THE PROBIOTIC POTENTIAL OF NON-TOXIGENIC BACTEROIDES FRAGILIS AND THE ROLE OF IL-22 IN COLITIS AND TUMORIGENESIS

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ABSTRACT - *Bacteroides fragilis* Co-infection

Polysaccharide A (PSA), a highly immunogenic capsular component of non-toxigenic *Bacteroides fragilis* NCTC 9343 (NTBF), has been shown to promote host mucosal immune system development and to suppress inflammation in the gut. In stark contrast, enterotoxigenic *Bacteroides fragilis* (ETBF) induces IL-17 and is highly associated with inflammatory bowel disease and colorectal cancer, rapidly inducing acute colitis and tumorigenesis in murine hosts. The interactions of these distinct *B. fragilis* molecular subtypes were studied to determine the impact of NTBF on ETBF disease. In specific pathogen free (SPF) C57BL/6 WT and Min$^{Apc716+/-}$ mice, we show that sequential treatment (NTBF then ETBF three days later) blunted colitis and decreased tumor development. Mice receiving simultaneous NTBF and ETBF treatment exhibited severe colitis and pronounced tumor formation. Abrogated disease severity in sequentially treated mice was attributed to NTBF strain dominance and decreased IL-17, but ETBF colonization prior to or simultaneous with NTBF mitigates the anti-inflammatory effect of NTBF. Surprisingly, NTBF-mediated protection was independent of polysaccharide A (PSA), as sequentially infected mice receiving ΔPSA NTBF exhibited similar levels of protection. Further, SPF WT and Min mice mono-associated with PSA-competent or PSA-deficient NTBF exhibit similar T cell-derived regulation, IL-17, and IFNγ responses. Daily NTBF probiotic treatment of mice stably colonized with ETBF failed to disrupt both ETBF strain dominance and niche occupation. Our findings demonstrate that NTBF may offer protection against ETBF disease but only under certain conditions. Disease outcomes are independent of PSA.

Significance: In a murine model, NTBF mediated protection against ETBF colitis and tumorigenesis solely through competitive niche exclusion and not PSA expression. Colonization with *Bacteroides fragilis*, that is not infrequently ETBF, is common in the general population. Our results suggest that the probiotic capacity of NTBF to limit inflammatory disease in humans may be limited by prior colon mucosal ETBF colonization.

ABSTRACT - IL-22 in enterotoxigenic *Bacteroides fragilis* disease

With purported roles in epithelial cell innate immunity and repair, the host-protective immune signaling molecule, interleukin-22 (IL-22), may have a paradoxical effect on the development of colonic dysplasia. As of yet, the role of IL-22 in enterotoxigenic *Bacteroides fragilis* (ETBF)-mediated disease is unknown. In C57BL/6 mice colonized with the human symbiote, ETBF, we show that an absence of IL-22 (IL-$22^{-/-}$) promotes increased epithelial cell shedding, intestinal permeability, and IL-17A and IL-11 pro-inflammatory signatures. Notably, ETBF-colonized Min$^{Apc716+/-}$ IL-$22^{-/-}$ mice exhibited increases in tumor burden compared with parental controls. Furthermore, IL-22 deficiency in ETBF-infected Min mice resulted in a further expansion of MHC class II low (ClassII^{low}) macrophages of the myeloid lineage. Herein, we describe a role for IL-22 that is protective against ETBF-initiated inflammation and tumor development.

PREFACE

My love of microbiology and immunology began with introductory courses while an undergraduate student at the University of Maryland. These experiences set in motion an ongoing fascination with the diverse array of microscopic organisms that surround us, and their contributions to human health and disease. Since that early time, I have been incredibly lucky to learn and to pursue my scientific interests under exceptional teachers and mentors. This work is a culmination of many years spent in pursuit of my scientific interests, of which many people have contributed to and supported.

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CHAPTER 1 - Introduction

The Human Microbiota

Current estimates for the ratio of bacterial cells to human cells is approximately 1.3 to 1, 39 trillion bacterial cells to 30 trillion human cells [1] . Although this ratio is somewhat less impressive than the 10 to 1 ratio the scientific literature had assigned in the past, it is still undeniable that the mutualistic relationships that have evolved between humans and the microbiota influence every aspect of our physiology. From odor to nutrition to our health status to a significant driver of disease, we are becoming increasingly aware of the microbiome's impact. Humans shed ~10^6, >0.5 μm diameter biological particles per hour that are likely to contain numerous bacteria[2,3]. Sampling airborne bacteria from air filters in a controlled environment, Meadow and colleagues recently showed that humans emit individualized microbial clouds and that individuals could also be identified by their specific microbial emissions [4] . Even the operational taxonomic units (OTUs) associated with the common vaginal microbes, *Lactobacillus crispatus* and *Gardnerella vaginalus*, were detected in the air surrounding the two female subjects, indicating the non-discreet nature of the personal microbial cloud leading to sex-relevant distinctions [4] . In turn, it is fascinating to consider the effects that our own microbiomes are having on the world around us, from our daily personal interactions to what signatures are left on what is termed the 'built environment microbiome', the community of microorganisms found in human constructed environments.

Role of Host Genetics in Defining the Gut Microbiome

Although there are many contributing factors, host genetics are likely at the crux of gut microbiome determination. For instance, a study comparing diverse mammalian fecal bacterial 16S ribosomal RNA profiles (from 13 taxonomic orders, including 60 species housed in 2 separate zoos or in the wild) found that gut microbiomes retained similarity among animals that were related by phylogeny[5]. Even in 16 sampled humans spanning three continents and ages (27-94 years old), their gut microbiome profiles were more similar to each other than to other mammals[5]. Thus, in spite of differing dietary patterns and environmental exposures, host genetics may exert the largest influence on the microbiome. Consistently, studies of human twins show significantly more similarity in the gut microbiome compositions of monozygotic twins compared with dizygotic twins[6–8].

Even though fluctuations in the microbiome commonly occur, for instance, throughout the day as dictated by circadian rhythm and feeding patterns[9], the long-term maintenance of microbial species appears to remain relatively stable over time within individuals[10]. Faith et al. showed that ~60% of total gut microbial strains were consistently detected in study subjects sampled over the course of 5 years with inferences made from a power law suggesting that harbored strains are likely maintained for decades or more[10]. Furthermore, another research study looked at the colonization of differentially-sourced microbes (e.g. zebrafish gut, termite hindgut, soil, and human feces) in germ free (GF) mice. It was found that the heterologous microbes could establish in GF hosts, yet co-housing of these mixed-microbiota mice with conventionally-colonized mice led to dominance by native mouse microbes and almost

an entire exclusion of the heterologous microbes[11]. This suggested an innate

predisposition for host-specific species.

Host-defined mechanisms and molecules also act to influence gut composition,

for instance through antimicrobial peptide (AMP), immunoglobulin A (IgA), and

microRNA secretion. For instance, 17 sampled human commensals, representative of the

dominant bacterial phyla of the gut, exhibited broad resistance to numerous

inflammation-associated AMPs, while common enteropathogens did not[12]. AMPs,

typically small (12 to 50 amino acids), cationic, amphiphilic or hydrophobic, are

generally thought to mediate cell damage by electrostatic binding to its target cell and

induction of cell membrane permeability and depolarization[13,14]. One prominent human

commensal, *Bacteroides thetaiotaomicron* (*B. theta*), has evolved to escape inflammation

AMP damage through cleavage of the 4' negatively-charged phosphate group of the

lipopolysaccharide (LPS) lipid A anchor found in its outer membrane. Encoded by the

lpxF gene, this cleavage neutralizes the bacterial negative charge, preventing AMP

binding to the membrane[12]. *lpxF* mutant *B. theta* was rapidly outcompeted by wild type

(WT) *B. theta* during experimental colitis in mice[12]. Suggestive of its mechanistic utility

in humans, all sequenced human Bacteroidetes were found to contain LpxF orthologues,

and all characterized lipid A LPS structures of this phylum were found to be under-

phosphorylated[12].

Another form of host control occurs through the production of antibodies, with

IgA being the most abundant Ig isotype found at the mucosal surface. In a study looking

at mice that were deficient in activation-induced cytidine deaminase (AID), the driver of

antibody hypermutation, the burden of intestinal segmented filamentous bacteria (SFB)

was strikingly enriched. It appeared that lack of functional IgA contributed to aberrant immune responses and increased bacterial burden[15]. SFB dysbiosis and immune activation in AID-deficient mice were both rescued by parabiosis[16] (a surgical union allowing for shared blood circulation) with WT mice. This IgA restoration prevented SFB mucosal localization[15].

Host microbiome regulation can also be perpetuated through production of circulating microRNAs (miRNAs), small (18 to 23 nucleotides), non-coding RNAs with complementary binding to mRNA transcripts typically resulting in their silencing. Liu and colleagues recently identified an abundance of fecal miRNAs in sampled human and murine feces and described their potential ability to enter and directly alter gene expression of bacteria. Specific miRNAs appeared capable of entering *Escherichia coli* and *Fusobacterium nucleatum*, co-localizing with bacterial nucleic acids, and leading to a promotion of bacterial growth[17]. Additionally, in mice with intestinal epithelial cell (IEC) specific loss of Dicer (*Dicer1$^{\Delta IEC}$*), the crucial miRNA processing enzyme, their gut microbiomes were distinct from Dicer-competent (*Dicer1$^{fl/fl}$*) littermates, and they were also more susceptible to dextran sulfate sodium (DSS)-induced colitis. Microbiome similarity and disease protection, presumably through the shaping of specific symbiotic bacteria that regulate intestinal barrier function, were restored after WT miRNA fecal transfer to *Dicer1$^{\Delta IEC}$* mice[17]. Altogether, these findings provide robust examples of host-driven selective pressures and gene regulation that can dynamically shape the gut microbiome.

