polymerase	0.5 µl
Forward primer	0.9 µl
Reverse primer	0.9 µl
H_2O	12.7 µl
Sample DNA (0.3 µg/µl)	2.0 µl (0.5 µl, for the *Prevotella spp.* PCR)

3.2. Mice

3.2.1. General and legal aspects

Animal experiments were conducted under permission of the health authority (Behörde für Gesundheit und Verbraucherschutz – Veterinärwesen/Lebensmittelsicherheit – der Freien und Hansestadt Hamburg). All animal experiments were conducted according to granted protocols under the experimental proposals Tierversuchsantrag (TVA) 13/17 and 12/17. Thereby, during the entire time of mice breeding and transferring, and during the experiments, the animal burden was reduced to a minimum. When mice fulfilled clearly defined criteria, those mice were euthanized. Euthanasia was carried out by a three-step approach consisting of anesthesia (with a oxygen/carbon dioxide gas), cervical dislocation, and confirmation of death. For all experiments careful considerations were taken to use only the minimal number of test animals required to answer the research questions. In accordance with the European Animal Welfare Directive, German law, Hamburg health authority, and University Medical Center Hamburg-Eppendorf (UKE) regulations, we based our experiments on the 3R principle of replacement, reduction, and refinement. My regulatory authorization was the UKE course for mouse research and the health authority approval (Ausnahmegenehmigung nach §16 Tierschutz-Versuchstierverordnung). We used age-matched litter mates, female and male mice for our experiments to exclude influence of age, sex, or batch effects.

3.2.2. Mouse lines

We used three lines: one *wt* line (C57B6/J) and two *Rag1*$^{-/-}$ lines which differed in their microbiota and were bred in individual colonies. All lines were on a genetic background of C57B6. The *wt* line (C57B6/J) was on a background from Jackson laboratories, and the *Rag1*$^{-/-}$ lines were on a background from National Institutes of Health, United States, respectively. *Rag1*$^{-/-}$ carry a homozygous knockout of the recombination activating gene 1. The recombination activating genes are crucial for V(D)J recombination of lymphocytes and are thereby the decisive genes leading to development of an adaptive immune system with T and B cells. T and B cells rely on V(D)J recombination for formation of highly variable antigen receptors, which can thereby recognize plentiful foreign antigens. *Rag1*, nowadays viewed as a domesticized transposon, is found in jawed vertebrates. (194, 195) This means that all these animals have the ability for V(D)J recombination and developing T and B cells.

By knocking out either *Rag1* or *Rag2*, no adaptive immune system can develop. *Rag*$^{-/-}$ mice are lymphopenic, meaning they lack T or B cells, and have underdeveloped lymphoid organs.

3.2.3. Animal husbandry

Both colonies, C57B6/J and *Rag1*$^{-/-}$, were kept in homozygous breeding at the breeding facility of the UKE. The mice were held under specific-pathogen-free (SPF) condition in individually ventilated cages (IVC). The excluded pathogens are for example the *cilia-associated respiratory Bacillus*, the colonic pathogen *Citrobacter rodentium,* and mouse hepatitis virus (MHV) (196). The individual ventilation preserves positive pressure in the cages with inwards flow of fresh air, which is free of infective agents. Because of the continuous inflow, the used air is passively removed from the cages through filter cloths in the IVC lid. This achieves more than 25 cycles of air exchange per hour, low ammonia concentrations, and minimizes the risk of spread of infectious agents or microbiota from one cage to another (197). Per cage up to five mice of either female or male sex were housed together. Animals were kept at a stable 12-hour light/dark cycle.

3.2.4. Antibody injections

As established by our laboratory earlier, injections of neutralizing anti-mouse *in vivo* antibody (clone AF582, see also Table 1) or isotype control (normal goat IgG control, see also Table 1) were performed intraperitoneally, 50 µg in 200 µl PBS (198).

3.3. Mb1 and Mb2 microbiotas

For our experiments we used two distinct types of transferable mouse microbiota (Mb). Mb1 is the homeostatic microbiota from *Rag1*$^{-/-}$ mice of our animal facility at the UKE, under specific-pathogen-free conditions (SPF). Mb2, on the other hand, comes from directly transferred *Rag1*$^{-/-}$ mice from the animal facilities of the laboratory of Richard Flavell, Ph.D., Yale School of Medicine, New Haven, United Sates. We kept these two microbiotas in distinct mouse colonies in IVC and stored in a frozen biobank. Mb2 is planned to be at times renewed by fresh import of *Rag1*$^{-/-}$ mice from the Flavell laboratory. The above discussed microbiota-dependent effect of IL-22 could be demonstrated in our laboratory with genetically identical mouse lines transferred with either of these two Mb types. While in mice that

received Mb1 IL-22 did not influence DSS colitis, Mb2 receiving mice where protected by presence of IL-22 (190).

3.4. Fecal microbiota transplant

For microbiota manipulations transferable microbiota was isolated from *Rag1*$^{-/-}$ either of the Mb1 or the Mb2 colony. These Mb1 or Mb2 aliquots were then transferred into recipient mice. The FMT was performed as described in Thiemann *et al*. (199). Briefly, at least eight-week-old donor mice were sacrificed and opened. The entire luminal content of colon and cecum was washed into thioglycollate differential medium (at 4 °C), and the thioglycollate tube was firmly closed. After homogenization (shaking and filtrating through a 70 µm filter), centrifugation (500 g, 10 minutes (min)), and resuspension in BHI media (3 ml), recipient mice were fed orally 200 µl each. The used technique of oral gavage is described elsewhere in full detail (200). For ensuring optimal engraftment of the transferred microbial organisms, recipient mice were young (four to five weeks old), starved (water only for two hours prior to gavage), and they were grouped into cages of the same microbiota after FMT.

3.5. DNA isolation from fecal samples

DNA was isolated from fecal samples using an organic extraction method, *i.e.*, phenol/chloroform/isoamyl alcohol (P/C/I). The extraction method is described in Turnbaugh *et al*. (92). Briefly, fecal samples were mixed with 500 µl P/C/I, 500 µl 2x buffer A, and 200 µl 20% SDS. Then, samples were homogenized and lysed using a homogenizer machine with sterile microbeads. The SDS aided lysis of the bacteria. Centrifugation (800 revolutions per minute (rpm), 4 °C, 3 min) led to formation of a lower phenol phase and an upper aqueous phase. The aqueous phase, which contains the water-soluble DNA, was transferred into a new tube and again mixed and centrifuged (800 rpm, 4 °C, 3 min) with 500 µl P/C/I. The aqueous phase was mixed with 1 ml isopropanol and 50 µl sodium acetate and incubated for one hour at -20 °C. Sodium acetate leads to precipitation of the DNA in the vicinity of isopropanol. After centrifugation (1200 rpm, 4 °C, 20 min), the precipitated DNA pellet was obtained from the bottom of the tube. The pellet was dried (room temperature (RT), one hour). Lastly, the DNA pellet was dissolved (50 °C, 30 min) in 20 µl pure tris/EDTA buffer and adjusted to DNA concentrations of 0.3 µg/µl for each sample. Concentrations were measured on a spectrophotometer (NanoDrop™). During the entire process, we used a

negative control that underwent all steps to exclude cross-contamination of bacterial DNA. This sample is marked as "-" in Figure 5.

3.6. Microbiota analysis

3.6.1. Conventional polymerase chain reaction

Polymerase chain reaction (PCR) was used to detect presence of signature bacterial species in our microbiota samples, which allowed distinction between the two types, Mb1 and Mb2, respectively. PCR was used to assess engraftment of microbiota two to four weeks after FMT. To this end, we used oligonucleotide primers specific for the 16S rRNA gene. In this gene, both highly conserved regions and species-specific regions exist. This allowed for either detecting all bacteria or detecting specific species (see, Table 4). The PCR program and reactions are stated in Tables 5 and 6, respectively. After amplification of the PCR product, amplicon bands were detected in DNA gel electrophoresis as per standard protocols. The gel was prepared with 2% agarose and one to two drops of 0.07% ethidium bromide. Bands were visualized using a UV transilluminator.

3.6.2. 16S microbiome sequencing

16S microbiome sequencing was used for validation of the PCR based assessment of microbial engraftment and for further analyses during or after the experiments. Details of the experimental procedures used have been published (201). Briefly, in all samples the V4 region of the 16S rRNA gene was sequenced. In a second step, very similar sequences (using a 97% similarity threshold) were grouped together into one operational taxonomic unit (OTU) based on the UCLUST reference. Further analysis assigned these to bacterial *taxa*, *e.g.*, on *phylum*, family, or species level (202). Sequencing was carried out on an Illumina MiSeq system. Sequencing and the initial data analysis, including calculation of alpha diversity in R, were carried out in kind cooperation by the laboratory of Prof. Till Strowig, Ph.D., at the Helmholtz Centre for Infection Research, Braunschweig, Germany (see, acknowledgements). We estimate alpha diversity as richness, meaning the count of singular OTUs per sample.

3.6.3. Metagenomic analysis

Shotgun metagenomic analysis sequences all DNA in the samples, including murine and viral DNA. This allows restoration of entire bacterial genomes and precise taxonomic

analysis (203). The used experimental procedures have been published (204). Briefly, a NEBNext Ultra DNA library was used on an Illumina HiSeq2000 machine. Sequencing and the initial data analysis, including calculation of alpha and beta diversity in R, were carried out in kind cooperation by the laboratory of Prof. Till Strowig, Ph.D., at the Helmholtz Centre for Infection Research, Braunschweig, Germany (see, acknowledgements). We estimate alpha diversity as richness, meaning the count of singular OTUs per sample.

3.7. Adoptive T cell transfer colitis

We transferred $CD4^+$ $CD25^-$ $CD45RB^{hi}$ naïve T cells from C57B6/J *wt* donors intraperitoneally into *Rag1*$^{-/-}$ recipients to induce a chronic colitis as outlined in the introduction. This IBD model is based on observations by Powrie *et al*. in 1993, and the method is described in detail in Ostantin *et al*. (178, 205). A brief description and our alterations from this protocol are stated in the following section.

3.7.1. Cell isolation from spleen and lymph nodes

C57B6/J female mice were sacrificed by our facility's three-step approach to ensure minimal distress for the animals. Mice were opened, and the spleen was taken out, prepared, and placed in RPMI media in a 12-well plate on ice. Peripheral and visceral lymph nodes were taken out, prepared, and placed in RPMI media in a 12-well plate on ice but handled separately from the spleens. Preparation of lymph nodes and spleens to remove any surrounding fat was carried out for obtaining high cell viability in downstream processing. Spleens were smashed through a 100 µm filter before centrifugation (350 relative centrifugal force (rcf), 4 °C, 10 min). The pellet was resuspended in 1x diluted ACK buffer and incubated at RT for two minutes. Splenocytes and cells from lymph nodes were pooled. After centrifugation (350 rcf, 4 °C, 10 min), the cells were resuspended in the MACS panel. Depending on the size of the experimental groups, roughly one donor was sacrificed to reconstitute four recipients with $CD4^+$ $CD25^-$ $CD45RB^{hi}$ naïve T cells.

3.7.2. Magnetic cell isolation

We used magnetic-activated cell sorting (MACS®) for isolation of $CD4^+$ cells of the cells following the manufacturer's instructions (206). Briefly, the pellet was resuspended in 1:10 anti-CD4 microbeads in MACS buffer and incubated (4 °C, 30 min). After moisturizing a fresh plastic column with MACS buffer and placing it in the magnet, the cell suspension was

placed though a 50 µm filter into the column. The column was washed three times with 4 ml MACS buffer each time. The column was taken out of the magnet and flushed with MACS buffer to obtain the $CD4^+$ cell fraction. Depending on the experimental schedule, the cells were either stored at 4 °C for up to two hours or directly centrifuged (350 rcf, 4 °C, 10 min) and resuspended in the antibody panel which is described in the following paragraph.

3.7.3. Cell sorting of $CD45RB^{hi}$ T cells

Having obtained $CD4^+$ cells from MACS, our aim was to isolate $CD25^-$ $CD45RB^{hi}$ cells by flow cytometric cell sorting, using the following gating strategy. We used the FACS Core Facility of our institution (cell sorter: BD FACSAria-IIIu).

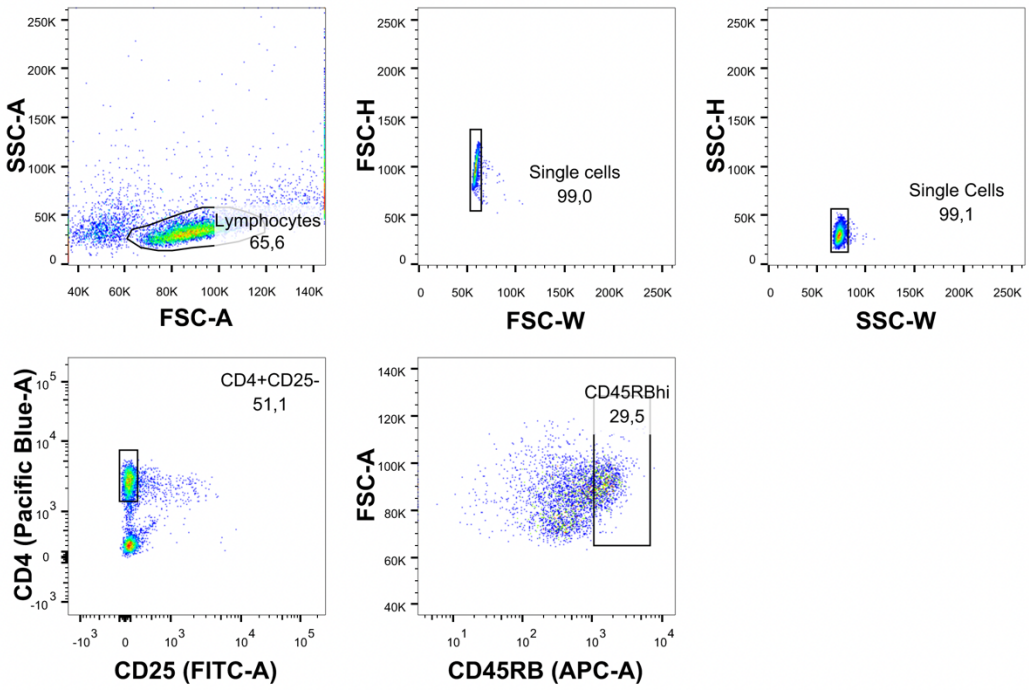


Figure 2: Gating procedure for sorting $CD45RB^{hi}$ cells. Hierarchy: all cells, lymphocytes, single cells by forward scatter (FSC), single cells by sideward scatter (SSC), and $CD4^+$ $CD25^-$. The percentage of the corresponding parent population of each selection is shown.

3.7.4. Cell counting, preparation, and injection

A cell suspension of sorted cells was collected in 5 ml round-button polystyrene tubes, counted, diluted, and injected into mice. All this was processed without delay to ensure high

viability of the sorted cells. 10 µl of the cell suspension were stained in 20 µl trypan blue and counted in a hemocytometer with a counting grid. The counting grid had squares of 1 mm length and 0.1 mm height, *i.e.*, volume = 0.1 µl. Living cells in four squares were counted and the average was calculated to obtain accurate estimates. The concentration of the suspension was calculated as the following:

$$viable\ cell\ concentration\ [cells\ per\ \mu l] = \frac{counted\ viable\ cells}{0.1\ \mu l \times\ dilution}$$

The dilution was at least 1/3 due to the staining, usually 1/10 for easier counting. Each mouse received 200,000 sorted cells diluted in pure, sterile PBS via intraperitoneal (*i.p.*) injection.

3.8. Sample collection

At the end of each mouse experiment, we collected samples for further analysis. We gently emptied the colon and stored a fecal sample in a screw lid 2 ml tube for microbiota analysis (first on dry ice, then at -20 °C). Colon length was measured with a centimeter ruler. We took a one cm colorectal whole sample and stored it in 4% paraformaldehyde sample for histology. The rest of the colon was placed in RPMI media in a 12-well plate on ice for downstream analysis of the cellular infiltrate. Next, mesenterial lymph nodes were collected, pooled, and placed in a well separate from the colon (RPMI media on ice).

3.9. Cell isolation for analysis

Cellular infiltrates of the colon and mesenteric lymph nodes were assessed separately. Colons were opened longitudinally. After washing off remaining luminal contents with PBS, samples were incubated with 1 mM DTT in HBSS (shaking, 37 °C, 20 min). After incubation, colons were taken out. The remaining cell suspensions contained IEL. For isolation of transmurally infiltrated immune cells, colon samples were incubated with collagenase (1 mg/ml) and DNase I (10 U/ml) in RPMI media (37 °C, 45 min). After digestion, remaining tissue was smashed through a 50 µm filter and resuspended in PBS 1% fetal bovine serum (FBS). The two cell suspensions (IEL and other infiltrated cells) were pooled for each initial colon sample and processed together in density separation for further enriching immune cells. This was performed with a Percoll® gradient as described in Bowcutt *et al*. with the alteration that we used a 40:60 gradient for higher purity (207). Cells were placed in 5 ml polystyrene tubes.