Establishment of Microbiome Symbiosis

Throughout development, humans and other mammals establish a complex symbiosis with microorganisms from numerous environmental exposures. This begins at birth, during exposure to the maternal vaginal microbiota[18] in the birth canal and continues throughout breastfeeding, where the infant gut becomes exposed to the milk-associated microbiota[19] . Infants are exposed to maternal antibodies that protect against harmful organisms[20], and the digestion of milk oligosaccharides also aids the selection of particular bacterial genera[21], such as *Bifidobacterium* and *Bacteroides*[22]. The transition to solid foods coincides with a shift towards a more adult-like microbiota (typically by the age of 3) with increased diversity and increased abundance of anaerobic Firmicutes[23]. Old age is also associated with microbial shifts accompanied by diminished diversity[24] , increased Enterobacteriaceae, and decreased Bifidobacteria[25]. Indicative of the vast co-evolution that has occurred between the human host and microbiota, the adult intestinal ecosystem is distinct from other evaluated microbial communities[26] and even contains many species not found anywhere else in nature.

Constantly bombarded with a milieu of microorganisms and other foreign products through food consumption, the mucosal immune system must establish a form of equilibrium. On the one hand, it must readily detect and defend against pathogens, e.g. enterohemorrhagic *E. coli* and *Vibrio cholerae*, yet robust immune responses to every microbe or product encountered in the gut lumen would lead to inflammatory reactions detrimental to the host. The research also shows that exposure to commensal microbes is essential for proper immune system maturation, thus the host requires a controlled immune response to these harmless and likely beneficial organisms[27]. This tolerant co-

existence allows us to reap the benefits of a healthy microbiota that aids in nutrient and

drug metabolism, intestinal barrier function, and competitive exclusion of pathogenic

organisms.

The analysis of fecal 16S rRNA sequences along with metagenomic sequencing

data shows that Firmicutes, Bacteriodetes, and Actinobacteria phyla are dominant in

healthy people, while Proteobacteria and Verrucomicrobia are present to a lesser extent[28].

Low abundance of Proteobacteria accompanied with high abundance of *Bacteroides*,

Prevotella, and *Ruminococcus* genera are also suggestive of a healthy state[29].

Furthermore, there are commonly detected 'phylotypes', which also include unculturable

microbial species, that colonize healthy individuals. A recent study analyzing the feces of

17 healthy adults yielded 66 abundant phylotypes, common to and detected in greater

than half of study subjects[30]. Comparison of these findings with other publicly available

data sets of the fecal microbiome from healthy humans show strong overlap, with 52 of

the phylotypes (78.8%) being detected in three out of four of these libraries. In spite of

individual subject variability, these data strongly suggest the existence of a phylogenetic

core of healthy human intestinal microbes.

Although the fecal microbiota of healthy adults appears stable over time, gut

composition fluctuations can be induced by dietary changes in the short-term and long-

term. For instance, a diet high in non-digestible carbohydrates induced microbial shifts

high in *Bifidobacterium* within 2 to 3 weeks[28]. Additionally, Wu et al. recently showed

that a long-term high fiber diet is associated with *Prevotella* spp., while a high protein

diet is associated with *Bacteroides* spp. in a study population of 96 adults[31].

Dysbiosis and Disease Implications

As previously mentioned, the intestinal microbiota has been shown to follow a diurnal rhythm, with oscillations occurring in the abundant Bacteroidales, Clostridiales, and Lactobacillalus taxonomic orders. However, in mice lacking a functional circadian clock (*Per1/2*[-/-]), these microbial oscillations were ablated[9]. Thaiss and colleagues found that internal clock-directed feeding patterns drove these microbial fluctuations. As nocturnal animals, WT mice fed primarily during the dark phase, yet *Per1/2*[-/-] mice fed constantly throughout the day. When *Per1/2*[-/-] mice were given controlled access to food only during the dark phase, proper microbial rhythms were restored. The initiation of jet lag in mice (an 8-hour time shift every 3 days) and observations in jet-lagged (an 8- to 10-hour time shift) healthy human subjects yielded disrupted intestinal microbial rhythms and microbial dysbiosis[9].

Fascinatingly, the fecal transfer from jet-lagged mice and humans to GF mouse recipients initiated metabolic disease, evident by increased weight gain and glucose intolerance compared with controls remaining on a normal circadian rhythm[9]. In this era of industrialization, disruptions to the human circadian clock have arisen from increased chronic shift work and the increased convenience of travel across different time zones. Such circadian misalignment has become associated with the development of numerous diseases, including obesity, cancer, diabetes, cardiovascular disease, and susceptibility to infection[32–36]. The findings of the described study provide evidence of a man-made synchronized circadian and microbial dysbiosis that may significantly impact human health.

In the context of intestinal disease, strong evidence exists for the role of the microbiota in both inflammatory bowel disease (IBD) and colorectal cancer (CRC). IBD is defined as a chronic inflammation of the gastrointestinal (GI) tract with periods of chronic remission and relapse, typically organized into Crohn's disease (CD) or ulcerative colitis (UC). Although the etiology of IBD is not fully understood, in genetically predisposed individuals, inflammation is attributed to aberrant mucosal immune responses to commensal gut bacteria[37]. Further meta-analysis showed improved remission rates in active CD and UC patients treated with antibiotics over the placebo-treated group[38].

Many IBD loci that have been discovered (e.g. *NOD2*, *STAT3*, and *ATG16L1*) are associated with innate immunity and defense against enteric bacteria[39,40]. Such genetic variants can contribute to the overgrowth of specific microbial species, likely playing a role in inducing an overzealous immune response[41]. For example, mutations in nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) are one of the strongest risk factors for IBD[42–44]. NOD2 senses muramyl dipeptide, a bacterial cell wall component, intracellularly, inducing NF-κB and MAPK signaling. This initiates production of AMPs, cytokines, and T cell co-stimulatory molecules leading to their activation[45,46]. Indeed, the absence of functional NOD2 in mice disrupts immune homeostasis, promoting aberrant mucosal immune responses to commensals and also increasing the bacterial load[47,48]. *NOD2*-deficient mice also displayed a predisposition to developing colitis induced by DSS and azoxymethane (AOM)[49]. Genetic alterations in known IBD loci (e.g. *NOD2, T-bet*, *Rag2*, *IL10*, and *Tlr5*) have also been shown to promote the development of a dysbiotic microbiome that, when transferred to healthy test

animals, initiates disease[50,51]. In the example of NOD2, administration of feces from *NOD2*-deficient mice to GF WT mice (*NOD2*$^{-/-}$→GF-*NOD2*$^{+/+}$) transferred the increased risk of colitis to recipients. Fecal transfer (*NOD2*$^{-/-}$→GF-*NOD2*$^{+/+}$; *NOD2*$^{-/-}$→GF- *NOD2*$^{-/-}$) was accompanied by microbiome composition shifts in *Bacteroides* (increased), *Butyrivibrio* (decreased), and *Lachnobacterium* (decreased) compared with recipients of specific pathogen free (SPF) WT feces[49]. This microbiota transfer appeared to sensitize the colons of WT mice to chemically-induced colitis through alterations in epithelial cell barrier function; for instance, enhanced claudin-5 (*Cldn5*) expression, a protein component of the tight junction strands, was observed in the colons of WT mice that had been co-housed with *NOD-2* deficient or *RIP2*-deficient mice. Notably, the transfer of WT feces to *NOD2*-deficient animals (*NOD2*$^{+/+}$→GF-*NOD2*$^{-/-}$) rescued them from disease development[49].

Parallel to the results of mouse models, microbiota shifts are also observed in humans afflicted with intestinal inflammatory conditions. For instance, an overall decrease in microbial diversity is observed in the microbiomes of IBD and CRC patients in comparison with healthy controls[52,53]. In IBD, decreased burden and diversity within the Firmicutes and Bacteroidetes phyla are noted, whereas increased *Enterobacteriaceae* (Proteobacteria phyla), specifically of adherent *E. coli*, are observed[52,54]. Of interest, a 474-person microbiome genome-wide association study (mGWAS) yielded that individuals with variants in the risk allele, *NOD2*, had specific enrichment in *Enterobacteriaceae*[55]. In addition, stool samples from CRC patients demonstrate an increase in the *Bacteroides* and *Prevotella* genera and associations with the *Actinomyces*,

Atopobium, *Fusobacterium*, and *Haemophilus* genera compared to colonoscopy controls[56,57].

In trying to elucidate microbial contributions to CRC development, three hypotheses exist: potential influences from 1) single microbes, 2) the microbial community, or 3) the interactions of single microbes with the microbial community directing a pathogenic microbiota[58]. In this composition, the contributions of single microbes to CRC development will be investigated. Through human association studies and animal models, several symbiotic microbes, including enterotoxigenic *Bacteroides fragilis*, *F. nucleatum*, *E. coli*, *Streptococcus gallolyticus*, and the *Enterococcus* spp., have a purported role in colorectal cancer[59]. These microbes may initiate or promote the progression of cancer indirectly or directly through the recruitment of inflammatory cells producing copious amounts of reactive oxygen species, or through bacterial-derived factors or toxins that can be highly damaging to DNA, alter cellular proliferation, and induce apoptosis[60]. Altogether, such findings suggest that targeted intestinal microbial manipulation of the host could lead to improvements in disease prevention and/or disease outcome for patients at risk or are undergoing treatment for CRC and IBD.

Enterotoxigenic *Bacteroides fragilis*

Bacteroides fragilis is a Gram-negative, obligate anaerobic bacterium known to typically reside in the lower gastrointestinal tracts of mammals and comprising only < 1 to 2% of the cultured fecal flora[61,62]. *B. fragilis* is a common commensal of humans; Zitomersky et al. found that 87% of adults were fecally colonized with *B. fragilis*[63]. Enterotoxigenic *B. fragilis* (ETBF) was originally discovered during study of the etiology

of newborn lamb diarrheal disease on ranches in the Rocky Mountain Region of the United States[64]. *B. fragilis* was isolated from the feces of young lambs with acute diarrhea, and enterotoxin activity was subsequently confirmed through the lamb intestinal loop (LIL) test. The LIL test utilized the creation of multiple isolated segments of the small intestine, created by surgical ligation, and inoculation of various test diarrheic feces, test bacteria, or controls into individual segments; the secretory phenotype of test samples was then measured by visually scoring the fluid accumulation within segments[64]. Inoculation with pure cultures of isolated fecal ETBF caused acute enteric disease in a subset of newborn lambs, and further ETBF-associated piglet, calf, and foal diarrheal illness would later be reported. A secreted heat-labile protein toxin was identified as the initiator of intestinal secretion.