3.10. Flow cytometry

We used flow cytometry (BD LSRFortessa) to characterize the isolated cell populations. First, cells were centrifuged (350 rcf, 5 min). The aqueous phase was decanted, and the cells were resuspended in the staining panels. We used two different staining panels: one for innate immune cells and one for T cells. To this end, the two cell suspensions (from colon and lymph nodes) of each sample were split in half. Either half was stained with the innate panel or with the T cell panel. Dilutions of the antibodies are listed Table 2.

For innate cells, first a live/dead staining was performed. To this end, cells were centrifuged (350 rcf, 5 min), the aqueous phase was decanted, and cells were stained in 1:1000 Zombie (fluorochrome UV 379) in PBS (dark, 4 °C, 20 min). Zombie dyes protein (amine-reactive). Therefore, no FBS was added to the staining panel.

Cells were washed and centrifuged (350 rcf, 5 min). Before the surface staining, cells were resuspended with FC-γ block (1:100) in PBS 1% FBS (4 °C, 10 min). After incubation, the cells were washed, centrifuged, and resuspended in the innate surface staining master mix. This panel included the following fluorochrome-coupled antibodies (see, Table 2): αCD3, αCD11b, αCD11c, αCD45, αlymphocyte antigen 6 complex, locus G (Ly6C), αlymphocyte antigen 6 complex, locus G (Ly6G), and αNK1.1 in 1x PBS 1% FBS. After incubation (dark, 4 °C, 20 min), cells were washed with 1x PBS 1% FBS, centrifuged (350 rcf, 5 min), resuspended in 1x PBS 1% FBS, and filtered through a 100 µm filter. Next, the samples were analyzed.

Concerning T cell staining, one additional staining (intracellular staining) was necessary. After incubation, washing, and centrifugation (350 rcf, 5 min), cells were restimulated to ensure high cytokine production for sufficient intracellular staining. Cells were resuspended in 1 ml Click's medium 1% penicillin G and streptomycin, 10% FBS, 50 ng/ml PMA, 1 mmol/l ionomycin, and 2 µmol/l monensin (5% CO_2, 37 °C, four hours). Click's is a full medium suitable for cell differentiation, containing various nutrients. Monensin ensures that the PMA-dependently and ionomycin-dependently synthesized cytokines stay in the cells and can be detected with intracellular staining. After incubation, cells were washed, centrifuged (350 rcf, 5 min), and the aqueous phase was decanted. The next step was the surface staining as described for the innate panel with the following antibodies: αCD3, αCD4, αCD45. After washing and centrifuging, cells were permeabilized for the intracellular

staining. First, for fixation of the surface antigens and their bound fluorochrome-coupled antibodies, the cells were incubated in 100 µl PBS 1% FBS 4% formaldehyde (RT, 20 min). After washing and centrifugation, cells were resuspended in 100 µl PBS 1% FBS 0.1% NP40 (RT, 4 min). NP40 is a detergent that permeabilizes cellular membranes so that the intracellular staining antibodies can enter the cells. After washing and centrifugation, cells were stained in the antibody master mix for intracellular antigens, containing αFOXP3, αIL-17, and αIFN-γ. After incubation, cells were washed, centrifuged, and filtered as performed for the innate panel. Gating procedures are shown in the results section.

Flow cytometric data was analyzed and visualized with FlowJo™ (software version 10).

3.11. Histology and staining

Distal colorectal samples of one cm size were stored in 4% paraformaldehyde for a minimum of one day and a maximum of four weeks until they were processed. The first processing step was dehydration and paraffine embedding of samples. Next, at a microtome, 4 µm sections of each sample were cut and placed on a microscope slide. Staining was performed according to standard protocols with hematoxylin and eosin (H&E). Briefly, the slide was placed in xylene for two minutes followed by a regressive series of ethanol baths. After washing with water, the sample was stained with hematoxylin. After another wash, the sample was stained with eosin. This was followed by a progressive series of ethanol baths and ended with placing the sample in xylene again for two minutes.

3.12. Weight measurements and endoscopy

According to animal protocols, mice were examined daily and weighed weekly – if necessary daily. Endoscopy was performed of the rectum and descending colon with a rigid rodent endoscope. We used the Karl Storz COLOVIEW® system with a light source, insufflation, and a camera. Upper regions of the gastrointestinal tract cannot be examined endoscopically in mice. The examiner was blinded to experimental groups. We used isoflurane to shortly anesthetize animals. All endoscopic procedures were strictly hygienic to limit risks of microbiota transfer between mice. This procedure is described in more detail in Becker *et al*. (208). We used the modified murine endoscopic score of colitis severity, which has been validated by Becker *et al.* and by Huber *et al*. (209, 210). Five parameters are assessed in the score: stool consistency, colon wall translucency, granularity, vascular pattern, and fibrin. Stool consistency accounts for inflammation-related diarrhea. Reduced translucency of

the colonic wall accounts for wall thickening, which is readily observed in transfer colitis. Granularity refers to cellular infiltration of the mucosa. Changes in vascular pattern can reach as far as gross bleeding due to tissue destruction. Fibrin presence accounts for fibrinous inflammation or wound healing after inflammatory damage to blood vessels. Zero to three points are given per category.

3.13. Statistics

All values are presented as means with standard errors except for the flow cytometry contour plots, for which means with standard deviations (*s.d.*) are stated. All statistical tests were preferred to use corrections for unequal variability of differences between groups (Geisser-Greenhouse correction). After careful consideration, we decided that the test that best answers our statistical question in the short-term neutralization experiment was a three-way ANOVA with Geisser-Greenhouse correction. For our long-term neutralization experiment there was one fewer variable (since there was only one type of microbiota, that is, Mb2). Therefore, here we used the two-way ANOVA with Geisser-Greenhouse correction. For analysis of differences in relative abundances of microbiotas we used the Kruskal-Wallis test or two-way ANOVA. If any given test yielded significant differences, multiple comparisons were conducted (for example in the long-term neutralization experiment). The significance level was set to p = 5%. So, results with p values smaller or equal 5% were accepted as significant. In the figures, significance was referred to as follows: "*" indicates $p < 0.5$, "**" $p < 0.1$, "***" $p < 0.001$, "****" $p < 0.0001$, and "ns" designates not significant. Box plots show median, 25th and 75th percentiles, and the whiskers display minimum and maximum values. All graphs and all automatic calculations were produced with the software GraphPad Prism ® Version 8.0.1. All manual calculations, for example standard errors for relative abundances of the microbiota data, were carried out in Microsoft® Excel® Version 16.0.13530.

4. Results

4.1. Microbiota types and engraftment

4.1.1. Composition of microbiota Mb1 and microbiota Mb2

Colonization of mice with Mb2 renders mice susceptible to colitis, which is ameliorated by endogenous IL-22. On the contrary, mice with Mb1 microbiota show less susceptibility to colitis, and IL-22 is dispensable (190). So, whether mice are colonized with Mb1 or Mb2 has remarkable consequences for the course of colitis. We hypothesized that these differences are due to differences in biodiversity of the two microbiotas. Since other colitogenic microbiotas have been shown to have high abundance of pathobionts (100), we hypothesized that abundance of specific *taxa* causes differences of Mb1 and Mb2. To this end, we performed 16S rRNA marker gene analyses of microbiota donors (Figure 3). Indeed, Mb2 showed higher biodiversity than Mb 1 (Figure 3 A). As expected, Mb2 was abundant in potential pathobionts, which were absent in Mb1. These included *Deferribacteraceae*, *Hel-icobacteraceae*, and *Prevotellaceae*. Indeed, some species of these families have been im-plied as typical colitogenic bacteria, for example *Prevotella spp*. Thus, we found differences in biodiversity and specific *taxa* between Mb1 and Mb2, which forms a basis for the differ-ences in phenotype of colitis. Figure 3 B displays successful engraftment of Mb2 into the donor after two weeks of engraftment.

4.1.2. Engraftment of Mb1 and Mb2

Next, we aimed at assessing the success of engraftment after FMT with another method than 16S rRNA sequencing. Based on the fact that some bacterial *taxa* are unique to Mb2, we used PCR to confirm engraftment of Mb2 (when these *taxa* were detectable) or Mb1 (when they were not), respectively. Indeed, PCR was able to confirm engraftment of either Mb1 or Mb2 in all biological replicates of FMT. However, the Mb1 and Mb2 signature *taxa* differed among experimental runs, so that the *taxa* used to discriminate between Mb1 and Mb2 in the second experimental cohort B differed from the ones of the first experimental cohort A (Figure 4 A and B). In group A (Figure 4 A) 17 mice underwent FMT (nine received Mb1, whereas eight received Mb2). Both groups were PCR positive for “all bacteria” (common 16S rRNA sequence), *H. typhlonius*, and *H. hepaticus*. The discriminating bacteria was *Prevotella spp*. This was only present in Mb2 recipient mice. Another group of

mice, cohort B, is depicted in Figure 4 B: 17 mice underwent FMT (nine received Mb1, whereas eight received Mb2). Both groups were PCR positive for “all bacteria” (common 16S rRNA sequence) and both were negative for *Prevotella spp*. In this case, *H. typhlonius* was the discriminating *taxon* and this was only present in Mb2 recipient mice. Taken together, based on the unique presence of specific *taxa* in Mb2, conventional PCR can be used to assess engraftment after FMT. However, variations in engraftment or imperfect sensitivity of the PCR limit this application. Therefore, the discriminating *taxa* can differ from experiment to experiment. FMT (using our method of one single oral gavage with previous starving but no antibiotic treatments) was very efficient. Mb1 is the baseline microbiota of all the mouse models used. This means Mb2 is dominant and engrafts into the mice that previously harbored Mb1. The experimental groups that showed successful Mb1 *vs.* Mb2 engraftment were used for colitis experiments later (Figures 6-13)

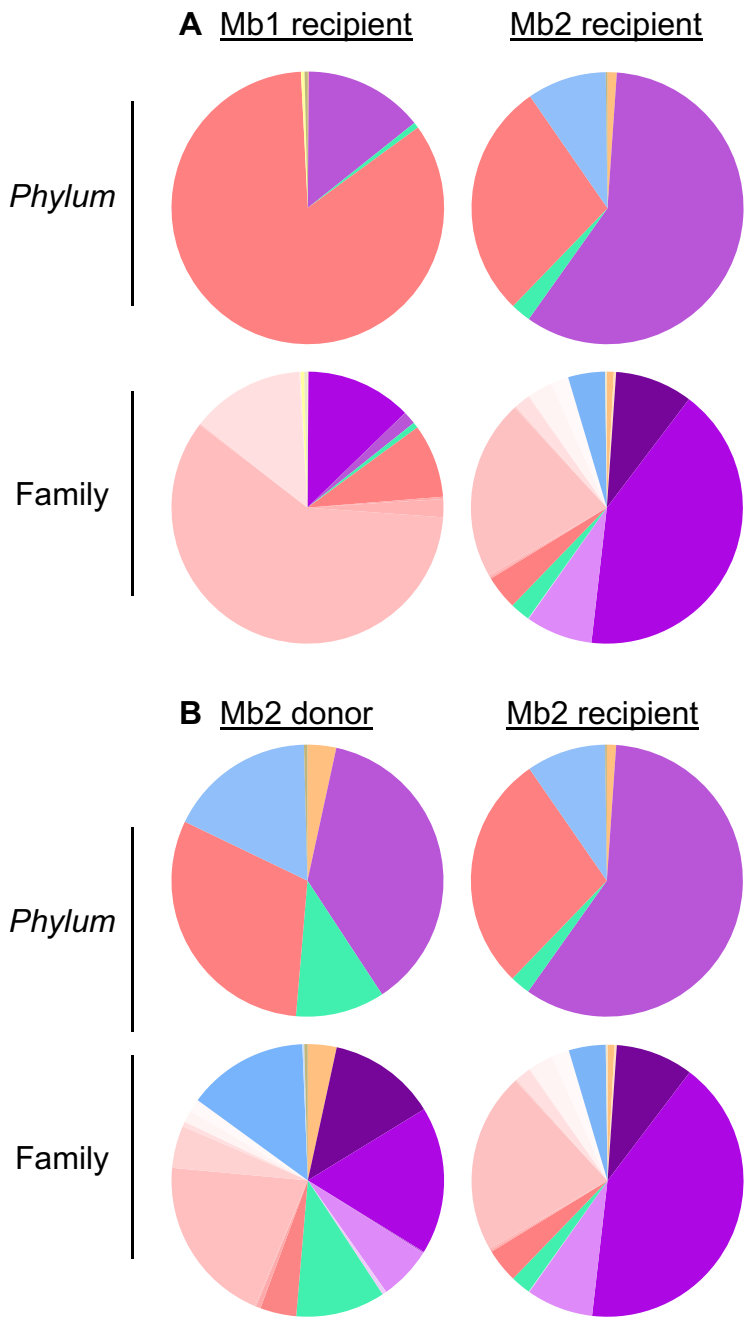


Figure 3 – legend and description on the next page

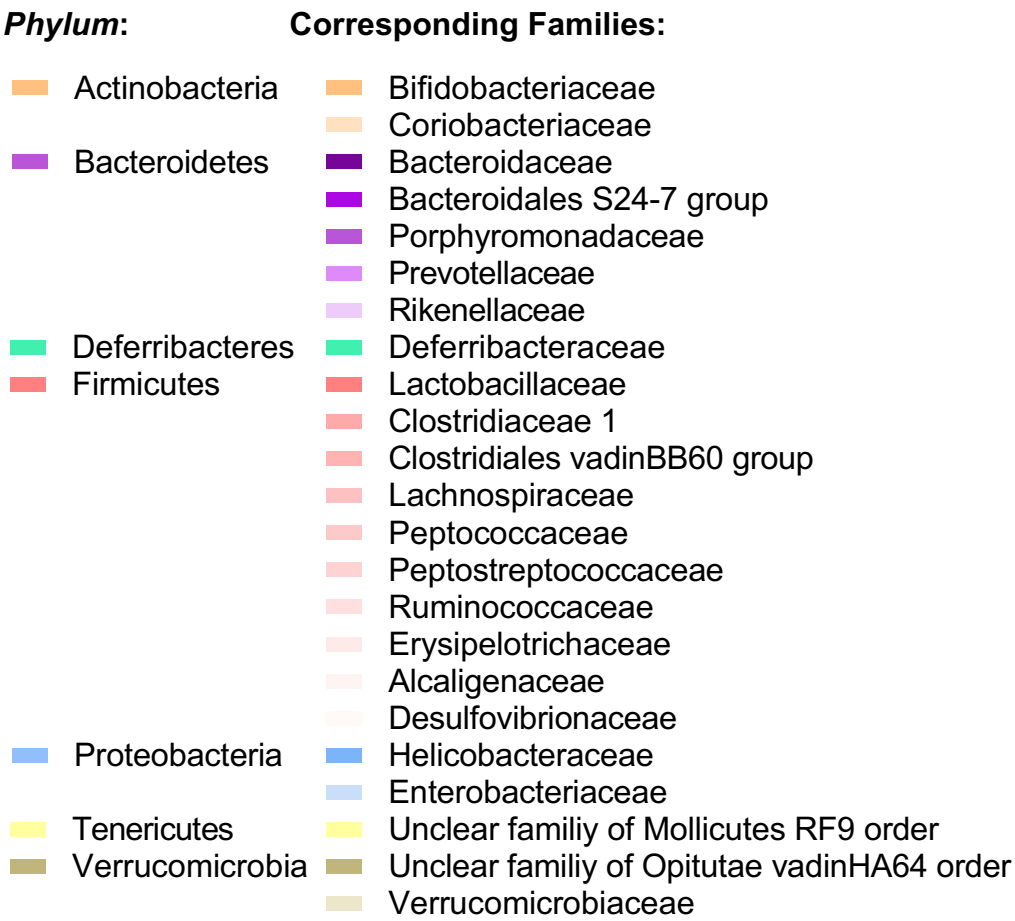


Figure 3: 16S rRNA sequencing, relative species abundances of microbiotas and Mb2 engraftment. The second row of pie charts shows the family abundances corresponding to the *phylum* abundances in the top row. A: comparison of Mb1 and Mb2 recipients. B: comparison of Mb2 donor and the corresponding recipient two weeks after FMT (engraftment check). n=1 in each group.

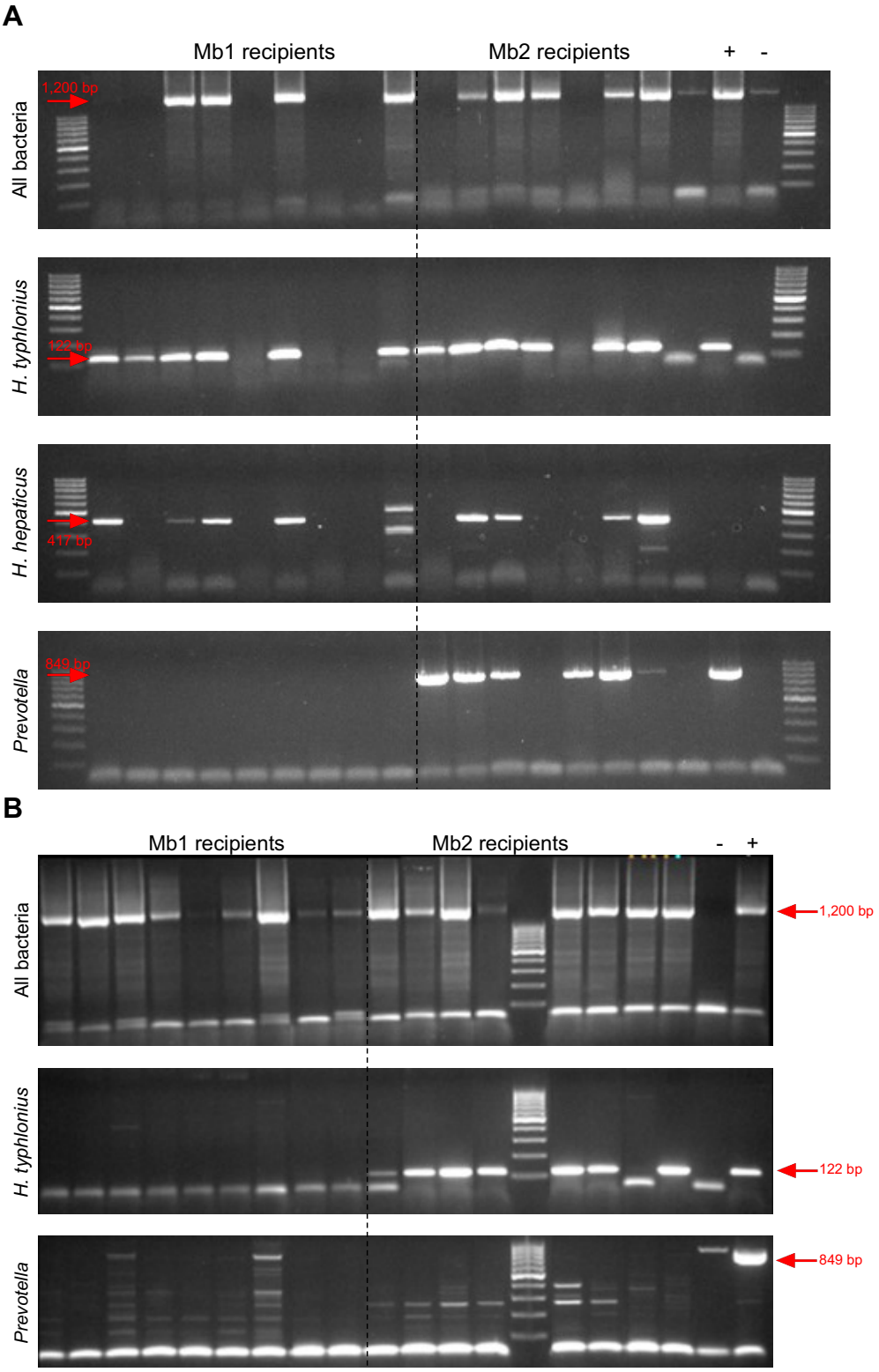


Figure 4: Gel electrophoresis showing correct engraftment two weeks after FMT. Red arrows: amplicon size. "-" and "+" are Mb1 and Mb2 donor controls, respectively. "Size marker in steps of 100 bp, from 100 (bottom) to 1000 bp (top). A: Mb1 recipients n = 9, Mb2 recipient n = 8. B: Mb1 recipients n = 9, Mb2 recipients n = 8.

Surprisingly, engraftment was unstable at times. In two experimental groups (Figure 5 A and B) all mice (irrespective of whether they had received Mb1 or Mb2) were PCR positive for Mb2 signature *taxa*. Figure 5 A shows a group of n = 16 mice. Eight received Mb1 on day zero (age four weeks), while the other eight mice received Mb2. On day zero all were negative for the Mb2 signature *taxon Prevotella spp.*, which shows that all had Mb1 baseline characteristics. Stunningly, at day 14 both groups were PCR positive for *H. typhlonius* and for *Prevotella spp*. One possibility for this might be a contamination of Mb2 components during the fecal collection, DNA isolation, and PCR process. However, this was ruled out through meticulous hygiene and because a negative control (denoted "-" in Figure 5) went through the entire process from feces collection until PCR and gel electrophoresis. Therefore, the most likely explanation is that actually all mice had developed Mb2 engraftment despite strict cage splitting and SPF conditions. Figure 5 B shows a similar observation in another experimental group. Here, six mice received Mb1, whereas four mice received Mb2. After engraftment time of 14 days, all mice were positive for the Mb2 signature *taxa H. typhlonius* and *H. hepaticus*. So, this fosters the concept that Mb2 is indeed a dominant microbiota which readily outgrows Mb1.

These "dominantly Mb2 engrafted mice" were not used for further experimental procedures.

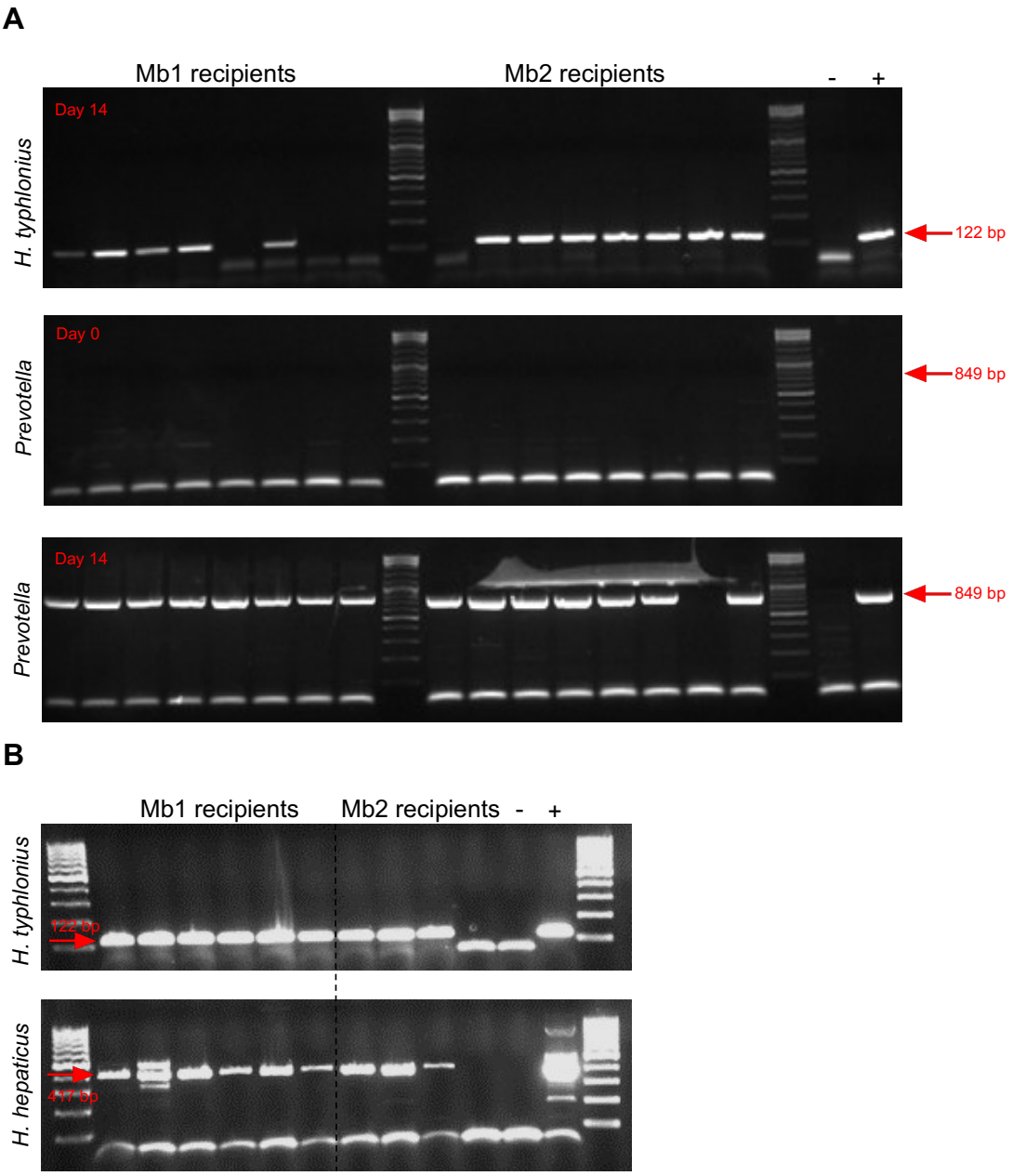


Figure 5: Gel electrophoresis showing dominant Mb2 engraftment two weeks after FMT and the baseline characteristics (A, middle). Red arrows mark the amplicon size. "-" is a negative control. "+" is an Mb2 donor control. Size marker in steps of 100 bp, from 100 (bottom) to 1000 bp (top). A: Mb1 recipients n = 8, Mb2 recipient n = 8. B: Mb1 recipients n = 6, Mb2 recipients n = 4.

4.2. T cell colitis and short-term blockade of IL-22 after FMT

Previous research found that *Il22*$^{-/-}$ mice colonized with Mb2 are more susceptible to colitis than *wt* mice. But Mb1 colonized mice had the same phenotype irrespective of IL-22 competency (190). From these experiments using knockout mice, it is suggested that IL-22 exerts its barrier-protective function rather selectively in Mb2 colonized mice. Knowing that this was shown in *Il22*$^{-/-}$ mice, we sought to find out if this is also true for short-term neutralization of IL-22. Figure 6 summarizes the experimental procedures. Four-week-old litter mate *Rag1*$^{-/-}$ mice were fed with Mb1 or Mb2 and split into new cages accordingly. After two to three weeks of incubation, correct engraftment of the Mb types was confirmed by PCR and validated later by 16S rRNA sequencing. Four weeks after FMT, all mice received CD4^{+} CD25^{-} CD45RB^{hi} T cells. On the morning of the same day as disease initiation, anti-IL-22 or isotype control (50 µg in 200 µl PBS) antibody injections were initiated. This was continued twice weekly until the end of the experiment.

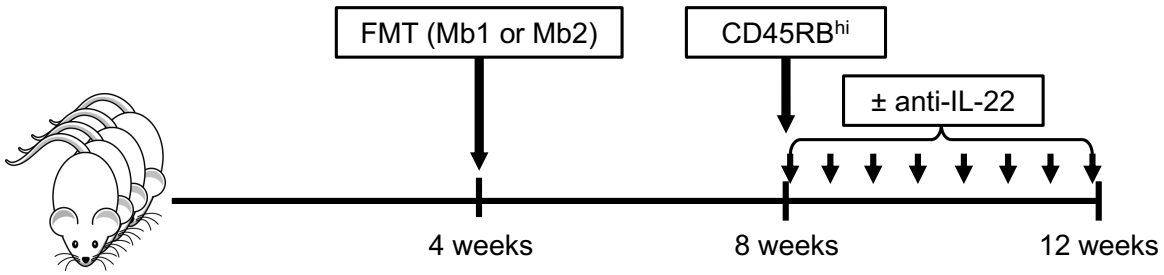
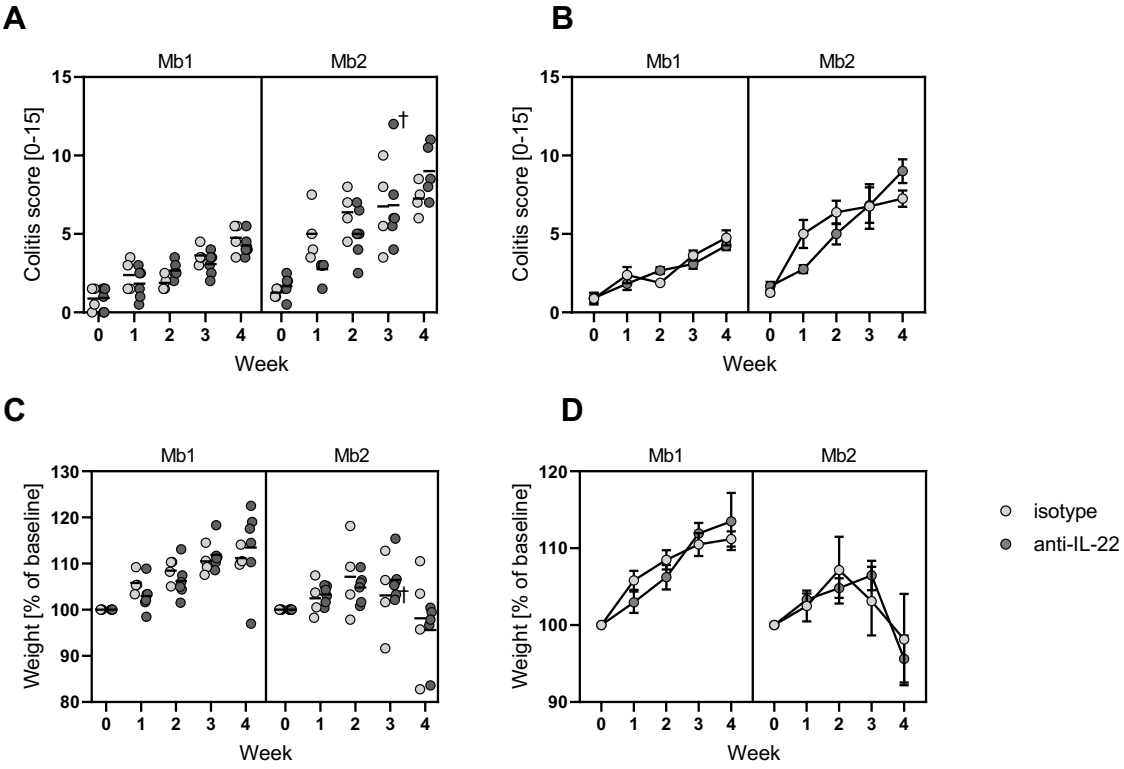


Figure 6: Outline of the short-term neutralization colitis experiment. One arrow (below the ± anti-IL-22 bracket) represents one injection of antibody (twice per week).

First, we aimed at assessing colitis severity in live mice. To this end we used weekly endoscopy. As expected, Mb2 colonized mice developed higher endoscopic score values ($p < 0.0001$), see Figure 7 A and B and Figure 9 E-H, when compared to Mb1. Interestingly, the endoscopic scores from Mb2 colonized mice also fluctuated more. This might be due to differences in colonization intensity or because in an Mb2 setting there is more biological variance. Our key experimental question was if IL-22 neutralization affects colitis scores depending on the Mb type. This is ideally reflected by a three-way ANOVA.

Surprisingly, there was no significant effect of IL-22 neutralization, neither alone ($p = 0.6007$) nor depending on Mb2 colonization ($p = 0.9053$), see Table in Figure 7. So, colitis scores measured by endoscopy showed no effect of IL-22 short-term blockade depending on Mb type. We also used another experimental group which was colonized with Mb2 only (Figure 8 A and B). Even in this simplified setting with a presumed higher experimental power, colitis scores did not differ between IL-22 neutralized and IL-22 competent mice ($p = 0.2269$). So, in contradiction of findings of experiments with *Il22*$^{-/-}$ mice, short-term blockade of IL-22 did not influence endoscopic colitis scores in a microbiota-dependent way.

Along our endoscopic evaluations, we asked if an effect of IL-22, depending on Mb2 engraftment, could be seen in weight loss of the mice. Weight loss reflects the wasting disease of transfer colitis. In accordance with endoscopic evaluations, Mb2 colonized mice lost more weight compared to Mb1 ($p = 0.002$, Figure 7 C and D). Importantly, there was, just as in colitis scores, no significant effect of IL-22 neutralization, neither on its own ($p = 0.8692$) nor depending on Mb type ($p = 0.9634$). So, the two key clinical methods assessed during the course of colitis – endoscopy and weight measurements – did not show the initially expected effect.



3-Way ANOVA of A-D			
	Tested groups	**Colitis score (A-B)**	**Weight loss (C-D)**
Microbiota	Mb1 *vs.* Mb2	p < 0.0001(****)	p = 0.0016 (**)
Treatment	anti-IL-22 *vs.* isotype	p = 0.6007	p = 0.8692
Microbiota x Treatment	Mb1 *vs.* Mb2 x anti-IL-22 *vs.* isotype	p = 0.9053	p = 0.9634

Figure 7: Endoscopic and weight measurements during T-cell colitis. Mb1 isotype n = 5, Mb1 anti-IL-22 n = 4, Mb2 isotype n = 4, Mb2 anti-IL-22 n = 6 (later n = 5; dead mouse is marked with a cross). In the three-way ANOVA table, the Microbiota x Treatment row reflects the experimental question: "Is there a microbiota-dependent effect of IL-22 on the variation of the data?"