In an uncontrolled study in 1987, ETBF was first isolated in diarrheic humans from Montana (10 subjects) and infants (34 subjects, 2-14 months old) from the Navajo Area Indian Reservation in Tuba City, Arizona[65]. ETBF isolates were detected in 8 of 44 subjects (2 adults and 6 children <5 years old)[65]. Three other uncontrolled clinical reports from Warsaw, Poland, provided further evidence of ETBF isolation from diarrheal patients: 1994 study in children (n=120; 16.7% of stools were *B. fragilis* positive; 2 children were ETBF positive)[66], 1999 study (n=50; 17/50 (34%) of stools were *B. fragilis* positive; 4/17 (23%) were ETBF positive)[67], 2003 study in children and adults (n=332; 50/332 (15%) of stools were *B. fragilis* positive; 9/50 (18%) were ETBF positive)[68]. In 1992, the first study including control subjects was conducted in pediatric outpatient clinics of the Apache Indian Reservation in Whitewater, Arizona; the study found ETBF isolates associated with diarrhea in children 1 to 5 years old but not in children < 1 year

old (n=275; overall, 12% diarrheic patients and 6% control subjects were ETBF positive), with strains isolated within families seemingly genetically related[69]. Although variable in nature, in total, the results of 12 of 17 controlled studies combining research in children and adults, showed associations with ETBF and diarrheal disease[62]. Collectively, the data suggest that ETBF is an etiologic agent of diarrheal disease in both children and adults with global distribution. ETBF illness in humans is typically characterized by an acute, self-limiting watery diarrhea with persistent diarrhea noted only in a small subset of patients. Even still, asymptomatic colonization with ETBF is common with earlier studies collectively reporting 2% to 30% detection[62], and a recent study reporting fecal detection in 40% of healthy individuals (6 out of 15 subjects in Boston, MA)[63].

Bacterial members of the enterotoxigenic *B. fragilis* subgroup were discovered to carry and secrete the *B. fragilis* toxin, or BFT, genomically encoded on a ~6 kb pathogenicity island[70]. Seminal work in human carcinoma cell lines showed that BFT, a 20 kDa zinc metalloprotease, severely alters the colon epithelium cell-to-cell adherens junctions through the cleavage of E-cadherin, disrupting barrier function and stimulating the Wnt signaling pathway to induce proto-oncogenes and other cell proliferative genes, such as *c-Myc*[71,72]. Later, ETBF was observed to cause marked colonic inflammation and tumor development in mice[73–75]. Moreover, a milieu of pro-inflammatory cytokines is produced, most notably IL-17A, required for ETBF pathogenesis[75]. Recent insights by our group have also been made into the complex interplay of IL-17, NF-κB, and STAT-3 signaling in the ETBF model. IL-17A directly influences colon epithelial cell (CEC) NF-κB signaling, inducing an NF-κB-dependent CXCL1 chemokine gradient leading to a critical homing of pro-tumoral myeloid cells to the distal colon region, the distinct site of

ETBF-induced tumors in genetically predisposed $Apc^{+/-}$ or Min mice (Chung et al. –
under review). Furthermore, STAT-3 activation in CECs is also required for colon
tumorigenesis, while IL-17A-producing cells (CD4^{+} and γδ T cells) and infiltrating
myeloid cells drive persistent colonic inflammation[76,77].

Non-enterotoxigenic *Bacteroides fragilis*

Another molecular subset of *B. fragilis* is designated as non-enterotoxigenic *B.
fragilis* (NTBF), which do not encode the pathogenicity island (BfPAI) containing the *bft*
gene. Beyond its association with diarrheal disease, historically, *B. fragilis* has been the
most commonly isolated obligate anaerobe in human infections including intra-
abdominal abscesses and bacteremia[78,79]. Intraperitoneal (i.p.) administration of
encapsulated *B. fragilis* or its capsular polysaccharide complex (CPC) along with sterile
cecal contents to rodents induced abscess formation[80]. Pathogenicity was later attributed
to a unique, zwitterionically charged component of the CPC (a repeating tetrasaccharide
unit with a balanced positively charged amino group and negatively charged carboxyl
group), capable of inducing T cell activation and prompting abscess formation[81].
Chemical modifications to either charge of the polysaccharide markedly reduced its
ability to induce abscesses, whereas an un-related *Salmonella typhi* polysaccharide
capsule (Vi antigen), containing one negative charge, could be modified to an abscess-
inducing form by introducing an additional positive charge[82]. In contrast, when *B. fragilis*
is allowed to remain in its primary ecological niche, the mammalian colon, its CPC
initiates a non-harmful, host-protective regulatory T cell response, a phenomenon that
will be later investigated in this work. Interestingly, rats exposed subcutaneously to the

high molecular weight polysaccharide A (PSA) component of the CPC prior to i.p. bacterial administration were protected from abscess formation in a T cell-dependent manner[83].

In lieu of the fact that bacterial polysaccharides have been strictly viewed as T-cell independent antigens, these findings were incredibly novel. Passive transfer of antibodies from CPC-immunized rats to un-immunized rats conferred protection against *B. fragilis* bacteremia but not abscess formation, while adoptive transfer of splenocytes from CPC-immunized rats did prevent abscesses[84]. Crucial *in vitro* experiments also showed that purified PSA could only induce CD4^{+} T cell proliferation in the presence of antigen presenting cells (APCs)[85]. Further computer modeling studies on the closely related molecule, PSA2 from *B. fragilis* clinical isolate 638R, showed a theoretical charged-based binding of PSA2 into the alpha-helical peptide groove boundaries of MHC class II molecules[86]. Confocal microscopy experiments further strengthened these data by showing the visualization of PSA at the immunological synapse between APCs (MHC class II) and T cells (αβ TCR)[87]. These studies and numerous others have continued to provide strong evidence for the non-traditional processing and presentation of polysaccharide A (PSA), the recognized immunodominant component of the CPC, to CD4^{+} T cells leading to their activation. Since the exciting discovery of these distinctive CPC immunochemical properties, the biological function of these structures in gut health has continued to be researched.

Although the *B. fragilis* CPC was initially thought to be comprised of only 2 high molecular weight molecules, the heavily studied polysaccharides A and B (PSA and PSB), genomic analysis later revealed that *B. fragilis* can produce at least 8 distinct

capsular polysaccharides (denoted A through H). This number is more than described for any bacteria outside of the *Bacteroidales* order; thus 133 kb of the *B. fragilis* genome is devoted to capsular polysaccharide synthesis[88]. Within a bacterial population *in vitro*, only a portion of the culture will be expressing any one polysaccharide with even dual or no expression observed when double-labeling experiments were performed. Analysis of sorted bacterial PSA-on and PSA-off populations after re-culturing yielded a mix of on and off populations within each subset, showing that polysaccharide phase variation exhibits a reversible on-off phenotype[88]. Excluding PSC, phase variation was shown to be regulated first through a novel system of invertible promoter regions located upstream of each set of polysaccharide synthesis loci, *upxY* (where *x* represents locus *a – h*, excluding *c*), rendering the promoter in the correct or incorrect orientation needed for transcription[88]. The second level of regulation occurs through UpxZ which acts to inhibit UpxY, a transcriptional anti-termination factor; UpxZ regulation allows for heterologous loci inhibition, ensuring that *B. fragilis* only synthesizes one polysaccharide at a time[89].

Thus, the previous observation of dual *B. fragilis* PS expression by labeling is likely a result of a phenotypic lag. And although expression of at least one polysaccharide is required for competitive colonization of the gut, the utility of multiple phase-variable polysaccharides is still being elucidated[90]. Longitudinal analysis of 15 healthy subjects over the course of a year revealed that IgA responses were not mounted to several *Bacteriodales* strains detected at a high burden in the host. In spite of the possible prospect that phase variation evolved as an IgA-dependent mechanism to evade the host immune response, similar, for example, to *Giardia lamblia*, there were no correlations observed between IgA responses and the elimination of strains or notable alterations in

the orientation of their respective invertible polysaccharide promoters[63]. Thus, it appears that the IgA response does not shape *Bacteriodales* composition in the adult gut but may have a role in compartmentalizing or keeping specific microbes at bay from the mucosal epithelium.

Bacteroides fragilis **bacteriocins**

The bacterial equivalents of antimicrobial peptides are typically referred to as bacteriocins. These antimicrobials or bacteriocins were first discovered about a century ago in *E. coli*, when it was observed that *E. coli* V could inhibit growth of *E. coli* S[91]. Initially designated as colicins, these bacterial products were later identified as proteins having activity on bacterial cells dependent on the presence or absence of specific receptors[92]. Since then, bacteriocins have been discovered among all major lineages of Bacteria with the suggestion by Klaenhammer et al., that 99% of bacteria may produce at least one[93]. Bacteriocins are distinct from classical antibiotics in that they are ribosomally synthesized and have a narrow killing spectrum, typically acting on closely related species or within the same species; specificity is likely receptor-mediated[14,94]. These proteinaceous toxins come in a variety of sizes, targets, and modes of action, typically acting in the same fashion as eukaryotic AMPs, through membrane permeabilization and nucleic acid degradation or inhibition mechanisms[14,95].

Having been used as an epidemiological typing method, numerous clinical isolates of *Bacteroides* produce and are sensitive to bacteriocins[96,97]. For instance, in a study of *B. fragilis* clinical isolates from hospitals in Athens, Greece, 94% of isolates (30 of 32) were discovered to inhibit at least one other *B. fragilis* strain, while one strain was

even discovered to inhibit 17 strains. Similarly, sensitivity to at least one other *B. fragilis* strain was found in 94% of isolates, while one strain was determined to be sensitive to 17 test strains[97]. An inverse relationship appeared to exist between the number of bacteriocins produced and the number of bacteriocins to which they were sensitive. Although not heavily studied within the *Bacteroides* field, one bacteriocin characterized from a human fecal isolate (Bf-1) uniquely inhibited RNA synthesis as its mode of action[98]. In another early study, in the human colon, researchers found that bacteriocin-susceptible *Bacteroides* co-existed in large numbers with bacteriocin-producers[99]. Seemingly counter to the hypothesis that bacteriocin production confers an advantage over non-producers, it is possible that bacteriocin production permits bacteria to persist at low levels or even gain access to a specialized microenvironment. It remains unclear what this array of natural antimicrobial responses means for human health, yet it seems that bacteriocins are contributing factors to gut microbial composition, stability, and dynamics.