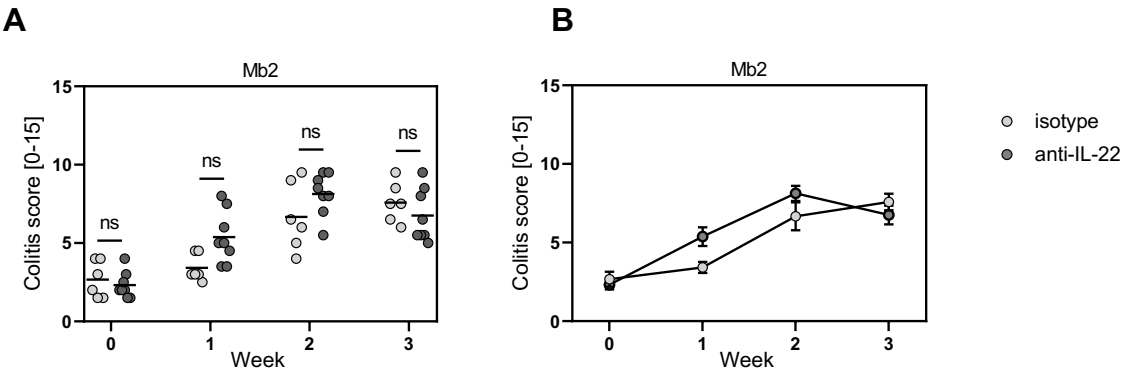


Figure 8: Endoscopic measurements during T-cell colitis. Isotype n=6, anti-IL-22 n=8, all Mb2

In addition to the clinical parameters (endoscopy and weight loss), we analyzed all mice at the end of the experiment, which we chose to be at the disease peak (in our case four weeks after colitis induction). We set this disease peak based on high endoscopic scores and weight loss. At the end of the experiment, we performed histologic analyses of colon samples and isolated cells from colon and mesenteric lymph nodes for flow cytometric phenotyping.

Regarding histology, we asked ourselves if the observed clinical impact of Mb2 colonization would manifest in higher tissue destruction and a more pronounced inflammatory infiltrate. Also, we asked if at the histologic level there was an Mb2-dependent effect of IL-22. To this end, we performed conventional H&E staining and qualitative analysis. Indeed, we observed higher tissue destruction and higher inflammatory infiltrates in Mb2 harboring mice. However, no effect of IL-22 (neither on its own, nor in conjunction with Mb type) was observed (Figure 9 A-D). Thus, the histologic results correspond tightly to our clinical parameters.

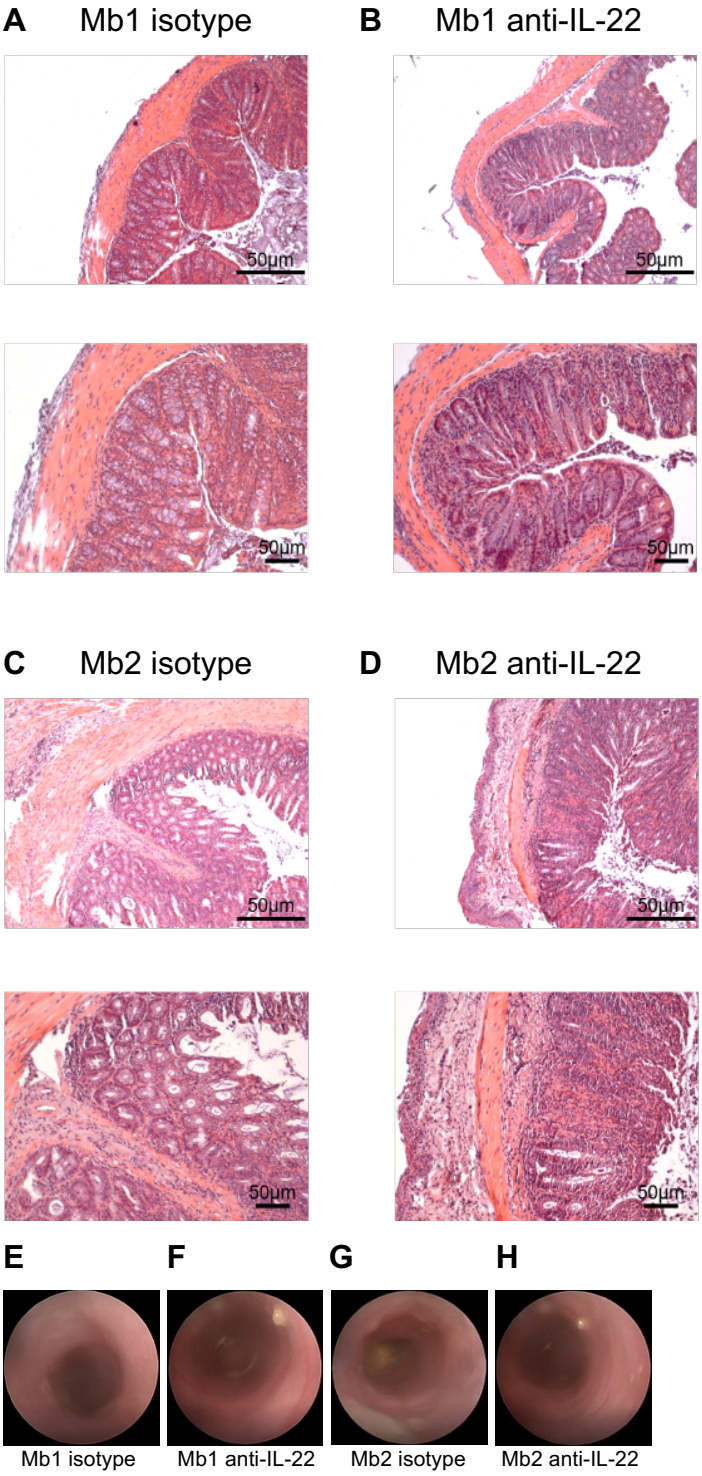


Figure 9: Representative pictures from colon histology at week four of colitis (A-D) and exemplary endoscopy findings at week two of colitis (E-H).

Regarding flow cytometric analysis of cellular isolations from colon and mesenteric lymph nodes, we asked ourselves if there were specific differences in immune cell

populations depending on IL-22. As per standard, we performed quality controls, both automatically in FlowJo and manually for each sample. We thus decided to analyze innate cell populations in the mesenteric lymph node infiltrates and T cells in the colonic infiltrates.

Our gating (Figure 10 A) was set on innate cells by FSC area and SSC area, single cells (gating out cells with relatively high signal width), living cells (Zombie negative), $CD45^+$ (leucocytes), and $CD3^-$ (gating out T cells). Then, populations of interest were defined. Natural killer (NK) cells were defined as $NK1.1^+$ (a common NK cell receptor). Neutrophils were defined as $NK1.1^-$ $Ly6G^+$ $CD11b^+$. Ly6G is a GPI-anchored protein found in mice on macrophages, monocytes, and neutrophils. CD11b (part of the complement receptor 3) mediates cellular adhesion and is most abundantly expressed on neutrophils and dendritic cells. (211) Macrophages were defined as $NK1.1^-$ $Ly6G^-$ $CD11c^-$ $CD11b^+$. Those with additional high Ly6C expression were defined as proinflammatory macrophages (212). Dendritic cells were defined as $NK1.1^-$ $Ly6G^-$ $CD11c^+$.

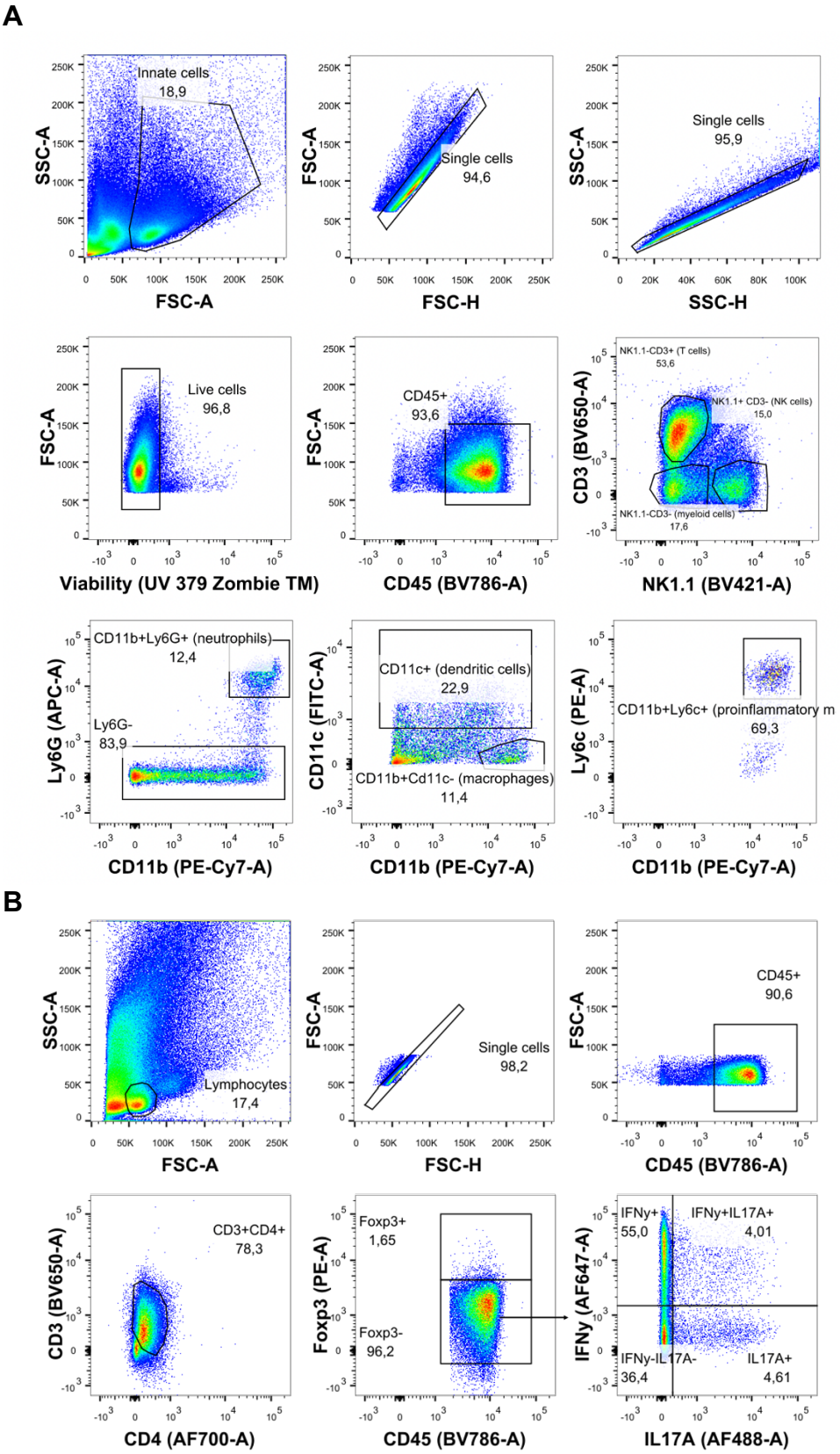


Figure 10: Gating for flow cytometric analyses. A: innate immune cells. B: T cells (exemplary samples).

We analyzed the draining lymph nodes of the colon for their innate immune cell composition. We measured $CD4^+$ cells, NK cells, neutrophils, dendritic cells, and proinflammatory macrophages. Figure 11 shows these populations as contour plots and provides the mean fraction of each population (as percentage of parent population ± *s.d.*). Regarding the contour plots, no large differences are visible between our four experimental groups. Figure 12 A-E provides frequencies of each population as percentage of $CD45^+$ cells. The following differences between Mb1 and Mb2 were statistically significant: In Mb2 (compared to Mb1), neutrophils, proinflammatory macrophages showed higher frequencies, whereas dendritic cells displayed lower frequencies. Taken together, our innate immune cell analysis of mesenteric lymph nodes provided no clear evidence to corroborate our hypothesis of an Mb2-dependent effect of IL-22 in this short-term setting.

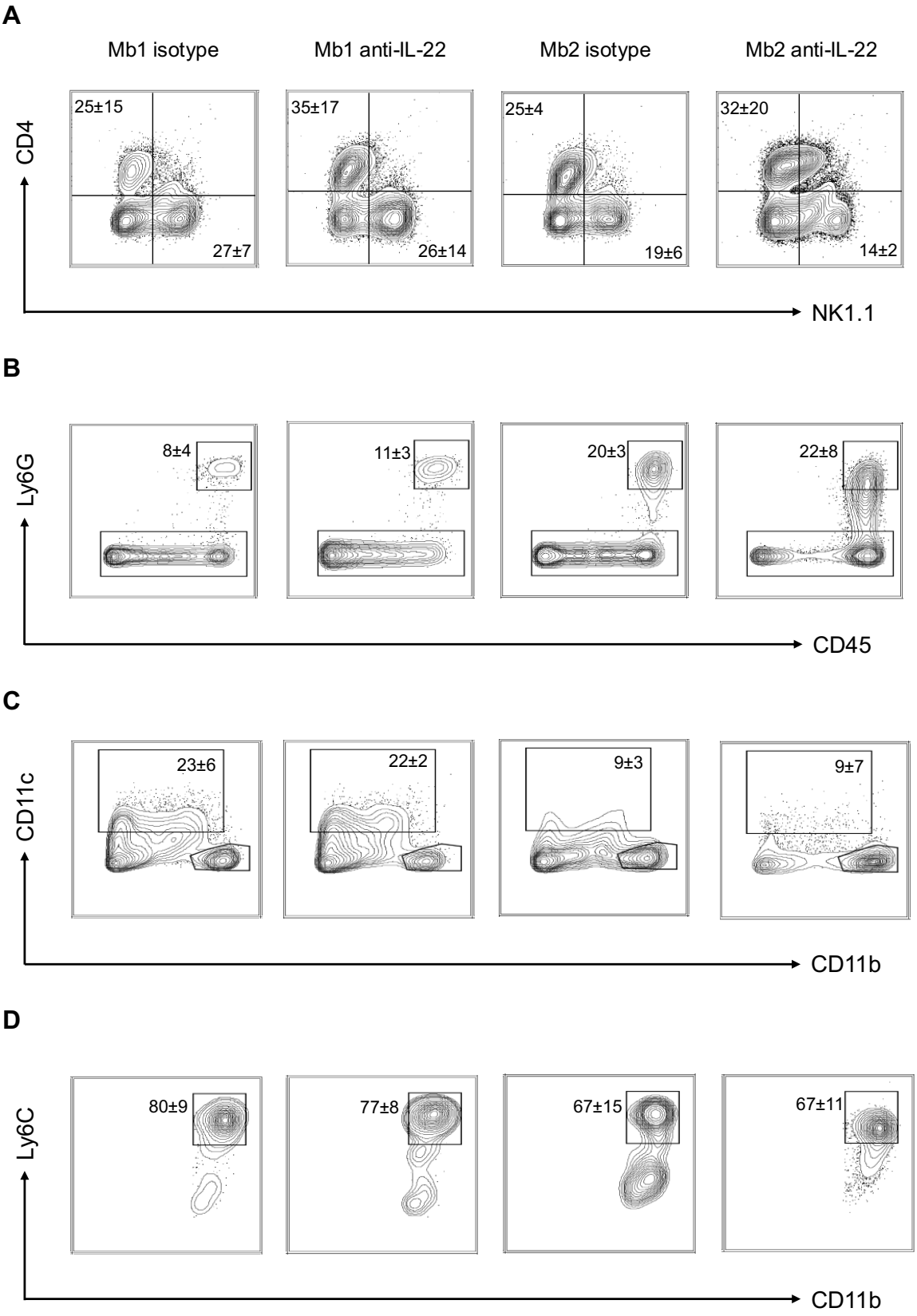


Figure 11: Frequency of innate cells in the mesenteric lymph nodes at week four of colitis. A-D: contour plots (means ± *s.d.*). Mb1 isotype n = 5, Mb1 anti-IL-22, n = 4, Mb2 isotype n = 4, Mb2 anti-IL-22 n = 4.

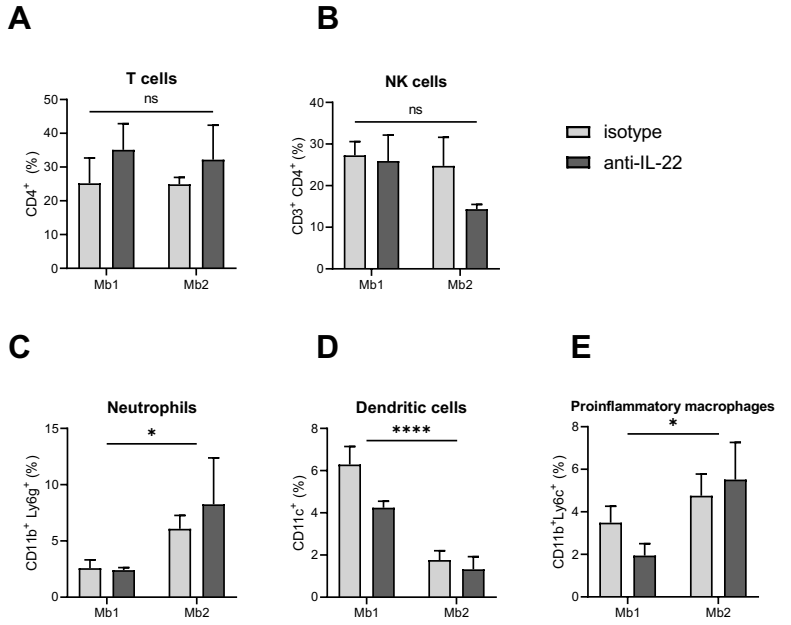


Figure 12: Frequency of innate cells of mesenteric lymph node infiltrates at week four of colitis (disease peak). A-E: bar charts of frequencies (as percentages of $CD45^+$ cells). Mb1 isotype n = 5, Mb1 anti-IL-22 n = 4, Mb2 isotype n = 4, Mb2 anti-IL-22 n = 4.

Subsequently, we analyzed the T cell composition in the colon. Flow cytometry gates were set according to Figure 10 B. Briefly, we gated on lymphocytes, single cells, $CD45^+$, $CD3^+$, $CD4^+$ and then defined our populations as $FOXP3^+$ T_{reg} and $FOXP3^-$ effector T cells with cytokine production of IL-17 and/or IFN-γ.

Since the adoptive transfer model of colitis is driven by T_H1 and T_H17 rather than T_H2, we examined these T helper lineages (see, introduction). Figure 13 A shows contour plots of these T helper subsets (T_H1, T_H17, double-producers, and double-negative). T_H1 are defined as IFN-γ producing, T_H17 are defined as IL-17A producing T helper cells. T helper cells producing IFN-γ and IL-17A are termed double producers, and those producing neither of the two cytokines are termed double negative. The average percentages in regard to the parent population ($CD3^+$ $CD4^+$ $FOXP3^-$) ± *s.d.* are provided. Basically, there is no difference among the contour plots. Only a slight trend can be seen towards lower frequencies of double producers. Figure 13 B-G shows percentages in regard to all $CD3^+$ $CD4^+$ cells; as so, also a meaningful comparison with T_{reg} is possible. There are significant differences between Mb1 and Mb2 (T cells, T_H1, double-producers, double-negative). Another statistically significant difference is a difference between isotype as such and anti-IL-22 as such within T_H17

cells ($p = 0.0342$). However, all these differences should be interpretated considering the higher immune cell infiltration in the colon of Mb2 harboring mice as seen in the histology. Taken together, the flow cytometric analysis on its own does not identify specific immunological differences in the different microbiota settings or in the presence of IL-22. However, it rather proves the significant difference in colitis phenotype between Mb1 and Mb2.

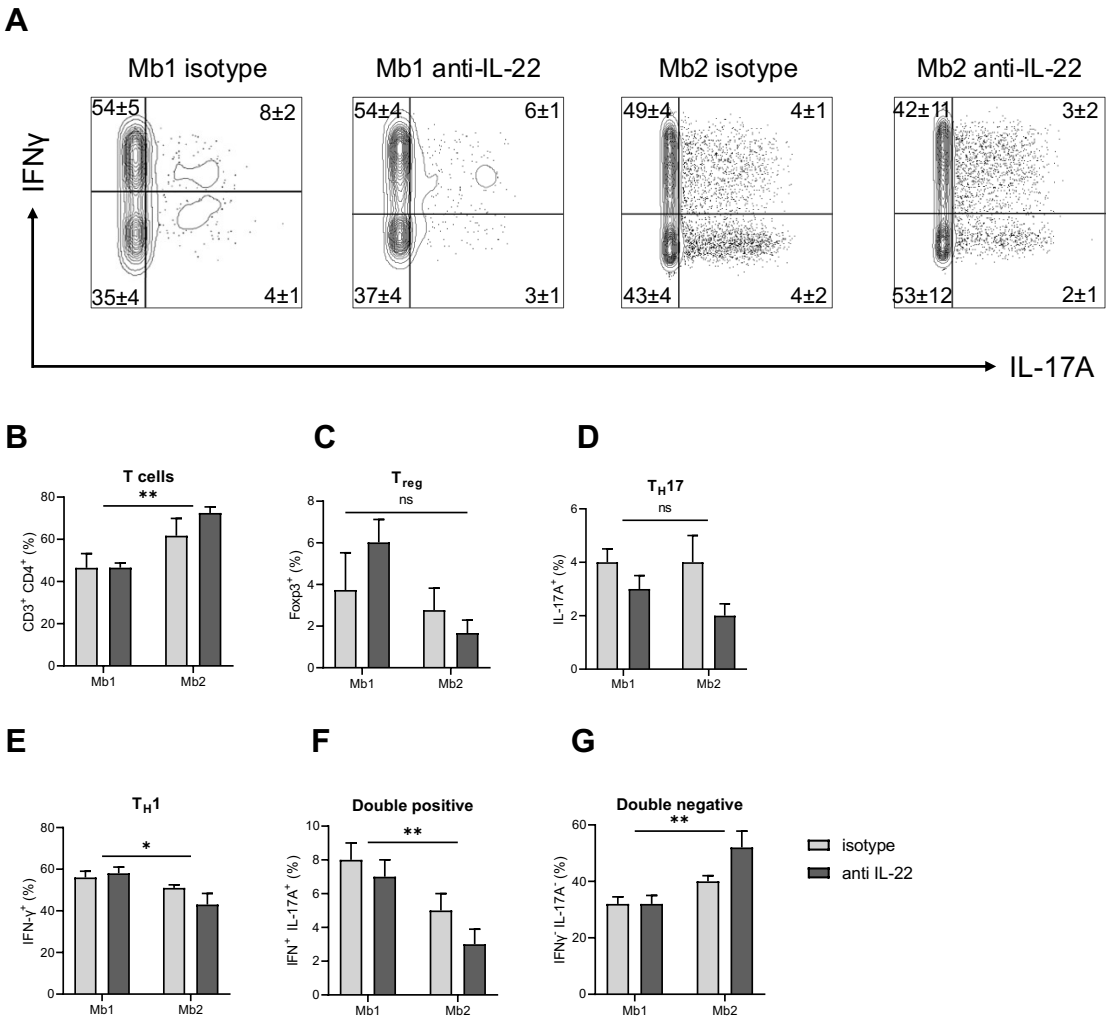


Figure 13: Frequencies of T cell infiltrates in the colon at week four of colitis (disease peak). A: contour plots with percentages of $CD3^+$ $CD4^+$ $FOXP3^-$. B-G: bar chart (showing percentages of $CD3^+$ $CD4^+$). Mb1 isotype n = 5, Mb1 anti-IL-22 n = 4, Mb2 isotype n = 4, Mb2 anti-IL-22 n = 4.

In summary, our findings of short-term blockade of IL-22 during T cell colitis following FMT of Mb1 or MB2, respectively, did not show the expected microbiota-dependent effect of disease aggravation due to IL-22 blockade. As expected, FMT of Mb2 was colitogenic, but, surprisingly, short-term blockade of IL-22 did not aggravate colitis in either Mb setting.

4.3. T cell colitis and long-term blockade of IL-22 after FMT

Since short-term neutralization of IL-22 did not lead to an observable Mb2-dependent effect on chronic T cell colitis, we next aimed at comparing it to long-term neutralization of IL-22 in a similar setting. Figure 14 A outlines the experimental procedure. Litter mates received FMT at the age of four weeks. After four weeks of engraftment (at the age of eight weeks), all mice received $CD45RB^{hi}$ cells as colitis induction. During FMT, all mice received Mb2. We did not include Mb1 in this setting because (based on our knowledge of *Il22*$^{-/-}$ experiments and our short-term neutralization experiment outlined above) we did not expect to see any phenotypical effect on colitis with long-term blockade of IL-22 in Mb1. Starting with the day of FMT (all Mb2), the *in vivo* antibody treatment was initiated. To that end, the mice were split in three groups. n = 3 mice (top) received an isotype control, n = 5 mice (middle) received anti-IL-22, and n = 4 mice (bottom) received isotype for the four weeks following FMT, while for the next four weeks (*i.e.*, starting with the day of colitis induction) they were changed to receiving anti-IL-22. This last group is very similar to the short-term blockade performed in the previous experiment, *i.e.*, neutralizing IL-22 during the course of colitis but with endogenous IL-22 in the weeks before colitis induction. All groups of mice were kept in strictly distinct cages to prohibit tranfers of microbiota between the three experimental groups. Also, during all manipulations (practical care for the animals, weight measurements, endoscopic procedures, or injections), strict hygienic measures were taken to inhibit the transfer of bacteria between the individual cages.

Endoscopic scores (Figure 14 B and D) showed an increase over time ($p = 0.0189$), and, interestingly, variation within the groups dimished over time. The mice started with slight differences in absolute weight (Figure 14 C). We normalized the weight to the day of colitis induction. The treatment of anti-IL-22 in the first four weeks after FMT did not influence weight gain of the mice in these weeks before the start of colitis. However, during colitis, the group that received anti-IL-22 the entire time (long-term) separated from the others, which became statistically significant on day 43 after FMT, during the disease peak ($p = 0.0238$), Figure 14 C. This shows that, with respect to weight loss (*i.e.*, wasting during colitis), long-term neutralization of IL-22 leads to more severe colitis in the Mb2 microbiota.

In line with the significantly higher weight loss of the anti-IL-22 long-term treated group, the colon length tends to be shorter when compared to both other groups (Figure 14 E). No flow cytometric or histologic analyses were performed.

Taken together, this experiment provides evidence that long-term blockade of IL-22 leads to a more severe colitis phenotype in Mb2. This is similar to the observed phenotype of *Il22*$^{-/-}$ mice. Interestingly, we did not observe this phenotype with short-term blockade or with switching from isotype to anti-IL-22. The only difference between the short-term and the long-term IL-22 neutralized mice are the four weeks following FMT. Since the treatment during the course of colitis is identical, the anti-IL-22 treatment in the four weeks prior to colitis induction (*i.e.*, during FMT engraftment) must render the mice somewhat susceptible to later colitis. The most probable hypothesis is that IL-22 acts upon the microbiota during the engraftment time. Blockade of IL-22 during this time would thereby lead to a more colitogenic microbiota which could then make the mice more susceptible to colitis. Therefore, we decided to next analyze the microbiota of our three groups at specific timepoints.

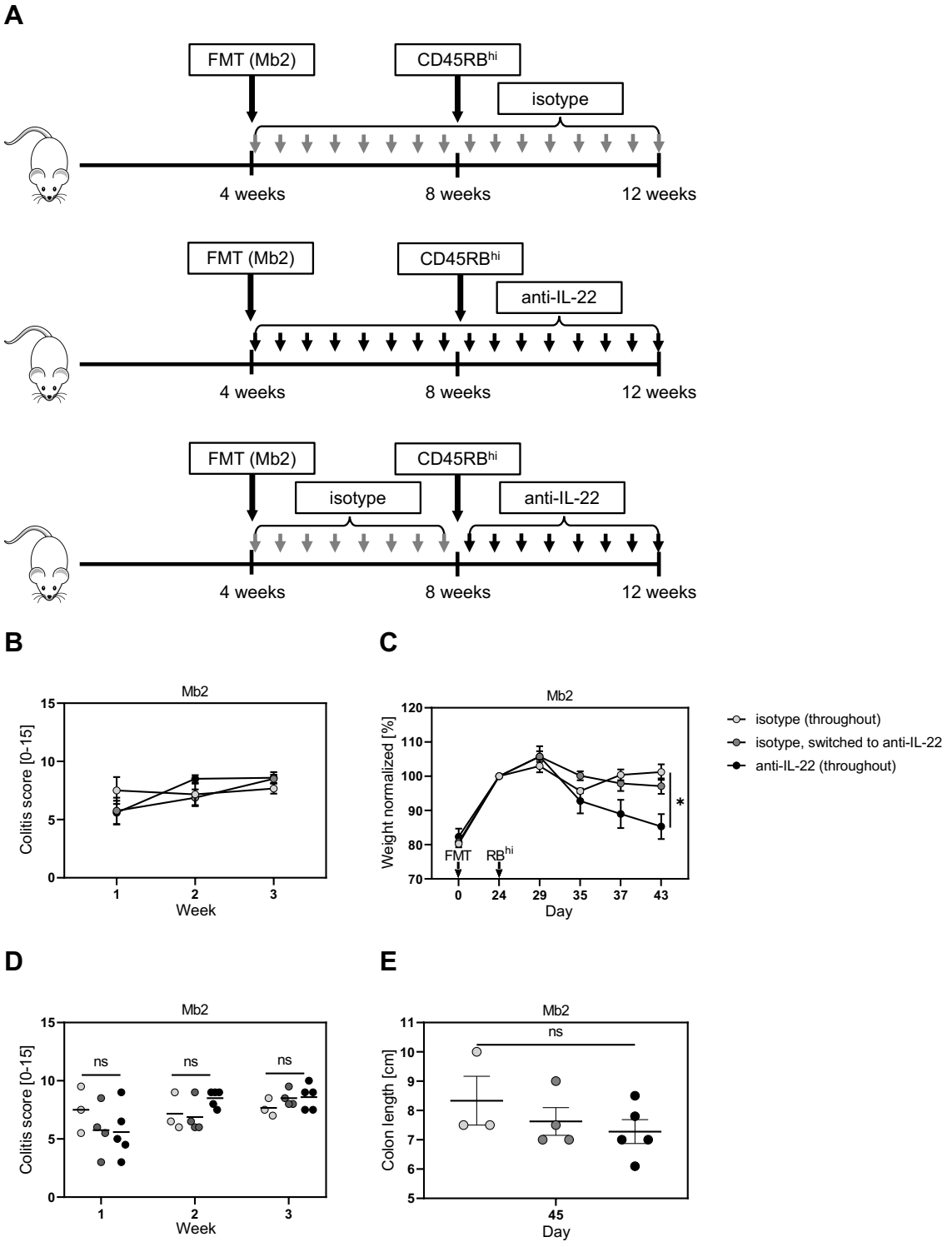


Figure 14: Long-term IL-22 neutralization experiment. A: outline, one arrow (below the isotype or anti-IL-22 brackets) represents one injection of antibody. B-E: endoscopic and weight measurements. Isotype (throughout) n = 3, isotype, switched to anti-IL-22 n = 4, anti-IL-22 (throughout) n = 5, all Mb2 microbiota.

4.4. Microbiota kinetics in response to colitis and to interleukin-22

We asked ourselves if there are differences between the microbiota of the three groups after colitis. To that end, we performed 16S rRNA sequencing at the endpoint of the experiment. Figure 15 shows alpha diversity of the three treatment groups (isotype, isotype switched to anti-IL-22, anti-IL-22). There is a trend towards higher alpha diversity in anti-IL-22 treated mice compared to the isotype and the short-term anti-IL-22 groups (Figure 15). Figure 16 A-C shows the relative abundances on *phylum* and on family level. Indeed, there are structural differences, albeit not statistically significant, probably due to the low n. Both anti-IL-22 treated groups (switched and throughout) show higher similarity to each other as compared to the isotype group. This can be seen in two aspects. First, there is a higher diversity among the *Firmicutes* in the groups that received anti-IL-22 compared to the isotype group. While the isotype group's *Firmicutes* are dominated by *Lachnospiraceae*, the anti-IL-22 group's *Firmicutes* also consist of, for example, *Clostridiales* vadinBB60, *Peptococcaceae*, and *Peptostreptococcaceae*. The second aspect that shows similarity between both anti-IL-22 groups is the smaller portion of the *phylum Bacteroidetes* when compared to isotype. As outlined in the introduction, alpha diversity, which is represented by the observed alpha diversity in Figure 15, can also be estimated as the F/B ratio. Both anti-IL-22 groups have a smaller portion of *Bacteroidetes* and a larger portion of *Firmicutes* in comparison to isotype (Figure 16). This leads to a higher F/B estimate of alpha diversity. Thus, the relative abundance pie charts correspond well with the calculated higher biodiversity of both anti-IL-22 groups (Figure 15). Taken together, we observed structural similarity – though not significant – among the switched and throughout neutralized IL-22 groups in contrast to the isotype group.