Their high potency, specificity, and fast-killing of targets, also make bacteriocins possible candidates for the treatment of bacterial infections. For instance, Casey et al.[100] and Walsh et al.[101] demonstrated that administration of milk that had been supplemented with bacteriocin-producing strains of lactic acid bacteria (LAB), helped to ameliorate *Salmonella*-induced diarrhea in pigs. An additional study by Corr et al. showed that colonization of mice with *Lactobacillus salivarius* UCC118 protected from *Listeria monocytogenes* infection, an invasive foodborne pathogen; protection was mediated through the *L. salivarius* production of a class IIb bacteriocin, Abp118, shown to have direct action on the pathogenic *Listeria* strains[102]. Furthermore, peptide engineering may

create bacteriocins that can be delivered even more efficiently. For example, pheromonicin AgrD1 is a recombinant peptide comprised of the *agrD1 Staphylococcus aureus* pheromone gene fused to colicin Ia[103]. Fusion to AgrD1 allows for colicin Ia passage through the cell wall of *S. aureus*, a non-native target, promoting killing of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive S. *aureus* (MSSA) *in vitro* and in a MRSA mouse infection model[103].

Colorectal Cancer Burden

In 2012, worldwide, there were an estimated 1.4 million new cases and 693,900 deaths resulting from colorectal cancer with the highest incidence levels occurring in North America, Europe, New Zealand, and Australia[104]. In the United States, CRC is the second leading cause of cancer-related deaths and is the fourth most common malignant neoplasm when both sexes are combined[105]. Yet, CRC cases are emerging in locations previously considered to be low risk, including South America, Eastern Europe, and Eastern Asia[104,106]. This rise in global incidence is likely, in part, due to globalization and its far-reaching impacts on changes towards more westernized dietary patterns, lifestyle, obesity, and other CRC risk factors[106,107].

As an example, since African Americans are disproportionately affected by CRC in the U.S., O'Keefe and colleagues conducted a series of studies analyzing diet, specific biomarkers, and polyp detection in African Americans compared with rural South Africans[108]. At baseline, African Americans had a diet 2 to 3 times higher in animal protein and fat compared with rural South Africans who had a diet high in carbohydrates and fiber, typically resistant starches; polyp detection and mucosal proliferation, as

measured by marker Ki67, were also distinctly higher in African Americans[108]. Interestingly, when African Americans were switched to an African diet (low fat, high fiber) for two weeks, putative anti-carcinogenic properties such as saccharolytic fermentation and butyrate production increased, while potentially cancer-promoting features such as secondary bile acid synthesis, epithelial cell proliferation, and colonic mucosal inflammation diminished[108]. Conversely, Africans put on an American diet (high fat, low fiber) for two weeks showed a reversal in all outcomes. Microbiota changes were also observed: both Africans and African Americans on an African diet exhibited higher levels of microbial functional genes associated with butyrate production (*bcoA*) and end-product hydrogen removal by methanogens (*mcrA*) and sulfate-reducing bacteria (*dsrA*); the crucial secondary bile acid synthesis gene, *baiCD*, was diminished in these groups.

Similarly, a study of Japanese migrants to Hawaii and California showed that CRC rates swiftly reached levels typical of Caucasian populations, within one generation[109]. The authors showed that high CRC risk in Japanese migrants was likely dependent on dietary changes, specifically the high intake of red meat combined with a rapid *N*-acetyltransferase (NAT2) variant predominant within the Japanese population (90% in Japanese vs 45% in Caucasians) coupled with a rapid N-oxidation (CYP1A2) variant. A combination of rapid CYP1A2 and NAT2 phenotypes may lead to more efficient metabolic activation and binding, respectively, of potentially genotoxic heterocyclic aromatic amines (HAAs) that are present in meats cooked at high temperature, to host DNA[109]. This study suggests a convergence of shifting diet and genetic factors contributing to increased CRC risk in a specific population.

Strikingly, by 2035, CRC incidence is expected to increase by 80%, projected to

cause 2.4 million cases and 1.3 million deaths[106]. Furthermore, IBD, characterized by chronic inflammation within the gastrointestinal tract, increases risk for development of CRC by 10-15%[110] with the overall risk of CRC in IBD patients (both Crohn's and ulcerative colitis) estimated as 1.7 times higher than the general population[111]. Most alarming, both CRC incidence and mortality in young adults (< 50 years old) has begun a distinct increase in the United States and elsewhere[112–114]. In spite of CRC age-specific risk having shown a steady decline in the first half of the 20th century (~1890 through 1950), recent analysis shows that CRC risk in the 1990 birth cohort, individuals now in their early 20s and 30s, has begun to approximate the CRC risk last observed in the 1890 birth cohort[115]. In other words, compared with individuals born in the year 1950, the 1890 birth cohort exhibited a doubled risk for colon cancer and a tripled risk for rectal cancer; in the same way, young people of the 1990 birth cohort now exhibit a doubled risk for colon cancer and a striking quadrupled risk for rectal cancer[115].

It is unclear what factors may be driving this resurgence in young people, although this increased CRC incidence appears to parallel increased obesity rates (both duration and degree of obesity) observed in recent birth cohorts[116]. There is evidence suggesting that every 5-unit increase in body mass index is associated with a 13% to 18% increased CRC risk[112,117–119]. Other preliminary studies note that young-onset CRCs typically occur in the distal colon and rectum, displaying specific histological distinctions from older-onset CRCs: poorly differentiated, mucinous, and signet ring features[120,121]; these findings may suggest that molecular differences exist between younger- and older-onset CRCs. Additionally, possibly due to lower screening rates and unrecognized symptoms in the population, younger-onset CRC is more likely detected at advanced

stages, e.g. stage III & IV., than older-onset CRC (respectively, in colon cancer: 63.4% vs 49.0%, p<0.01; respectively, in rectal cancer: 57.3% vs 46.2%, p<0.01)[120].

Furthermore, although the risk of dying from CRC has generally decreased in the overall population, it has begun to rise in those aged 20 to 54 years old: from 3.9 deaths per 100,000 in 2004, to 4.3 deaths per 100,000 in 2014[122]. These surprising early findings suggest that the burden of CRC is shifting towards the younger population, and further research will be necessary to determine the underlying causes of this increased disease risk and mortality. Collectively, it is clear that augmented, population-accessible approaches to CRC prevention are needed.

CHAPTER 2 - *Bacteroides fragilis* Co-infection

<u>Background</u>

Probiotics, defined as living microorganisms that confer a health benefit to the host when administered in adequate amounts[123], are proposed for both disease prevention and therapy. Clinical studies assessing the effectiveness of probiotics as supplemental treatments in IBD and CRC have shown promise. For instance, single bacterial species, such as *E. coli* Nissle 1917, or multispecies cocktails, such as VSL#3 (containing *Streptococcus salivarius* and multiple species of *Lactobacilli* and *Bifidobacteria*), aid in the prevention or recurrence of post-operative pouchitis and in the therapy of active ulcerative colitis[124]. In CRC, oral treatment with *Lactobacillus casei* Shirota resolved atypia of colon tumors in patients who underwent colon resection[125]. Perioperative treatment in CRC resection patients with combination probiotics led to reductions in serum zonulin (a marker of intestinal permeability), bacterial translocation, and septicemia, while Yang et al. further described reductions in both gut-related complications and recovery time to bowel function[126–128]. The human colon symbiote non-toxigenic *Bacteroides fragilis* (NTBF type strain NCTC 9343) has been proposed as a probiotic due to its anti-inflammatory actions including the initiation of regulatory T cell expansion and production of IL-10, a characteristic immunosuppressive cytokine[129], as well as to limit pathogenic Th17 development[130]. These activities have been ascribed to expression of an NTBF-derived capsular component, polysaccharide A (PSA), also proposed as a potential probiotic molecule.

In contrast, another molecular subgroup of *B. fragilis*, enterotoxigenic

Bacteroides fragilis (ETBF) that secretes the *B. fragilis* toxin, BFT, induces marked

colonic inflammation and tumor development in mice[73–75]. BFT, a 20 kDa zinc

metalloprotease, severely alters cell-to-cell adherens junctions in the colon epithelium

through the cleavage of E-cadherin, disrupting barrier function and stimulating the Wnt

signaling pathway to induce proto-oncogenes and other cell proliferative genes, such as

c-Myc[71,72]. Moreover, a milieu of pro-inflammatory cytokines is produced, most notably

IL-17A, required for ETBF pathogenesis[75]. It is an enticing prospect that probiotic

organisms may have the potential to disrupt the cycle of ETBF pathogenic signaling,

lessening the burden of disease.

Humans are commonly colonized with *B. fragilis*, a bacterium with known

proclivity for the colon mucosa. Thus, we tested the hypothesis that the

immunoregulatory probiotic, NTBF, can protect against ETBF-mediated disease. We

found when NTBF colonized at high levels in the colon, prior to ETBF treatment, the

NTBF strain limited inflammation and tumor development in mice. However, this

protective effect was independent of NTBF-encoded polysaccharide A or direct host

immune system modulation, and rather, appeared to be solely mediated by competitive

exclusion and mucosal NTBF strain dominance. Daily NTBF therapeutic treatment of

mice stably infected with ETBF failed to diminish colitis or ETBF strain dominance.

Together our results suggest that the capacity of NTBF to act as an anti-inflammatory

probiotic in humans may be limited.

Materials and Methods

Bacterial Strains and Growth

B. fragilis strains NCTC 9343 (NTBF) and 86-5443-2-2 (ETBF) were used for all experiments[74,75,131]. The PSA-deficient isogenic mutant, ΔPSA NTBF, was graciously provided by L. Comstock (Harvard University). All strains were clindamycin-resistant: ETBF strains (naturally) and NTBF strains (pFD340-transformed to confer resistance). Strains were grown anaerobically at 37°C with brain-heart infusion (37 g/L; BD Bacto) culture media or agar plates (1.5% BD Bacto Agar) supplemented with yeast extract (5 g/L; BD Bacto), L-cysteine (50 mg/L; Sigma), hemin (0.5 mg/L; Sigma), and vitamin K (0.1 mg/L; Sigma). For all mouse experiments, glycerol stocks of NTBF and ETBF were streaked onto BHI clindamycin (10 μg/L; Research Products International) plates with pure, isolated colonies sub-cultured in BHI clindamycin media for ~24 hours. For mouse inoculums, bacterial pellets were washed twice and resuspended with 1X Dulbecco's phosphate buffered saline (1X PBS free calcium chloride & magnesium chloride).

For individual growth curves, strains were grown to mid-logarithmic phase simultaneously, diluted to an OD_{600} of 0.1 to a fresh pre-warmed, pre-reduced anaerobic culture tube (BHI clindamycin) and monitored every hour for 8 hours and at 24 hours. Bacterial turbidity was monitored by spectrophotometer (600 nm) and subcultures performed (48 hours incubation) to quantitate viable colony forming per unit volume (CFU/ml). For *in vitro* co-inoculation assays, NTBF and ETBF were grown to mid-logarithmic phase and either introduced to a fresh culture tube (BHI clindamycin) at equal parts or at varying ratios (NTBF to ETBF: roughly 10:90, 50:50, 60:40, 90:10) and

cultured for up to 48 hours. At each sampling time point, subcultures were performed to allow random selection of ten colonies to test for colony identification (NTBF vs ETBF) using strain-specific PCRs (**Supp. Table 1**)[132,133]. Percentages of NTBF and ETBF positive colonies within the *in vitro* culture were then determined. All assays were run in triplicate.

For agar diffusion assays, NTBF and ETBF strains were grown separately in BHI media (no antibiotics). After 48 hours, bacterial suspensions were centrifuged (13,000 rpm, 5 minutes) and culture supernatants sterile filtered (0.22 μm). 500 μl of the indicator strain, NTBF, was mixed with 12 ml of BHI soft agar (0.7%); after drying, hole punches (6 mm) were created, sealed with 35 μl of soft agar, and inoculated with 100 μl of cell-free supernatants. Plates were grown anaerobically overnight and observed for an inhibition zone. Treatments of supernatants consisted of trypsin (200 μg/ml, 2 hours, 37°C), proteinase-K (100 μg/ml, 2 hours, 37°C), and heat inactivation (15 minutes, 100°C).

Mice and Infection Model

Specific pathogen free (SPF) C57BL/6 WT mice and C57BL/6 Min$^{Apc716+/-}$ mice (obtained from Dr. David Huso, Johns Hopkins University, Baltimore, MD) were used for all experiments. C57BL/6 WT mice were originally purchased from Jackson Laboratories and strictly maintained in house. Min$^{Apc716+/-}$ heterozygosity was maintained with C57BL/6 WT female breeders. Mice at 3 to 4 weeks of age were placed on antibiotic water bottles (clindamycin 0.1 g/L and streptomycin 5 g/L) (Hospira and Amresco) for 5 to 7 days and discontinued. Mice were then gavaged with ~10^8 CFU of

NTBF and/or ETBF (100 μl total volume in 1X PBS) either singly, sequentially, or simultaneously. Sham controls were treated with 1X PBS. Treatment groups included: 1) single/monocolonization with only one *B. fragilis* strain (NTBF or ETBF), 2) sequential colonization with initial strain at day 0 and challenge strain at day 3 (NTBF→ETBF or ETBF→NTBF), and 3) simultaneous colonization with both *B. fragilis* strains (NTBF+ETBF). Fecal colonization of the initial strain was confirmed for all sequentially treated mice prior to administration of the challenge strain. ΔPSA NTBF denotes the isogenic PSA deletion mutant of *B. fragilis* NCTC 9343[131]. For co-infection colitis experiments, mice were sacrificed 2 weeks after infection with the initial bacterial strain in WT mice, at 2 to 4 weeks in Min mice for the microadenoma experiments, and at 12 weeks in the Min mice for the macroadenoma experiments. For germ free (GF) monocolonization experiments, C57BL/6 GF mice were gavaged at 6 to 10 weeks old and sacrificed at both 2 weeks and 8 weeks post-colonization.

For therapeutic treatment of SPF co-infected mice, mice were colonized with a single strain for 2 weeks (NTBF or ETBF). Fecal colonization of the initial strain was confirmed for all mice prior to administration of the second strain. At 2 weeks, mice were gavaged with NTBF (when pre-colonized with ETBF) or ETBF (when pre-colonized with NTBF) daily, for 9 days. Control NTBF or ETBF only mice were gavaged with 1X PBS. Mice were rested for 2 days after consecutive treatments and then sacrificed at 24 days after initial strain gavage. The Johns Hopkins University Animal Care and Use Committee approved all experimental protocols. SPF mice were held in specific pathogen free conditions in accordance with the Association for the Assessment and Accreditation

of Laboratory Animal Care International. GF mice were bred in the gnotobiotic mouse facility at Johns Hopkins University Medical Campus, Baltimore, MD.

Fecal Colonization and Mucosal Adherence

Mouse stools were weighed, homogenized (10^{-1} dilution), and serially diluted (10^{-5}, 10^{-6}, 10^{-7}) in sterile 1X PBS. At sacrifice, mouse distal colon tissue (~1 cm in length) was harvested, weighed, and washed twice (30 seconds, vortex setting 7) with 0.016% dithiothreitol (Thermo Scientific) in 1 ml of 1X PBS to quantitate bacteria that were tightly adherent to the mucosa. Washed tissue pieces were transferred to 350 μl 1X PBS, homogenized, and serially diluted (10^{-1}, 10^{-2}, 10^{-3}). Dilutions of stool and colon tissue were plated onto BHI clindamycin agar and grown anaerobically for ~48 hours for determination of viable bacterial CFUs. Data are presented as *B. fragilis* CFUs per gram of stool or tissue.

Quantitative RT-PCR

Distal colon whole tissue RNA was extracted using the Qiagen RNeasy Mini Kit according to manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA using the Superscript III Reverse Transcriptase kit (Invitrogen) according to manufacturer's instructions. For each sample, two technical replicates, 1 μl of cDNA per reaction, were run with Taqman Gene Expression Mix. According to standard amplification protocols, samples were run on the 7500 Real-Time PCR System (Applied Biosystems) for 40 cycles. Primer/probe sets for respective cytokines were purchased from Applied Biosystems. Relative quantification (ΔΔCt) of indicated genes was

determined by subtraction of endogenous control 18S rRNA from all samples and then subtraction of normalized uninfected control values from colonized test values. Fold change was determined by $2^{-\Delta\Delta Ct}$.

Fecal DNA was extracted using the Fast Stool DNA Kit (Qiagen). Whole tissue DNA was extracted using the QIAamp DNA Mini Kit using the pathogen detection protocol. Using the Taqman system, supplemented with bovine serum albumin, 100 ng of stool DNA was measured with three technical replicates per sample and 40 cycles (AB 7500). Multiplex *bft/16S* qPCRs[133,134] were run optimally at 58°C; singleplex 9343-Tn qPCRs were run optimally at 59°C[132] (**Supp. Table 1**). Bacterial strain quantification was determined by standard curves developed from serially diluted (10^{-1}) 9343 and 86-5443-2-2 gDNA (using total genome copies from 10^8 to 10^2).

Histology, Microadenoma, and Macroadenoma Counts

Colons were dissected, Swiss rolled, and fixed with 10% buffered formalin for ~24 hours. Histology examination and scoring was done on hemotoxylin and eosin (H&E) stained 5 μm sections. Disease scoring was done as previously described by a pathologist (Dr. Sarah Beck)[75]. Swiss rolled H&E stained sections of Min$^{Apc716+/-}$ mice were examined for microadenomas throughout the distal colon by light microscope at 20X magnification. At late time points, Min$^{Apc716+/-}$ colons were flushed, opened, and fixed with 10% buffered formalin for ~24 hours. After fixation, colons were stained with methylene blue solution (0.2% in saline) (Sigma) for 1 hour. Macroadenomas were counted using a dissecting scope (Leica ES2).

Immunofluorescence and FISH Staining

Un-flushed colons of co-infected mice were fixed in formalin for 24 hours, suspended in saline, and paraffin-embedded (FFPE). Tissue sections were stained with Periodic acid-Schiff (PAS) to confirm mucus preservation. Deparaffinized histological sections (5 μm) were subjected to a standard IF protocol as follows: 1) initial block with 1X PBS (5% goat serum, 1% BSA) for 1 hour at RT, 2) incubation overnight at 4°C with primary antibodies: adsorbed NTBF rabbit serum (1:100) and adsorbed ETBF rat serum (1:100), 3) two washes, 1X PBS, 4) incubation at RT for 1 hour with secondary antibodies: goat anti-rabbit AF488 (1:100) (Sigma, A-11034) and goat anti-rat AF568 (1:100) (Sigma, A-1107), 5) two washes, 1X PBS, 6) incubation with DAPI 5 minutes, 7) two washes, dH2O, 8) mounted with ProLong Gold Antifade (Invitrogen) reagent, 9) cured for 48 hours before imaging with Zeiss LSM 780 confocal microscope. Rabbit serum generated to NTBF was graciously provided by Dr. Laurie Comstock (Harvard University). Rat serum generated to ETBF was commissioned by Covance Inc.

Successive sections were hybridized with a *B. fragilis* bacterial probe (IDT) (**Supp. Table 1**)[135] and stained with DAPI. Probes were applied at a concentration of 2 μM in pre-warmed hybridization buffer (900 mM NaCl, 20 mM Tris pH 7.5, 0.01% SDS). Slides were incubated at 46°C for 2 hours. Slides were washed three times for 5 minutes with FISH wash buffer (215 mM NaCl, 20 mM Tris pH 7.5, 5 mM EDTA). Slides were washed in distilled water, dipped in 100% ethanol, and air-dried. Coverslips were mounted with ProLong Gold Antifade (Invitrogen) reagent. Slides were imaged with a Zeiss LSM 780 confocal microscope. Paired images were taken and analyzed under identical parameters.