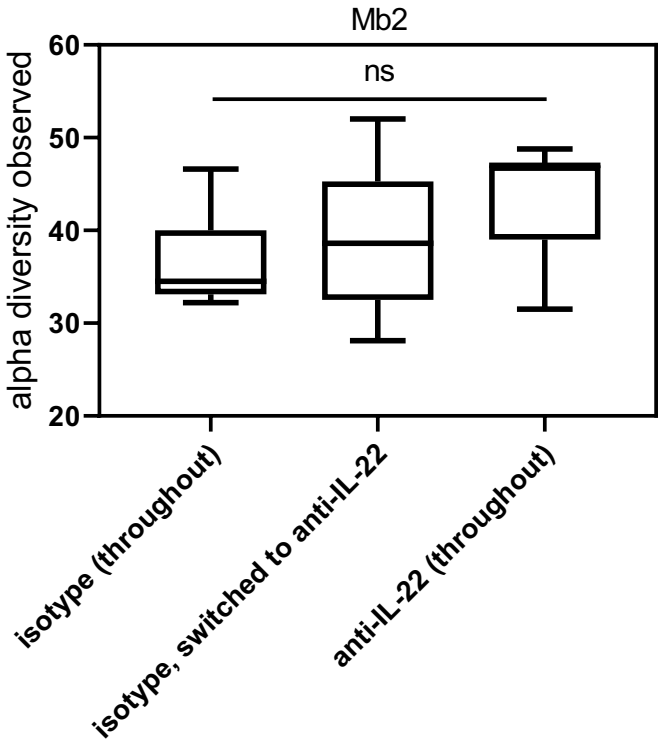


Figure 15: Comparison of the three experimental groups (isotype, isotype ⇒ anti-IL-22, anti-IL-22) at the end of colitis. 16s rRNA sequencing, observed alpha diversity. Isotype (throughout) n = 3, isotype, then anti-IL-22 n = 4, anti-IL-22 (throughout) n = 5, all Mb2 microbiota.

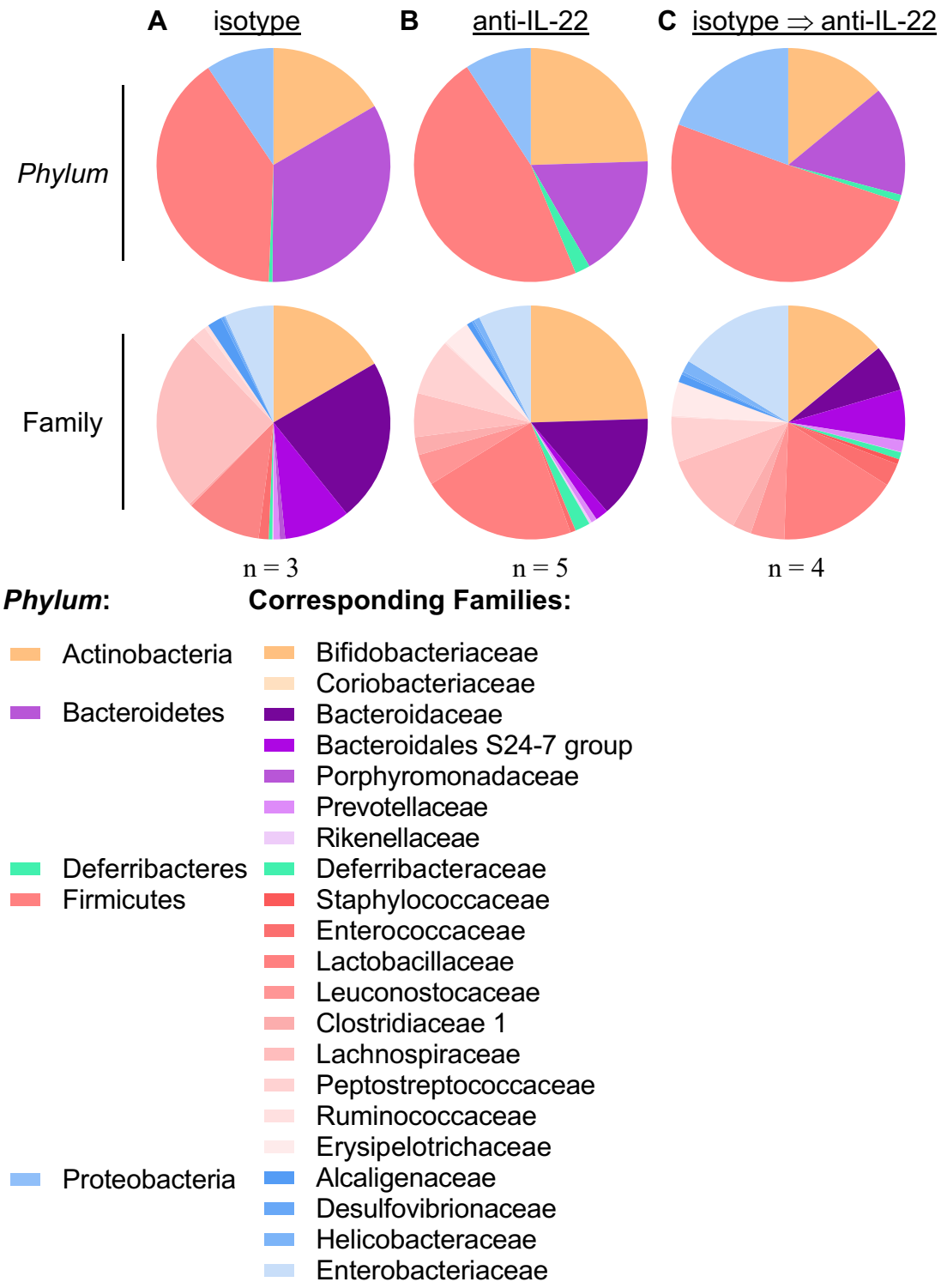


Figure 16: Comparison of the three experimental groups (isotype, isotype ⇒ anti-IL-22, anti-IL-22) at the end of colitis. 16S rRNA sequencing, relative abundances. The second row of pie charts shows the family abundances corresponding to the *phylum* abundances in the top row. Isotype (throughout) n = 3, isotype, then anti-IL-22 n = 4, anti-IL-22 (throughout) n = 5, all Mb2 microbiota.

Next, we asked ourselves to what extent the microbiota is changed during colitis. We compared relative abundances of baseline Mb2 microbiota (Figure 3 A) with the isotype group after colitis (Figure 16 A). For better comparison, both are depicted next to each other in Figure 17 A and B. Limitations to this comparison are that the measurements come from different experimental groups and that Mb2 baseline only measured n = 1 mouse. Nevertheless, one structural trend might become apparent: On *phylum* level *Bacteroidetes* are reduced after colitis, while *Actinobacteria* are increased. On family level it can be observed that the decrease in *Bacteroidetes* is mainly balanced out by an increase in *Bifidobacteriaceae* and *Enterobacteriaceae*. So, colitis appears to be accompanied by a decline in *Bacteroidetes*, probably *Bacteroides*, which is *a priori* the most common commensal *genus*. Taken together, we observed some structural changes that are possibly caused by colitis pathology.

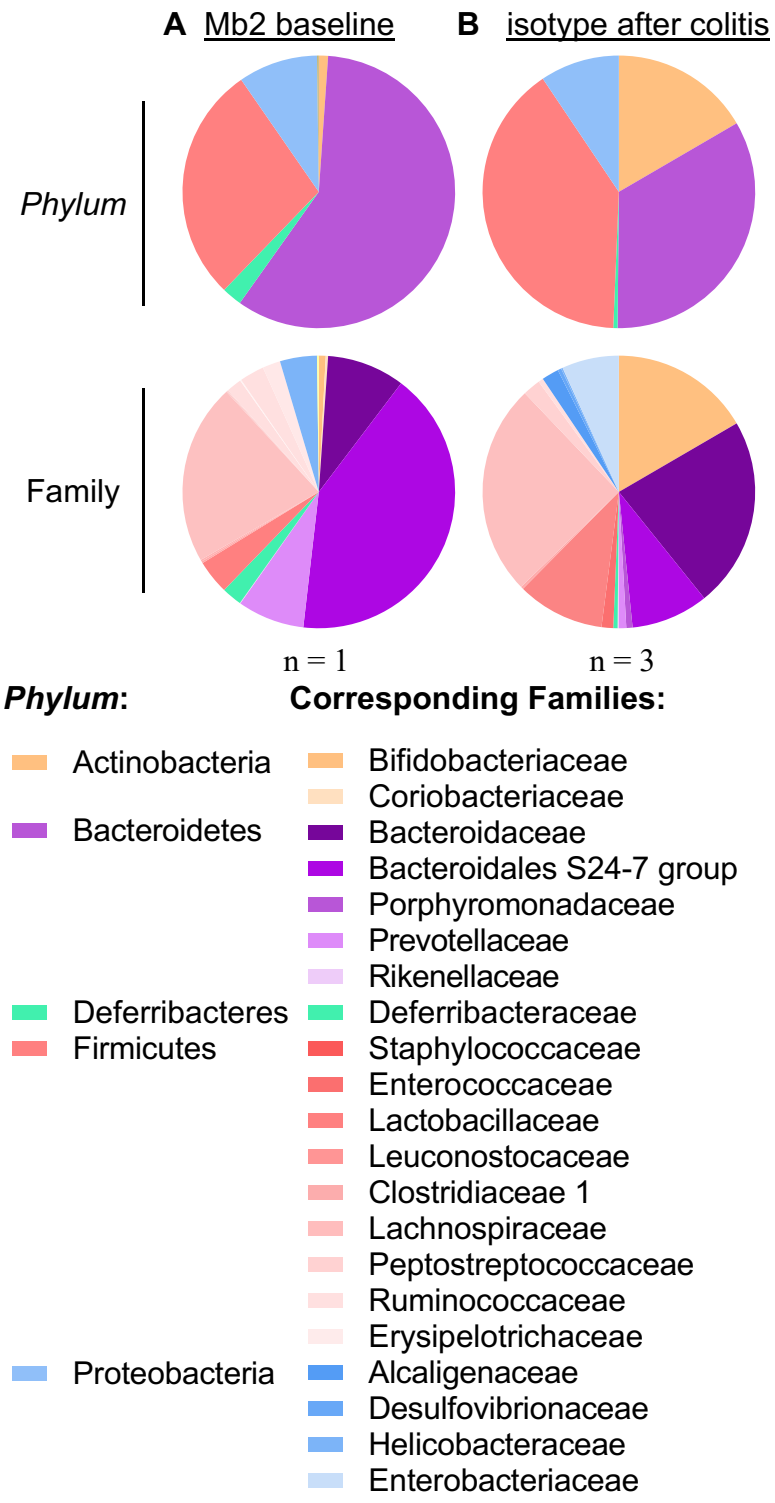


Figure 17: Comparison of microbitoa before (A) and after (B) colitis. The samples stem from (A) baseline microbiota (n = 1) and (B) the isotype group from the long-term neutralization experiment (n = 3), all Mb2. 16S rRNA sequencing, relative abundances. The second row of pie charts shows the family abundances corresponding to the *phylum* abundances in the top row.

We also aimed at investigating how far the microbiota of the group that received isotype during the four weeks of engraftment and anti-IL-22 during the four weeks of colitis changes during this course. Based on our evidence that microbiota changes both depending on IL-22 (Figure 16) and, possibly, depending on colitis (Figure 17), we supposed to observe an effect in comparing the isotype ⇒ anti-IL-22 group before and after colitis. To that end, we compared 16S rRNA sequencing of two timepoints: Fecal samples were taken on the day of colitis induction (before injection of anti-IL-22 or CD45RB^{hi} cells) (Figure 18 A) and at the end of the experiment (*i.e.*, after anti-IL-22 had been injected for four weeks during colitis) (Figure 18 B). Indeed, we observed structural differences, although these were not statistically significant. On *phylum* level we saw a reduction in *Bacteroidetes*, while on family level we additionally observed higher biodiversity among *Firmicutes*. These two findings were consistent with our observations due to anti-IL-22 or due to colitis on its own. At this point it is hard to tell if these changes are more due to the anti-IL-22 treatment or due to the colitis. However, we observed typical changes (lower *Bacteroidetes*; higher diversity of *Firmicutes*) after colitis and anti-IL-22 treatment which were consistent with our other findings (Figures 16 and 17).

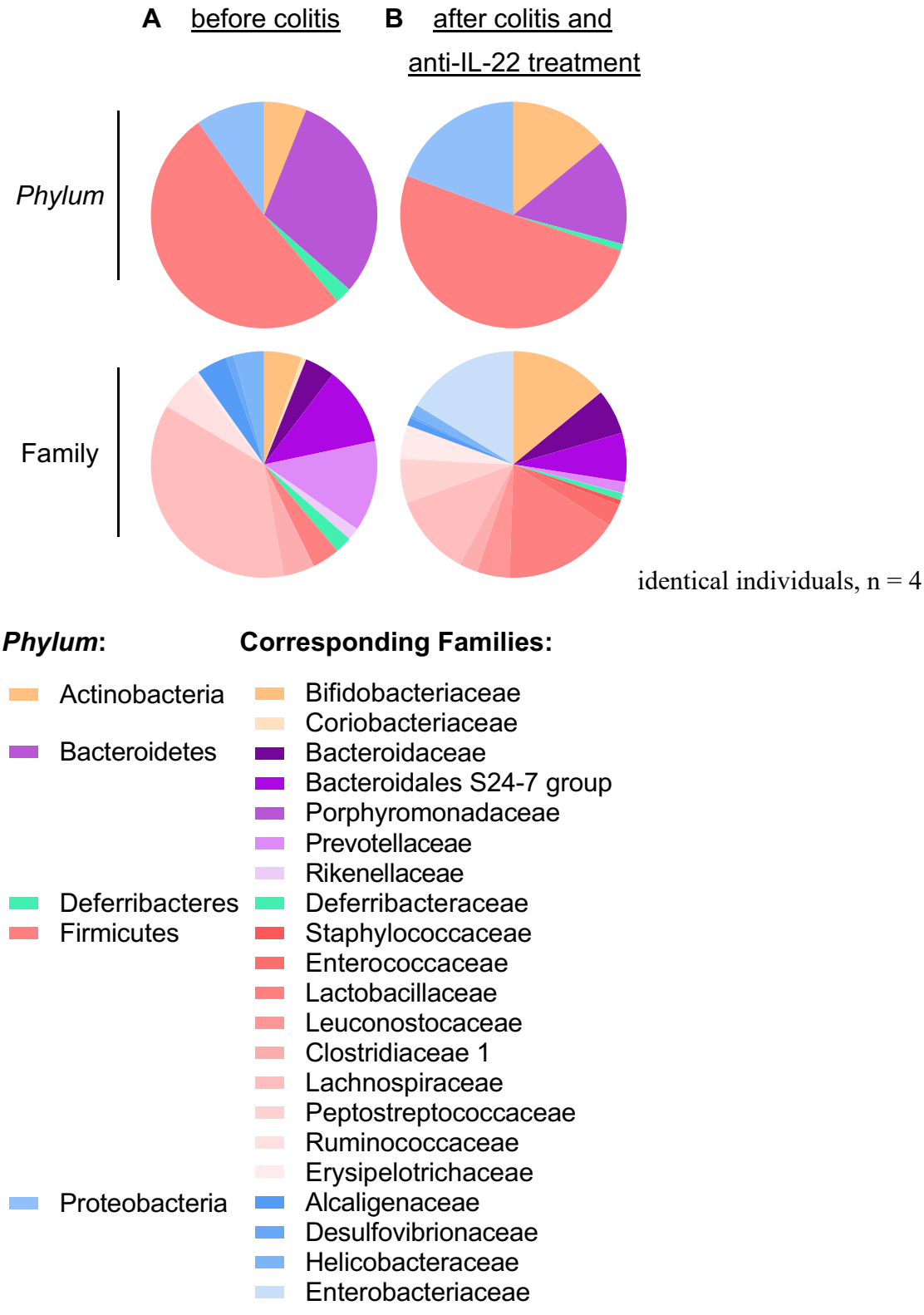


Figure 18: Comparison of microbiota before and after colitis. The mice received isotype for four weeks of Mb2 engraftment followed by a four-week course of T cell colitis with anti-IL-22 (n=4). 16S rRNA sequencing, relative abundances. The second row of pie charts shows the family abundances corresponding to the *phylum* abundances in the top row.

Our main hypothesis from the long-term experiment was that our experimental groups (Figure 14 A) differed in microbiota before the induction of colitis. The group that received anti-IL-22 could have a more colitogenic microbiota than the isotype groups. Therefore, later anti-IL-22 treatment in the short-term anti-IL-22 group would have no effect on colitis since the microbiota was already less colitogenic and would not require further IL-22. So, we compared the microbiota of mice after four weeks of engraftment. We performed a comprehensive metagenomic sequencing. We chose ten mice, five of which had received anti-IL-22 from the start of FMT, five of which had so far received isotype. Downstream analyses of metabolic pathways or gene expression did not appear reliable – owing to low n. However, Figure 19 provides results of alpha and beta diversity calculations, which is the most robust read out for our question (were there differences between isotype and anti-IL-22 treated mice before colitis?). Indeed, a lower alpha diversity is observed in the anti-IL-22 group compared to isotype ($p = 0.0093$, Mann-Whitney test). Curiously, this opposes our 16S rRNA sequencing-based observation of trends towards a higher alpha diversity in our colitogenic microbiotas (*e.g.*, Mb2 *vs.* Mb1 or anti-IL-22 treated *vs.* isotype groups; Figures 3 A and 15). It should be kept in mind that these are all trends without statistical significance. Moreover, Figure 19 provides a more in-depth sequencing which cannot be easily compared to the 16s rRNA sequencing data, particularly, because different mice were analyzed. To more thoroughly examine if the two microbiotas, isotype treated and anti-IL-22 treated, were different, we performed an analysis of dissimilarity (beta diversity). To this end, the ten samples underwent a principal coordinate analysis (Figure 19 B). Indeed, a trend towards clustering dissimilarly along the first principal coordinate axis (comprising 32.8% of the variation of the data), can be seen. However, also along the second axis differences can be seen which do not refer to differences in antibody treatment. Therefore, beta diversity does provide clear evidence that anti-IL-22 treatment changes the microbiota, but it would need a reexamination with a larger n.