LPL, Spleen, and MLN Isolations

Dissected distal colons (defined as everything distal of the proximal colon striated or feathered architecture) were flushed. Colons were cut to <0.5 mm pieces and washed in 1X sodium bicarbonate buffer supplemented with EDTA (2 mM), FCS (10%), HEPES (25 mM), and HBSS for 20 minutes at 37°C, three times. Tissues were digested in RPMI supplemented with FCS (5%), liberase (400 units/ml; Roche Diagnostic), and DNAse 1 (0.2 mg/ml; Roche Diagnostic) for 30 minutes. Percoll gradient (20%, 40%, 80%) (GE Healthcare Life Science) separation was used to isolate mononuclear cells. Harvested spleen and MLNs were each pooled and digested in RPMI (5% FCS), with liberase and DNAse 1 for 30 minutes. Accu-Prep (Accurate Chemical) cell separation media was used to isolate mononuclear cells.

Flow Cytometry

For SPF and GF experiments, LPLs from individual colons, 4 to 5 mice per group, were isolated. Isolated mononuclear cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with FCS (5%) with Cell Stimulation Cocktail (containing protein transport inhibitors) (eBioscience) in 96-well plates for 4 hours. Cells were washed with 1X PBS, stained for cell surface markers, fixed, and permeabilized. ICS was done for IL-17A, IFNγ, and Foxp3. Flow cytometry was performed with the BD LSRII. Data was analyzed with BD FACSDiva 6.1.3 software.

Statistical Analysis

        The nonparametric two-tailed Mann-Whitney U test was used to compare means of all treatment groups. A p value of <0.05 was denoted as significant. For correlation analysis, the nonparametric two-tailed Spearman test was used. An rs value of $\geq$0.7 was considered a strong positive correlation.

Results

NTBF Subverts Inflammation and Adenoma Development

ETBF infection of specific pathogen free (SPF) C57BL/6 WT ($Apc^{+/+}$) mice induces an acute, self-limiting inflammatory diarrhea and robust mucosal IL-17A response[73,74]. After 3 to 5 days, the mice recover from the diarrhea but maintain subclinical, chronic Th17-mediated colitis for about one year after infection[74,76]. As NTBF-derived PSA has been shown to be protective in the 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) and *Helicobacter hepaticus* SPF experimental models of colitis[129,136], we sought to disrupt ETBF-induced colitis by sequentially introducing NTBF followed by ETBF (NTBF→ETBF: NTBF given first and ETBF given 3 days later) or by simultaneously introducing NTBF along with ETBF (NTBF+ETBF: NTBF and ETBF given at the same time) (**Supp. Fig. 1** Colitis Protocol). The same co-infected treatments were replicated with the PSA mutant strain of NTBF (ΔPSA NTBF) (**Supp. Fig. 2**). Two weeks post-infection (p.i.), we found that both NTBF→ETBF and ΔPSA NTBF→ETBF treated mice exhibited a significant decrease in *IL17A* transcription in comparison with NTBF+ETBF or ETBF only control mice as measured by whole colon qRT-PCR (**Fig. 1A**). However, both NTBF→ETBF and ΔPSA NTBF→ETBF treated mice exhibited increased *IL17A* expression levels compared to NTBF or ΔPSA NTBF monocolonization. Nonetheless, consistent with decreased inflammatory *IL17A* expression, NTBF→ETBF, and to a larger extent ΔPSA NTBF→ETBF mice, showed a decrease in histopathological inflammation and hyperplasia in comparison with ETBF only controls (**Fig. 1B-C**). These findings suggest that NTBF, irrespective of PSA expression, may offer protection

against Th17-driven colitis.

A prior report indicated monocolonization of germ free (GF) mice with ΔPSA NTBF induces a pathogenic Th17 cell accumulation, a response suppressed by exogenous treatment with purified PSA[130]. Notably, in our SPF mice, there were no differences in whole tissue *IL17A* expression between NTBF and ΔPSA NTBF groups, either singly or sequentially treated, suggesting that the absence of PSA did not drive a microenvironment high in IL-17A (**Fig. 1A**). NTBF has been shown to induce CD4 T cells to produce IFNγ and IL-10, indicative of its putative role in contributing to the Th1/Th2 balance and restriction of inflammatory responses in the gut[129,136,137]. In evaluating cytokines typical of Treg and Th1 responses in our SPF infection model, there were no distinct increases in transcriptional expression of the genes encoding IL-10 or IFNγ in SPF mice monocolonized or co-infected with WT or ΔPSA NTBF; modest increases were observed in singly infected ΔPSA NTBF mice that were significant compared to monocolonized NTBF mice, although these results remained close to baseline representing less than a 2-fold increase (**Fig. 1A**). Long-term assessment of SPF mice colonized with WT NTBF yielded no changes in characteristic helper T cell cytokines (**Supp. Fig. 3**). These data suggest that within a more complex host (e.g. SPF), immune system balances already in place are not impacted by the introduction of PSA in the form of NTBF colonization. Protection from ETBF colitis is not mediated through the NTBF-derived PSA molecule.

We next evaluated the role of NTBF in the context of ETBF tumorigenesis (**Supp. Fig. 1** Microadenoma/Macroadenoma Protocol). ETBF infection of SPF Min ($Apc^{+/-}$) mice induces rapid colon tumorigenesis with microadenoma (detected by microscopy)

onset at 5 days and quantifiable as early as 2 weeks, and visible macroadenomas noted as early as 4 weeks after colonization[75]. Consistent with the results of the colitis model, SPF Min mice infected sequentially (NTBF→ETBF) showed a significant decrease in macroadenoma formation in comparison with NTBF+ETBF or ETBF only control mice at 12 weeks (**Fig. 2A-B**). There were no differences in macroadenomas noted between WT and ΔPSA NTBF treatment groups, either singly or sequentially treated. Furthermore, ΔPSA NTBF colonization of Min mice did not augment macroadenoma formation in Min mice not infected with ETBF, reinforcing the absence of a mucosal Th17 response following colonization with ΔPSA NTBF (**Fig. 1A, Fig. 2A-B**). When evaluating microadenomas at 2 to 4 weeks, we found that NTBF→ETBF mice exhibited no significant reduction in counts and microadenoma morphology and size remained variable, consistent with ETBF controls. (**Fig. 2C-D**). Yet, it was observed that the blunting of visible macroadenomas was maintained at 8, 12, and 16 weeks p.i. (**Supp. Fig. 4**). Although these results suggest that tumor initiation between co-infected groups (sequential or simultaneous) is similar, the NTBF-mediated protection from tumor progression appears highly durable and suggests protection may be at the level of tumor growth versus tumor initiation. It should be noted that distinct outliers could be observed in Min mice that were sequentially treated, e.g. mouse #312 and mouse #238 produced 51 and 10 macroadenomas, respectively (**Fig. 2B** & data inset), a finding that prompted us to evaluate the relative burden of each *B. fragilis* strain in individual mice. Both WT and ΔPSA NTBF appear to prevent ETBF-induced tumor progression.

PSA Does Not Impact *B. fragilis* Fecal or Mucosal Colonization

Within the GF mouse mucosa, *B. fragilis* was reported to specifically reside within the colonic crypts with PSA as the purported mediator of this colonization through the suppression of antibacterial Th17 responses[130,138]. Thus, in GF mice, ΔPSA NTBF exhibited diminished mucosal colonization in comparison with the PSA-competent parental strain (NTBF). This colonization deficiency was restored through treatment with exogenous PSA or IL-17A neutralizing antibodies[130]. We used fluorescence in situ hybridization (FISH) to localize *B. fragilis* in the mucosa of monocolonized SPF and GF mice. Both NTBF and ΔPSA NTBF strains were observed to colonize in the lumen and at the mucosal surface in SPF and GF mice, with a small subset visualized to reside in the mucus layer (**Fig. 3A** arrows). *B. fragilis* penetration into the mucus layer appeared patchy and limited, while a few instances of possible crypt invasion were also observed. In either housing condition, both bacterial strains exhibited the same colonization pattern. This finding is consistent with reports that *B. fragilis* can bind to, degrade, and utilize intestinal mucins as a nutrient source[139–141].

Because ΔPSA NTBF monocolonized GF mice were reported to display a significant decrease in the abundance of tissue-associated bacteria, suggestive of the PSA requirement for the close association of *B. fragilis* with the host mucosa[130], the distal colon tissue and feces of SPF and GF monocolonized mice were homogenized and cultured to quantify viable CFUs of bacteria closely associated with the epithelium (mucosal colonization) and in the stool (See Methods). No differences in the viable bacterial burden were observed in the feces or the mucosa at early or late time points (2 or 8wk) post-infection (**Fig. 3B**). Thus, PSA deficiency did not change bacterial burden

and localization, indicating that PSA does not routinely impact the parameters of *B. fragilis* niche occupation. Routine assessment of *B. fragilis* colonization in SPF mice also yielded similar fecal and mucosal colonization levels among NTBF, ΔPSA NTBF, and ETBF-treated mice (**Supp. Fig. 5**), with each strain achieving a similar colonization burden.

Disease Outcome is Determined by an ETBF Critical Threshold

ETBF monocolonization induces rapid lethality, within 3 days, in GF mice (129S6/SvEv[74] and C57BL/6 (P. Fathi and C. Sears; unpublished data)). GF mice (129S6/SvEv) colonized with NTBF and subsequently exposed to ETBF survived ~2 weeks, but ultimately succumb (S. Wu, B. Sartor, and C. Sears; unpublished data). Similarly, ETBF IL-17A-mediated colitis and tumorigenesis was significantly abrogated in SPF sequentially infected mice (**Fig. 1-2**). We investigated whether *B. fragilis* strain competitive exclusion could be playing a role in disease prevention. In viable bacterial colony screenings of stools harvested from WT NTBF→ETBF or NTBF+ETBF treated mice, we found that one dominant strain was established, as early as 1 week after infection. NTBF→ETBF mice became colonized predominantly with NTBF (90% of mice), while NTBF+ETBF mice became colonized predominantly with ETBF (100% of mice) (**Fig. 4A**). Using quantitative PCR (qPCR) to detect low-level colonization, the relative proportions of both *B. fragilis* strains were determined in the feces of co-infected mice. At 2 weeks p.i., WT NTBF→ETBF and ΔPSA NTBF→ETBF mice both showed NTBF dominance in the feces (defined as $\geq 10^5$ - 10^8 detected copies) (purple and orange circles, **Fig. 4B**), whereas NTBF+ETBF mice showed ETBF dominance in the feces

(green circles, **Fig. 4B**). Similarly, at ≥ 3 months p.i., Min NTBF→ETBF and ΔPSA

NTBF→ETBF mice exhibited NTBF dominance in the feces (purple and orange circles,

Fig. 4D), whereas NTBF+ETBF mice exhibited ETBF dominance in the feces (green

circles, **Fig. 4D**). Of note, we also observed that the high tumor-producing Min

NTBF→ETBF mice, #312 and #238, showed complete NTBF displacement, indicative of

the capability for host strain replacement by ETBF with modification of disease

trajectory. Strain quantification in distal colon mucosal tissue by qPCR mimicked the

fecal results, although approached the limits of detection (not shown).