Taken together, we provide insights that make it likely that anti-IL-22 treatment altered the microbiota during the engraftment phase before colitis induction.

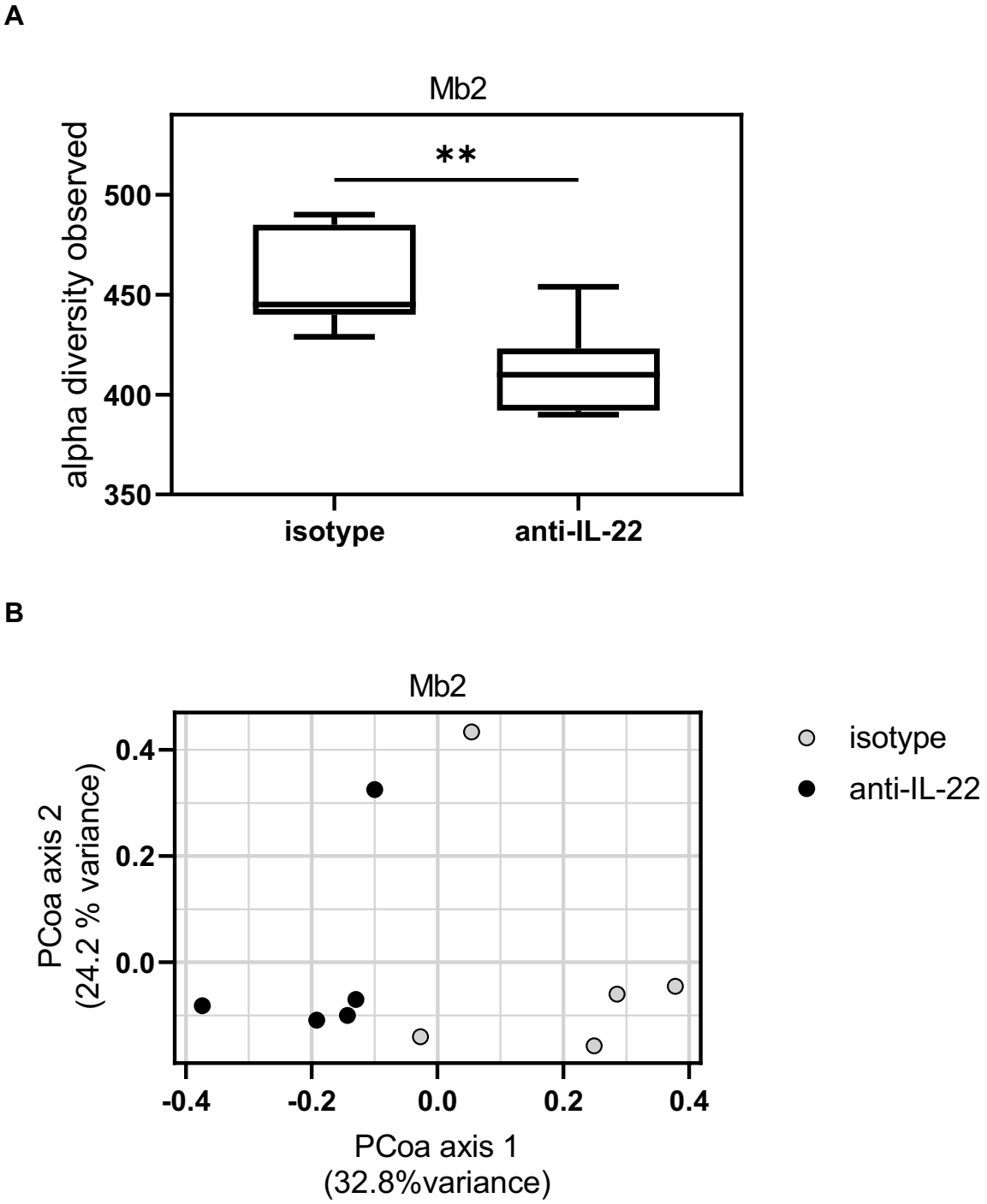


Figure 19 Comparison of differentially treated mice after Mb2 FMT into treatment-naïve recipients. During the four weeks of engraftment, n = 5 mice received isotype, and n = 5 mice received anti-IL-22. Shotgun metagenomic sequencing, alpha diversity (observed) and beta diversity (Bray-Curtis dissimilarity) as principal coordinate analysis.

Summing up our experimental results, three potentially colitogenic microbiotas (Mb2, anti-IL-22 microbiota, and microbiota after colitis) shared similarities. Short-term neutralization of IL-22 did not affect colitis severity in a microbiota setting that needs IL-22 for protection from colitis (Mb2). Then again, long-term neutralization aggravated disease. So, presence of IL-22 before colitis induction is sufficient to ensure its protective function. We assume this to be due to a direct impact of IL-22 on the microbiota. After the engraftment period, we found differences in Mb2 depending on IL-22. Here, 16S rRNA sequencing showed lower *Bacteroidetes* and higher biodiversity among *Firmicutes* in anti-IL-22 treated mice. A confirmatory metagenomic sequencing substantiated the notion of differences depending on IL-22 as seen by somewhat separate clustering in a beta diversity principal coordinate analysis. These differences in microbiota could correspond to phenotypical consequences during colitis. This means if IL-22 is present during the microbiota engraftment, the microbiota is ameliorated, and later IL-22 is dispensable.

5. Discussion

Previously, IL-22 has been found to protect from DSS-mediated and from T cell transfer colitis (160–164). Previous experiments from our laboratory have shown that under certain microbiota conditions, IL-22 does not significantly impact colitis susceptibility. While the mechanism of IL-22-mediated protection remains yet to be elucidated, IL-22 has been shown to influence the gastrointestinal microbiota composition. Here, we confirm that the presence of IL-22 ameliorates disease, and we provide further insight into the effect of IL-22 on the microbiota. Interestingly, we find IL-22-mediated protection does not require active IL-22 production after colitis onset, pointing to an anti-colitogenic effect that happens prior to the induction of colitis. Our adoptive transfer colitis model suggests that already produced IL-22 induces changes in the microbiome that protect from colitis even in the absence of IL-22 later on. Taken together, our research connects the complex picture of IL-22-dependent pro-tection from colitis with its containment of microbiota.

5.1. Importance of quality controls during FMT

Essentially, mice within one hygienic barrier (in our case within an individually ventilated cage) harbor very similar intestinal microbiotas. This cage effect is natural because mice ingest fecal material via coprophagy or contamination of feed within in the cage. Thereby, different microbiotas of mice put together into one cage will eventually adjust within two to four weeks. This phenomenon is the basis for cohousing experiments. (213) A key question is what the resulting microbiota would be after merging of two microbiotas. The first possibility would be that both microbiotas become more similar to one another, so that the resulting microbiota is “in between” the two original ones. In the Bray-Curtis dissimilarity this would mean clustering of the merged microbiota right between the original microbiotas. This mostly symmetric merging occurs if the two merged microbiotas are quite similar (213). Another possibility would be that one microbiota outgrows the other, so that the resulting microbiota is similar to only one of the original microbiotas. So, one microbiota would be dominant over the other. In fact, exactly this is observable with our colitogenic/dysbiotic microbiotas. Via FMT Mb2 engrafted very efficiently into mice that initially harbored Mb1. So, Mb2 is dominant over Mb1. Hence, neither antibiotic pre-treatment of the recipients nor multiple Mb2 donations were needed. Moreover, some experimental groups that had received Mb1 still developed an Mb2 microbiota – probably via minor

contaminations during the FMT process. This confirms the dominance of Mb2 over Mb1. Mb1 is a simple, very hygienic microbiota of low observed alpha diversity. On the other hand, Mb2 includes pathobionts that are absent in Mb1. Therefore, these Mb2 signature bacteria probably easily find a biological niche ("a vacant spot") in the simple microbiota of Mb1 harboring mice. Similarly, it has been shown that cohousing of *wt* mice with mice harboring a dysbiotic microbiota leads to both predominance of the dysbiotic microbiota after some engraftment time and transmission of the colitogenic effect of the dysbiotic microbiota onto the *wt* mice (100, 101). This fosters our view that more colitogenic microbiotas are also more dominant when it comes to FMT or cohousing. A key limitation of these models is that laboratory mice (particularly, in SPF conditions) have a simple microbiota compared to physiological microbiota of wild mice (214). Within a more complex microbiota it might be far more difficult for *taxa* from the dysbiotic microbiota to find a biological niche, *i.e.*, "all spots are already taken". Therefore, it remains questionable if dysbiotic microbiotas are generally dominant over healthy ones, for example in the setting of physiological (*i.e.*, wild) murine microbiota or human microbiota. Taken together, we deem quality controls, such as PCR, following FMT very important to validate correct engraftment since microbiotas can be exceptionally dominant.

5.2. Short-term IL-22 blockade has no effect on T cell transfer colitis

As known from experiments with *Il22*$^{-/-}$ mice, IL-22 protects mice from colitis in certain microbiota settings – but not in the setting of very hygienic laboratory microbiota (190). In parallel, others have found a similar relation with the proinflammatory T_H1 transcription factor T-bet: In some microbiotas, T-bet is required for induction of adoptive T cell colitis. With another microbiota the proinflammatory action of T-bet was not needed to induce colitis. (215) Interestingly, this microbiota was more biodiverse and included pathobionts, similar to our Mb2 microbiota. Consequently, the concept that some molecules of the immune system might only be important in certain microbial surroundings had already been developed. We found that neutralizing IL-22 during the course of T cell colitis has no effect on colitis severity. This was true in both of our microbiota conditions, Mb1 and Mb2, respectively. Mb2 was derived *in vivo* from the mouse colonies used by Zenewicz *et al*. (101, 162). However, one has to point out the experiments by Zenewicz *et al*. were done more than ten years ago, so that microbiota changes have likely occurred during this time and prior to the

import to Hamburg. During transfer and breeding of multiple generations of mice in Hamburg, our Mb2 has probably developed also some unique characteristics. However, in this microbiota (essentially, our Mb2 source) Zenewicz *et al*. have shown that IL-22 is protective in two colitis models (DSS and T cell colitis) (162). So, initially, it was surprising that short-term IL-22 blockade had no effect – even in this Mb2 setting. However, Zenewicz *et al*. had shown that host-derived IL-22 is sufficient to carry out the IL-22-dependent tissue protection. This was observed by two different transfer experiments. Transferring either *wt* or *Il22*$^{-/-}$ CD45RB^{hi} T cells into *Il22*$^{-/-}$ *Rag1*$^{-/-}$ mice showed more severe colitis in those mice that received *Il22*$^{-/-}$ CD45RB^{hi} T cells. On the other hand, when transferring either *wt* or *Il22*$^{-/-}$ CD45RB^{hi} T cells into *Rag1*$^{-/-}$ (*i.e.*, single knockout) mice there was no difference in colitis phenotype between those that received *wt vs.* those that received *Il22*$^{-/-}$ T cells. Thus, IL-22 produced by immune cells of the host is sufficient for carrying out the protective function of IL-22. Because host mice were *Rag1* deficient, this IL-22 must be derived from the innate compartment. Zenewicz *et al*. argue that it is probably produced by NK cells. One might now think that our short-term neutralization had no effect because of host-derived IL-22 just as in this transfer experiment from Zenewicz *et al*. However, with our short-term blockade during the colitis also the host-derived IL-22 was neutralized. Therefore, it follows that host-derived IL-22 that is secreted before initiation of the neutralization (*i.e.*, during the weeks from birth of the mice until induction of colitis) is sufficient to carry out the effect of IL-22. This might be true then even for future episodes of colitis. Prerequisites for this would be, firstly, that IL-22 acts on the intestinal mucosa and, secondly, that this early host-derived IL-22 has a lasting effect. This lasting effect must be at least four weeks long because we did not observe more severe colitis in the anti-IL-22 group for this duration. However, it might well be that this effect does not hold forever. Firstly, our data indicate that at week four there is a slight trend towards more colitis severity in the anti-IL-22 group. Secondly, our adoptive model could not run longer because of the burden of the mice, but it might be possible that after a longer duration of disease the protective effect of the early host-derived IL-22 wears off. Therefore, one sensible follow-up experiment would be to choose an even longer model of colitis. This could be carried out with a chronic DSS model with low doses and numerous rounds of DSS in the drinking water. Another option would be to use our adoptive model of colitis but inject fewer CD45RB^{hi} T cells as colitis induction. The next question we asked ourselves was how IL-22 might act on the intestinal mucosa to produce this lasting effect. Sugimoto *et al*. showed that local injection of an IL-22 gene delivery

system led to swift activation of IL-22 downstream genes, such as MUC1 and MUC3, and diminished local inflammation in a genetic model of colitis (160). However, due to the rapid turnover of IECs and mucus, there must be an additional mechanism how this might lead to a long-term effect. We proposed that changes in the IECs subsequently act on the microbiota. This altered microbiota might then be functionally stable for some weeks and convey the protective effect of IL-22 during future episodes of colitis. And, lastly, this would open up a concept that (at least part of) the effect of IL-22 on colitis is carried out in an indirect way via the microbiota: IL-22 ⇒ IL-22-dependent gene transcription ⇒ change in the microbiota ⇒ less inflammation. The logical follow-up experiment was carried out, and it is discussed in the next paragraph.

5.3. Long-term IL-22 blockade aggravates T cell transfer colitis in Mb2 microbiota

The follow-up experiment was to perform long-term blockade of IL-22. Increased colitis severity in this long-term group would also be more similar to the knockout experiments, and we expected to see an effect on colitis here. Following our hypothesis that IL-22 acts via a change of the microbiota, we took young mice (four weeks old) and chose to perform long-term blockade during colitis and prior to colitis induction (during engraftment of Mb2). This was compared with short-term blockade and isotype. We found that long-term blockade of IL-22 leads to increased colitis severity, which bolsters the concept that endogenous IL-22 plays a protective role and IL-22 during colitis is *per se* dispensable. These findings are in line with the results from Zenewicz *et al*., who used a transfer experiment to prove that host-derived innate IL-22 leads to sufficient IL-22 action and protection from colitis severity (162). Our experiment adds a new perspective to this with respect to two aspects: First, we used therapeutic antibodies instead of germline knockout mice to confirm the concept of innate-derived IL-22 being sufficient. Second, we add new insights into the role of the microbiota (discussed in the next paragraph). Based on our observations, one follow-up experiment would be of particular interest. In our long-term experiment we included three treatment groups (① isotype, ② anti-IL-22, ③ isotype ⇒ anti-IL-22). It would be useful to add a fourth group (④ anti-IL-22 ⇒ isotype). In this group, IL-22 would be neutralized only during the engraftment phase (four weeks) of Mb2. This could answer the question if early IL-22 is more important than IL-22 during the colitis. From our experiments it is already

clear that early IL-22 is sufficient, *i.e.*, its lack leads to colitis aggravation. However, it remains elusive if the presence of IL-22 exclusively during the course of colitis can compensate for blockade of the early host-derived IL-22.

5.4. The protective effect of IL-22 is at least partly mediated by alteration of the microbiota

We found that microbiota from mice treated with an anti-IL-22 antibody during the engraftment of Mb2 was altered and potentially more colitogenic. To sufficiently prove our deduction that this altered microbiota leads to aggravated colitis, two more experiments would be needed. Firstly, the experiment should be repeated with a fourth group that only receives anti-IL-22 during engraftment but regains IL-22 competency after that (as explained above). The second experiment to provide more evidence for this hypothesis would be to isolate feces from isotype treated and anti-IL-22 treated mice and transfer these to germ free mice. This gnotobiotic approach could ultimately show if the changes in colitis phenotype which we observed during our long-term blockade experiment were due to the microbiota. Gnotobiotic experiments have the highest value in establishing causality in microbiota research because all other confounding factors are deleted. There, a specifically transferred microbiota is the only difference between the experimental groups. We think that this would be quite promising as earlier work has already provided evidence that IL-22 prohibits dysbiosis and this might carry out the effect of IL-22 on colitis (101, 216, 217).