The consistency of the bacterial viability (**Fig 4A**) and the qPCR (**Fig 4B and

4D**) measures of strain dominance suggest two points: 1) NTBF, given adequate time to

colonize, prevents ETBF from establishing dominance; and 2) ETBF exhibits a

competitive advantage over NTBF during simultaneous co-infection. The percentage of

WT and Min mice sequentially infected (NTBF→ETBF) and displaying a high NTBF

copy number was 92.9% and 86.7%, whereas those with a high ETBF copy number was

28.6% and 20.0%, respectively (**Tables 1 and 2**). These percentages exceed 100%

because a subset of mice acquired high level colonization ($\geq 10^5$ copies) with both NTBF

and ETBF, counter to a prior report[138]. The percentage of WT and Min mice

simultaneously infected (NTBF+ETBF) with a high NTBF copy number was 44.4% and

36.4%, whereas those with a high ETBF copy number was 100% and 90.9%, respectively

(**Tables 1 and 2**). These data suggest that once a *B. fragilis* strain is established in the

host, it is infrequently altered or supplanted by the introduction of another strain. Notably

in our SPF model, we show that a secondary *B. fragilis* strain introduced through

subsequent challenge has the ability to maintain persistent colonization (up to 4 months),

but at appreciably lower levels, on average, a thousand‑fold less than the initial

colonizing strain (**Fig. 4B and D**; **Tables 1 and 2**). We also observed that ETBF strain

dominance was predictive of inflammatory and tumor responses. Strong positive

correlations were observed between ETBF copy number with *IL17A* expression

(rs=0.7469) and tumor development (rs=0.7899) (**Fig. 4C and E**). Of significance, in

individual co-infected mice (NTBF+ETBF or ETBF→NTBF) when the critical disease-

triggering ETBF burden (~10^7 copies) was achieved, relatively high levels of NTBF co-

colonization (~10^5-10^6 copies) did not abrogate disease (***** denotes individual mice with $\geq$

10^5 copies of both ETBF and NTBF, **Fig. 4B-E**). Additionally, we observed the

infrequent occurrence (2 out of 15 mice) of complete NTBF displacement by ETBF in

long-term infected NTBF→ETBF Min mice (purple circles: mouse #312 & #238, **Fig.**

4D-E). Of note, these data points correspond to the high tumor-producers noted in **Fig.**

2B.

Collectively, these findings suggest that NTBF has the ability to prevent, yet not

rescue, pathogenic ETBF from reaching disease-inducing levels within the host. Given

the relatively high frequency of NTBF and ETBF co-colonization observed, we

postulated that NTBF and ETBF may display distinct mucosal colonization patterns.

Thus, we sought to identify the colonic locale of both *B. fragilis* strains, NTBF and

ETBF, using immunofluorescence (**Supp. Fig. 6**). Our results suggest both NTBF and

ETBF colonize the lumen and at the mucosal surface, with patchy mucus layer

penetration (**Fig. 4F** top row) (**Supp. Fig. 7**). Due to lower level colonization of the non-

dominant strain leading to imaging limitations (4×10^4 bacteria per ml are required to

visualize one bacterium per 400X magnification observation field) in co-colonized mice,

only the dominant *B. fragilis* strain could be clearly visualized. *B. fragilis* in either

NTBF-dominant or ETBF-dominant co-infected mice colonized the distal colon with a

similar pattern (**Fig. 4F** bottom row) (**Supp. Fig. 7**).

Therapeutic Treatment with NTBF Fails to Disrupt ETBF Dominance and Disease

 To further assess whether NTBF could abrogate disease in a therapeutic manner,

WT mice were stably infected with ETBF for 2 weeks and then gavaged with NTBF

daily, for nine days (ETBF→NTBF^R) (**Supp. Fig. 1** Probiotic Protocol). Mice were

sacrificed after two days of rest from NTBF probiotic treatment. In a subset of

ETBF→NTBF^R mice (3 out of 5), we were able to introduce low-level stable

colonization of NTBF with therapeutic treatment (**Fig. 5A**, red circles-mouse ID: 62, 61,

59). Interestingly, low-level NTBF integration into stably colonized ETBF mice led to no

impact seen on ETBF colonization or clinicopathology. Namely, ETBF→NTBF^R mice

exhibited small, contracted ceca containing blood clots and diminished cecal content, a

response to cecal injury, comparable to ETBF→PBS^R controls; cecum weights and

splenomegaly (an indicator of chronic inflammation in our model) were indistinguishable

between these groups (**Fig. 5B-D**). Consistent with our results regarding the

establishment of strain dominance, even with repeated exposure to ETBF,

NTBF→ETBF^R mice maintained the appearance of healthy ceca, displaying

characteristic weight and content, with no noted spleen enlargement, comparable to

Sham→PBS^R and NTBF→PBS^R controls (**Fig. 5B-D**). Of interest, although our previous

experiments suggested that ETBF had the capability to oust stably colonized NTBF, in

this series of experiments, we found that NTBF→ETBF^R mice (5 out of 5) did not

develop stable ETBF colonization (**Fig. 5A**, purple circles). Overall, these data are consistent with the results of our colitis and tumor experiments, suggesting that NTBF may only be beneficial as a preventative measure.

NTBF Modulation of the Immune System

To further examine whether NTBF modulates immune responses in SPF mice, both WT and Min mice were colonized with NTBF or ΔPSA NTBF for 2 weeks. For this experiment, the lamina propria was sampled specifically from the distal colon, the colitis and tumor-prone locales in the WT and Min ETBF models. Comparisons of Sham, NTBF, and ΔPSA NTBF mice yielded unchanged IL-17A$^+$, Foxp3$^+$, or IFNγ$^+$ T cell populations as well as IL-17A$^+$ Foxp3$^+$ lymphocyte populations whether analyzed by percentage or cell density (**Fig. 6**, **Supp. Fig. 8**: filled circles=WT; open circles=Min). Though the colon lamina propria represents the anatomical site most reflective of and meaningful to the immune responses initiated by intestinal microbial exposure, similar results were also obtained in the mesenteric lymph node and spleen (**Supp. Fig. 9**). These data were further consistent with the analysis of whole tissue transcriptional expression of *IL17A*, *IL10*, and *IFNG* cytokines in SPF WT mice (**Fig. 1A**).

We extended these results to GF WT mice (6 to 8 weeks of age) that were monocolonized with NTBF or ΔPSA NTBF for either 2 or 8 weeks. We first examined both the splenic morphology and histological sections of control and monocolonized GF and matched SPF mice. Spleens from mice across all treatment groups and housing backgrounds exhibited a normal size and weight, while histological sections revealed normal appearing lymphoid development in the form of well-defined follicles comprising

the white pulp (**Supp. Fig. 10**). Surprisingly, flow cytometric analysis of the GF colon LP

yielded no differences in the accumulations of Th17, Treg, Th1, or total IL-17A

populations between WT and ΔPSA NTBF-colonized mice at early or late time points as

reflected by absolute cell number (cell density) (**Fig. 7A**). In parallel, we analyzed the

transcription of cytokines of functional relevance to the described immune cell

populations. Again surprisingly, we were unable to observe any differences in the

stereotypical Th17 or Treg cytokines, IL-17A and IL-10, in WT versus ΔPSA NTBF-

treated mice. However, we did observe that monocolonized mice exhibited increased

IL17A expression compared with un-infected GF controls at 2 weeks but not at 8 weeks.

We also evaluated the gene expression of characteristic Th1 and Th2 cytokines, IFNγ and

IL-4, and found no differences among groups (**Fig. 7B**). Accumulations in IL-17A-

producing or IFNγ-producing γδ T cells were also comparable between groups (**Supp.

Fig. 11**). However, the total IFNγ-producing cell population was significantly less in

mice colonized with ΔPSA NTBF for two weeks and then significantly greater at 8 weeks

when compared to PSA-sufficient NTBF (**Fig 7A**), suggesting a possible role of PSA in

maintaining the balance of interferon in the GF host. However, this result was not

confirmed by whole colon tissue expression analysis (**Fig. 7B**). Taken together,

colonization with PSA-sufficient NTBF does not modulate SPF or GF host immune

responses in a manner protective against ETBF-induced colitis.

ETBF Secretes an Intraspecies Inhibitory Molecule

        Competition for limited resources can be broadly categorized into scramble

competition, where one organism rapidly utilizes a limiting resource with no direct

contact with a competing organism, or contest competition, where antagonistic

interactions occur between competing organisms[142]. Such mechanisms are vital to

microbial establishment and persistence. Our data show that ETBF consistently

outcompetes NTBF during simultaneous co-infection in mice (**Fig. 4A, B, and D**). Many

factors, including growth rate, nutrient utilization, colonization factors, and possible

antimicrobial secretion likely contribute to ETBF fitness[142].

To evaluate strain differences in growth and nutrient utilization, bacterial growth

and recovery was monitored. Individually, NTBF and ETBF strains exhibited similar

growth rates (**Fig. 8A**). Intriguingly, when strains were co-cultured at equal starting

points, the percent recovery of NTBF was severely diminished after 24 to 48 hours (**Fig.

8B**). Finally, when strains were co-cultured at varying input ratios for either 24 or 48

hours, NTBF could only be recovered when it comprised the majority of the input strain

(**Fig. 8C**). Since we did not observe any individual growth differences among strains, we

hypothesized that ETBF was secreting an antimicrobial compound leading to NTBF

growth inhibition.