In 2013, Zenewicz *et al*. showed that microbiota is altered in *Il22*$^{-/-}$ mice (101). This study from Zenewicz *et al*. has been criticized since no litter mate controls were being used (218). However, we believe the effect of IL-22 on dysbiosis to be very robust: First, it corresponds to our observations depending on anti-IL-22 treatment. Second, the impact of IL-22 on the microbiota might only be observable in already slightly dysbiotic microbiotas, *i.e.*, when it is required for containment of pathobionts (219). Furthermore, in the 2013 study from Zenewicz *et al*. the dysbiotic microbiota of *Il22*$^{-/-}$ mice was transmissible and passed on the colitogenic phenotype onto *wt* mice. Thereby, these *wt* mice were more susceptible to DSS colitis. Even though this has been shown in another model of colitis and with large variability in phenotype, this is a good basis for our hypothesis that IL-22-dependent microbiota change renders mice susceptible to colitis. Zenewicz *et al*. measured the effect of IL-22 on the microbiota by performing analysis of alpha diversity, beta diversity (between *wt* and

Il22$^{-/-}$ mice), and comparisons of relative abundances using 16S rRNA sequencing. In these studies, alpha diversity was found to be increased in *Il22*$^{-/-}$ mice. This only corresponds well with a part of our results. We show by 16S rRNA sequencing that alpha diversity was (as general trend) increased in the long-term anti-IL-22 group after colitis. However, our metagenomic sequencing displays a decrease in alpha diversity in mice that received anti-IL-22. This might be due to low n and because different mice are being compared. Interestingly, observed alpha diversity as examined by number of species was higher in Mb2 compared to Mb1. So, in the laboratory setting (where "normal" microbiota is extremely low complex and free of many *taxa* due to strictly hygienic animal facilities), one might argue that higher alpha diversity tends to be typical for more colitogenic microbiota. This is probably no effect of alpha diversity as such because wild caught mice typically have a much more divers microbiota with high alpha diversity (220). Rather, Mb2 and *Il22*$^{-/-}$ microbiota might be colitogenic due to the presence of pathobionts that are not balanced out by the rest of the *taxa*. Another variable analyzed by Zenewicz *et al*. was beta diversity. With respect to this data, *wt* and *Il22*$^{-/-}$ microbiotas differed significantly. This is partly supported by our metagenomic results as there was some differential clustering in the Bray-Curtis dissimilarity. Next, Zenewicz *et al*. examined relative abundances on different levels of depth. On family level (which is the one most suitable to compare) there were starkly reduced *Bacteroidaceae* and *Lactobacillaceae*. This was balanced out by an enrichment in potential pathobiont families: *Prevotellaceae* and *Helicobacteraceae*. Our data (albeit providing only a general trend) fit well into this picture. After colitis, anti-IL-22 treated groups had reduced *Bacteroidaceae* and slightly increased *Prevotellaceae* and *Helicobacteraceae*. However, *Lactobacillaceae* were not reduced by anti-IL-22 treatment. Comparing the microbiota changes caused by adoptive T cell colitis with the microbiota changes caused by anti-IL-22 treatment, it becomes clear that these two types of dysbiosis share similarities. In both cases we observed a shift of relative abundances from *Bacteroidetes* to *Firmicutes* alongside a reduced alpha diversity (in our 16S rRNA data). Peculiarly, this is opposite to the typical dysbiotic changes observed in human IBD patients (see, introduction). One possibility is that there is a very different "normal" cohort to compare the dysbiotic changes to. Whilst our murine microbiota was compared to a rather low complex microbiota, the human IBD cohorts were compared to healthy individuals with massive complexity and biodiversity. Therefore, we might observe an increase in alpha diversity (alongside a B $\Rightarrow$ F shift) because our baseline microbiota is simpler than a physiologic gut microbiome. This hypothesis is fostered by the fact that

an enrichment in potentially pathological *taxa* is seen both in our experiments and in human IBD patients. Both the murine dysbiosis and human IBD dysbiosis are characterized by enrichment in *Prevotellaceae* and *Helicobacteraceae*.

5.5. Outlook

5.5.1. Future studies

Three experiments can be carried out to further consolidate our hypothesis. First, a gnotobiotic colitis experiment: Fecal recipients should be Mb1 *Rag1*$^{-/-}$ that receive Mb2 via FMT from *wt* mice that have been treated with isotype or anti-IL-22 treatment four weeks prior to the stool donation. Second, addition of a fourth experimental group to our long-term neutralization experiment (group ④ anti-IL-22 during engraftment ⇒ isotype during colitis). Third, a specific metagenomic analysis of microbiota engraftment: Mb1 *Rag1*$^{-/-}$ should receive Mb1 or Mb2 simultaneous with either isotype or anti-IL-22 treatment during engraftment. Then, microbiota should be analyzed after four weeks. This would function as a test to verify that IL-22 does not affect Mb1. Our hypothesis is that IL-22 is not needed in Mb1 settings because there are essentially no pathobionts which would need to be contained by IL-22. Therefore, the above-mentioned experiment would be suitable to closely observe if there are any effects of IL-22 on this less colitogenic microbiota.

5.5.2. Possible therapeutic potential

Since IL-22 protects from colitis and from IBD, some pharmacologic interventions for promoting IL-22 have been proposed and even tested in phase I or II clinical trials. For example, small molecules that induce IL-22 have been suggested, and IL-22 fusion proteins have been tested for ulcerative colitis. So far, none of these attempts has been successful. Moreover, IL-22 can have negative effects, namely it can foster inflammation or promote tumor development. IL-22 driven inflammation occurs in the skin or at joints. (221, 222) A function in promoting tumor growth has been debated regarding colon cancer: On the one hand, IL-22 can secure a physiologic apoptotic reaction of IEC in response to carcinogenic mutagens (43). On the other hand, unregulated IL-22 (*i.e.*, due to loss of the physiologic negative regulation by IL22BP) can promote tumor development (166, 167). Thus, pharmacological promotion of IL-22 should be studied under careful observation, and probably the cancer risk needs to be further investigated and precluded before clinical studies can be considered safe enough. Our results propose another way of using such pharmacological

concepts, which is to choose pharmacological treatments depending on a patient's gut microbiota. This personalized approach has already been proposed in other disease contexts, such as melanoma (189). Regarding pro-IL-22 treatment for IBD patients, it could also be valuable: It is known that the extent of dysbiosis differs vastly among IBD patients (148), and we provided evidence that the protective function of IL-22 on colitis is largely due to amelioration of dysbiotic microbiotas. On that account, future trials of pro-IL-22 drugs could be conducted in highly dysbiotic IBD patients. Such a specific approach for dysbiotic IBD patients might improve effectiveness and tolerability and could be an example of more personalized medicine.

6. Summary

Inflammatory bowel disease (IBD) affects circa half a percent of the Western world population. IBD is characterized by recurring flares of abdominal and, in some cases, systemic symptoms. While the causes for IBD remain unknown, different cytokines have been assigned a central role for IBD pathogenesis. Interestingly, during intestinal inflammation, cytokine effects appear to differ among individuals. For example, IL-22 protects from IBD and murine colitis in most settings but not all. In the presence of certain microbiotas, *e.g.*, Mb2, IL-22 is protective, while in others, *e.g.*, in Mb1, it has no effect on colitis. We hypothesized that IL-22 ameliorates colitis in mice by correcting a colitogenic microbiota. Consequently, we expected IL-22 to fail to alleviate colitis in mouse models with an *a priori* less colitogenic microbiota. To test our hypothesis, we used mice with defined and distinct microbiotas (fecal microbiota transplant of Mb1 and Mb2, respectively) and a murine colitis model ($CD45RB^{hi}$ T cell transfer). For neutralizing IL-22, we intraperitoneally injected a monoclonal anti-IL-22 antibody. 16S rRNA sequencing revealed structural differences among Mb1 and Mb2 microbiotas. Some *taxa* were exclusively present in Mb2 microbiota, *e.g.*, the notoriously colitogenic *Prevotella spp.* and *H. typhlonius*. Mb2 microbiota rendered mice more susceptible to T cell transfer colitis. Unexpectedly, short-term blockade of IL-22 after $CD45RB^{hi}$ T cell transfer into $Rag1^{-/-}$ did not affect colitis severity. This was independent of the microbiota (Mb1 or Mb2). In comparison to short-term blockade, IL-22 long-term neutralization aggravated colitis in Mb2. Long-term neutralization was initiated at the time of fecal microbiota transplant, and it was continued throughout the engraftment and the later colitis phase. These findings indicate that IL-22 that is released from innate immune cells during the engraftment affects the mucosal homeostasis, *i.e.*, the microbiota, which in turn protects from later episodes of colitis. In accordance with our hypothesis, metagenomic analysis unveiled differential engraftment of Mb2 microbiota depending on IL-22 presence or absence. We show alterations in the microbiota of IL-22 neutralized mice, which might be indicative of a higher colitogenic potential. However, in order to fully establish this causal link, further gnotobiotic experiments are necessary.

Taken together, we provide evidence that in an IBD model system IL-22 confines a colitogenic microbiota and that the protective effect of IL-22 on colitis is at least partly via this modulation of the microbiota.

7. Zusammenfassung

Etwa ein halbes Prozent der westlichen Weltbevölkerung leidet an einer chronisch-entzündlichen Darmerkrankung (CED). Gekennzeichnet sind CED durch wiederkehrende abdominelle Beschwerden, etwa Diarrhöen und Bauchkrämpfe, und teils durch systemische Symptome, zum Beispiel Fatigue oder Gewichtsverlust. Während die Ursachen für CED unbekannt sind, wird Zytokinen des Immunsystems eine zentrale Rolle für die Pathogenese zugeschrieben.

Interessanterweise gibt es Hinweise, dass sich die Wirkungen einzelner Zytokine während einer Darmentzündung je nach Situation unterscheiden. Beispielsweise schützt Interleukin (IL)-22 in Gegenwart bestimmter Mikrobiome, zum Beispiel Mb2, vor muriner Kolitis, während es zum Beispiel in Mb1 keine Wirkung auf den Verlauf der Kolitis hat. Wir stellten die Hypothese auf, dass IL-22 murine Kolitis abmildert, indem es kolitogene Mikrobiome korrigiert. Folglich erwarteten wir, dass IL-22 die Kolitis in Mäusen mit einem *a priori* nicht kolitogenen Mikrobiom nicht lindern würde. Um unsere Hypothese zu prüfen, untersuchten wir Mäuse mit definierten Mikrobiomen (durch Stuhltransplantation von Mb1 bzw. von Mb2) in einem Kolitismodell (CD45RB^{hi} T-Zell Transferkolitis). Um IL-22 zu neutralisieren, injizierten wir einen monoklonalen anti-IL-22-Antikörper intraperitoneal.

Eine 16S-rRNA-Sequenzierung zeigte strukturelle Unterschiede zwischen Mb1 und Mb2 auf. Einige *taxa* waren ausschließlich im Mb2 Mikrobiom vorhanden, wie etwa die als kolitogen bekannten *genera Prevotella spp.* und *H. typhlonius*. Dazu passend waren Mäuse mit dem Mb2 Mikrobiom anfälliger für T-Zell Transferkolitis als solche mit Mb1. Anders als erwartet hatte die kurzfristige Blockade von IL-22 nach CD45RB^{hi} T-Zell Transfer in *Rag1*$^{-/-}$ Mäusen keinen Einfluss auf die Schwere der Kolitis. Dies war unabhängig vom Mikrobiom (Mb1 oder Mb2). Im Vergleich zur kurzzeitigen Blockade führte eine längerfristige IL-22 Neutralisation zu einem schwereren Verlauf der Kolitis in Mb2. Diese längerfristige IL-22 Neutralisation wurde zum Zeitpunkt der Stuhltransplantation eingeleitet und sowohl in den nachfolgenden Wochen (Einwachsen des Transplantates) als auch in der späteren Kolitisphase fortgesetzt. Diese Ergebnisse weisen darauf hin, dass IL-22 während der Zeit des Einwachsens des Stuhltransplantates durch Zellen des angeborenen Immunsystems freigesetzt wird und die Schleimhauthomöostase, insbesondere das Mikrobiom, beeinflusst und dadurch vor späteren Kolitis-Episoden schützt. In Übereinstimmung mit unserer Hypothese

enthüllte eine metagenomische Sequenzierung ein unterschiedliches Einwachsen des Mb2 Mikrobioms in Abhängigkeit von der Anwesenheit oder Abwesenheit von IL-22. Unsere Analysen zeigten Veränderungen im Mikrobiom von IL-22 neutralisierten Mäusen, die auf ein höheres kolitogenes Potenzial hinweisen könnten. Um diesen kausalen Zusammenhang vollständig nachzuweisen, wären jedoch weitere, gnotobiotische Experimente notwendig.

Zusammenfassend liefern wir Beweise dafür, dass IL-22 in einem Modellsystem für CED ein kolitogenes Mikrobiom abmildert und, dass die Schutzwirkung von IL-22 weitgehend über diese Modulation des Mikrobioms erfolgt.

8. Abbreviations

Where appropriate, abbreviations or acronyms were used (introduced when a term is used initially). We generally used murine orthologs (*e.g.*, REGIIIγ instead of REGIIIα).

Abbreviation used	**Meaning**
[…]-H	[…] signal height
[…]-W	[…] signal width
[…]-A	[…] signal area
ACK	ammonium-chloride-potassium
APRIL	a proliferation-inducing ligand
ASCA	anti-saccharomyces cerevisiae antibodies
ATG16L1	autophagy related 16 like 1
B.	*Bacteroides*
BAFF	B cell-activating factor
BHI	Brain Heart Infusion
bzw.	Beziehungsweise
C.	*Clostridium*
Cbir1	anti-Cbir1 flagellin antibody
CCL	CC-chemokine ligand
CCR	chemokine receptor
CD[number]	cluster of differentiation[…]
CD	Crohn's disease
CED	chronisch-entzündliche Darmerkrankung(en)
CXCR	C-X-C chemokine receptor
DAG	diacylglycerol

DEGS	differentially expressed genes
DNA	deoxyribonucleic acid
DSS	dextran sulfate sodium
DTT	dithiothreitol
E.	*Escherichia*
e.g.	*exempli gratia*
EDTA	ethylenediaminetetraacetic acid
et al.	*et alii*
etc.	*et cetera*
F.	*Faecalibacterium*
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FMT	fecal microbiota transplant
FOXP3	forkhead box P3
FSC	forward scatter
GALT	gut-associated lymphoid tissue
GWAS	genome-wide association studies
H.	*Helicobacter*
HBSS	Hank's balanced salt solution
H&E	hematoxylin and eosin
i.e.	*id est*
IBD	inflammatory bowel disase
IEC	intestinal epitehlial cell
IECs	intestinal epitehlial cells
IELs	intraepithelial lymphocyte(s)

IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL10RB	IL-10 receptor beta subunit
Il22$^{-/-}$	IL-22 deficient
IL22RA1	interleukin-22 receptor, alpha 1
IL22BP	IL-22 binding protein
ILC	innate lymphoid cell
IPEX	immune-dysregulation polyendocrinopathy enteropathy X-linked
IVC	individually ventilated cage
ln.	*lymphonodus*
lnn.	*lymphonodi*
LTi cell	lymphoid tissue inducer cell
Ly6C	lymphocyte antigen 6 complex, locus C
Ly6G	lymphocyte antigen 6 complex, locus G
M cell	microfold cell
MACS	magnetic-activated cell sorting
MAIT cell	mucosal associated invariant T cell
MATE	microbial adhesion-triggered endocytosis
Mb	microbiota
min	minutes
mTNF	memrbane-bound TNF
MUC	mucin
n	number of biological replicates

N	populations size
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NKT cell	natural killer T cell
NLRP6	NOD-like receptor family pyrin domain containing 6
NOD2	nucleotide-binding oligomerization domain-containing protein 2
OmpC	anti-E. coli Outer membrane porin C precursor
OTU	operational taxonomic unit
P/C/I	phenol/chloroform/isoamyl alcohol
p-ANCA	perinuclear antineutrophil cytoplasmic antibodies
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
Rag	recombinase activating gene
RANKL	receptor activator of NF-κB ligand
rcf	relative centrifugal force
Ref.	reference
REGIIIγ	regenerating islet-derived protein 3 gamma
RORγ	RAR-related orphan receptor gamma
rpm	revolutions per minute
RT	room temperature
SCFA	short-chain fatty acids
SCID	severe combined immunodeficiency
s.d.	standard deviation

SDS	sodium dodecyl sulfate
SFB	segmented filamentous bacteria
SPF	specific-pathogen-free conditions
SSC	sideward scatter
TCR	T cell receptor
TFH	T follicular helper cell
TGFβ	transforming growth factor-beta
T_H1	T helper cell type 1
T_H17	T helper cell type 17
T_H2	T helper cell type 2
T_H22	IL-22 producing T helper cells (term is not mutually exclusive in regard to other T helper cell subtypes)
TVA	Tierversuchsantrag
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF (TNFα)	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1
Tr1	type 1 regulatory T cells
T_{reg}	regulatory T cell
tris	tris(hydroxyethyl)aminomethane
TRM	tissue-resident memory T cell
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis
UKE	University Medical Center Hamburg-Eppendorf, Germany
vs.	*versus*
wt	*wild type*

XBP1	X-box binding protein 1

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11. Curriculum Vitae

Der Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

12. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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