Bacteriocins differ from classical antibiotics in that they are synthesized

ribosomally and have a narrow killing spectrum, acting on closely related species or on

variants of the same species[95]. We carried out agar diffusion assays with cell-free ETBF

supernatants harvested from stationary phase cultures and found that, indeed, ETBF-

derived culture supernatants could inhibit the growth of NTBF and other closely related

strains in a BFT-independent fashion. Protease treatment with trypsin, proteinase-K, and

heat inactivation at $100^{o}C$ eliminated the activity of the ETBF supernatant, indicating that

the inhibitory molecule is likely a protein (**Fig. 8D**, **Supp. Fig. 12**). Chatzidaki-Livanis *et*

al. recently showed membrane attack complex/perforin (MACPF) bacteriocidal activity among *B. fragilis* strains[143]. Interestingly, ETBF carry an identical copy of the identified Bacteroidales-secreted antimicrobial protein 1 (BSAP-1) gene originally characterized in *B. fragilis* strain 638R. Indeed, qRT-PCR analysis revealed that *BSAP-1* was highly expressed in ETBF but not in NTBF (**Supp. Fig. 13A**). Therefore, the secretion of a pore-forming molecule is one likely candidate contributing to ETBF's competitive advantage over NTBF, leading to the dramatic growth inhibition of NTBF.

Recently, a novel *Bacteroides* polyssacharide utilization locus regulating species saturation was described[138]. Mutations in this commensal colonization factor locus (*ccf*) altered *B. fragilis* niche occupation in mice, becoming permissive to challenge colonization by the same species. We hypothesized that differential gene expression in *ccfA* and *ccfB*, the sigma/anti-sigma factor pair, would contribute to an ETBF competitive advantage. Distal colon tissue *ccfA* and *ccfB* expression levels were similar between NTBF and ETBF-monocolonized mice, suggesting that *ccf* gene regulation was similar between both *B. fragilis* strains (**Supp. Fig. 13B**). This result does not rule out differential gene expression occurring when competing strains are simultaneously in contact within the murine host.

Discussion

Basis for NTBF Probiotic Usage

The colon symbiont, NTBF, was first discovered to influence gut immune homeostasis in germ free mice by directing lymphoid organogenesis, restoring the splenic CD4$^+$ T cell compartment, and rectifying the Th2-skewed imbalance[137,144]. Indeed, purified spleen CD4$^+$ T cells from ΔPSA NTBF mice showed an overproduction of IL-4 and diminished IFNγ as measured by ELISA; the reverse was seen in mice colonized with WT NTBF[137]. After this initial groundbreaking study, the functional role of the PSA molecule has been further expanded upon. GF mice monocolonized with NTBF or treated orally with purified PSA initiate Foxp3$^+$ Treg accumulation in the colon LP and MLN[129]. This accumulation is accompanied by Treg-derived IL-10 production as measured by LPL ICS and by whole colon tissue and purified CD4$^+$ Foxp3$^+$ T cell (MLN) qRT-PCR[129]. Regarding inflammatory development, ΔPSA NTBF GF mice exhibit a noted Th17 cell accumulation in the colon LP, accompanied by increased *IL17A* and *RORC* transcription as measured by qRT-PCR in purified CD4$^+$ T cells of the MLN[130]. This Th17 signature even interferes with the strain's ability to establish on the colonic mucosa, its main ecological niche[130]. Amazingly, this immune phenotype and colonization defect is completely reversed when ΔPSA NTBF mice are exogenously treated with purified PSA, evidence of a crucial immunomodulatory function for PSA in mucosal immune system development[130].

Certainly, previous studies have shown that these effects translate over to a more complex host [129,136]. Rag-deficient SPF mice co-infected with WT NTBF or treated with

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purified PSA were protected from CD45RB^{high} T cell transfer/*Helicobacter hepaticus*-induced colitis, whereas ΔPSA NTBF co-infected or vehicle-treated mice were not[136]. Furthermore, SPF mice treated orally with PSA were also protected from a chemically-induced (TNBS) form of colitis, and this was attributed to the PSA inducement of Foxp3^{+} Treg MLN expansion and IL-10 production as measured by expression in whole colon, whole MLN, and purified CD4^{+} T cells of the MLN[129,136]. In the prevailing working model, NTBF is thought to promote immune tolerance by producing outer membrane vesicles (OMVs) containing PSA, which are then recognized by dendritic cells (DC) via TLR2[145]. This gives rise to DC-derived IL-10 production which promotes Treg responses, which produce additional IL-10, leading to the suppression of T helper cell-derived inflammation[145].

To discern the probiotic potential of NTBF in gut health, we studied how NTBF impacted an ETBF-driven disease model of colitis and tumorigenesis. Specific pathogen free C57BL/6 WT and Min[*Apc*716+/-] mice were used in a NTBF and ETBF co-infection model where mice were sequentially or simultaneously treated. We showed that NTBF limited ETBF disease severity, significantly reducing the IL-17A-mediated inflammatory signature and blunting the development of colon tumors. However, this finding was limited to mice that had been sequentially infected, receiving NTBF first and ETBF three days later (NTBF→ETBF), suggesting the potential of NTBF prophylaxis. Mice simultaneously infected (NTBF+ETBF) or sequentially infected, where ETBF was given first and NTBF three days later (ETBF→NTBF), continued to exhibit severe disease, even in mice who became co-colonized with comparable levels of NTBF and ETBF (**Fig. 4B-D**). Notably, although prophylactic NTBF blunted pro-inflammatory IL-17A gene

expression, it failed to fully block IL-17A production in the colitis setting (**Fig. 1A**). Consistent with this finding, we observed the development of microadenomas in NTBF→ETBF mice that did not progress to visible macroadenomas, even after 16 weeks (**Fig. 2, Supp. Fig. 4**). Though our results show that NTBF partially subverted both ETBF-induced colitis and tumorigenesis, surprisingly, we discovered that this effect was not dependent on NTBF-encoded polysaccharide A (PSA). Namely, we observed disease prevention in both mice co-infected with the wild type NTBF parental strain as well as in mice co-infected with the PSA deficient NTBF strain.

We demonstrated that SPF mice singly colonized with WT or ΔPSA NTBF displayed neither an inherent difference in inflammatory or regulatory T cell levels in the LP (IL-17^{-}, IFNγ[+], Treg), nor differences in cytokine expression in whole colon tissue (*IL17A, IFNG, IL10*), contrasting with the studies described above. Geis et al. recently described a mechanism by which Foxp3^{+} regulatory T cells initiated the inflammatory Th17 response during ETBF infection[146]. We analyzed the accumulation of this IL-17A^{+} Foxp3^{+}T cell population and found no differences among groups. Altogether, these data demonstrate that the absence of PSA neither diminish NTBF's protective capability, nor primes SPF mice for inflammatory Th17 polarization.

It is well established that gut immune homeostasis in SPF mice is vastly different from that of GF mice, which exhibit many immune deficiencies[147,148]. Accordingly, we evaluated the impact of NTBF colonization in GF mice. In contrast to previous reports[137,149], we did not observe a PSA-inducement of lymphoid organogenesis in the spleen. Furthermore, to our surprise, PSA competent NTBF did not affect the accumulation of LP Treg or Th1 cellular subsets or alter the accumulation of Th17 cells

observed in ΔPSA NTBF colonized mice at early (2wk) or late (8wk) time points. The parallel assessment of *IL10, IFNG, IL4*, and *IL17A* cytokine expression also yielded no differences between groups; yet, suggesting that bacterial colonization alters the immune system of GF mice, monocolonized mice with either WT or ΔPSA NTBF showed increased *IL17A* gene expression compared with uninfected controls at an early time point. Beyond the CD4$^+$ T cell compartment, there were also no differences seen in the recruitment of IFNγ-producing or IL-17A-producing γδ T cells. Although we observed an accumulation in total IFNγ-producing cells in ΔPSA NTBF mice at 8 weeks, this finding contrasted with previous reports describing the reverse, where PSA-competent NTBF induced IFNγ and reduced IL-4 production in stimulated splenic T cells[137]. Finally, in contrast with a previous study, ΔPSA NTBF-colonized SPF and GF mice did not exhibit diminished mucosal colonization levels or differential mucosal niche occupation.

A key feature of our findings is that they represent the analysis of cells and tissues isolated from the colons of mice on the C57BL/6 background. The earliest discoveries showing NTBF-directed immune system maturation and lymphoid development were made in the spleens of GF Swiss-Webster mice obtained from Taconic[136,137]. Importantly, our GF C57BL/6 mice were bred and maintained in sterile isolator conditions in house at JHU. Previous analyses of *B. fragilis* niche occupation and species resistance also utilized GF Swiss-Webster mice[138,139]. Finally, because we studied direct NTBF colonization in SPF and GF mice, we cannot exclude the role of purified PSA in directing gut immune development. For disease modeling, these data suggest the importance of sampling the most clinically relevant experimental sites, as well as taking into account the effects of differences in mouse strain and housing conditions.

Mucosal Niche and Barrier Development

Another important aspect of disease control in the gut comes from the anatomical separation of the luminal contents from colon epithelial cells that is maintained by gel-forming mucins. In mice, similar to humans, the mucus layer of the colon consists of an impermeable, firm inner layer (~50 μm thick) devoid of bacteria and a loose outer layer (~100 μm thick) containing high numbers of bacteria[150]. Bacterial penetration and persistence within the inner mucus layer could be indicative of a dysbiosis contributing to disease.

It is possible that the resident microbiota may temper the effects of colitis. For instance, ETBF induces rapid lethality in GF mice[74], while GF mice colonized with NTBF prior to ETBF exposure, exhibit extended survival (S. Wu, B. Sartor, and C. Sears; unpublished data). Similarly, Chiu et al. showed that NTBF colonization of GF mice ameliorated DSS-induced colitis[151]. Although we did not observe a direct immunological or anti-inflammatory component to NTBF colonization, we hypothesize that NTBF's presence in the host can act as an anatomical barrier from pathogenic organisms present in the gut lumen. In our model, the pattern of colonization burden in NTBF→ETBF sequentially treated mice showed clear evidence of NTBF competitive exclusion of ETBF, likely diminishing epithelial cell damage or bacterial invasion that could be induced by the latter. In contrast, NTBF+ETBF simultaneously treated mice typically exhibit competitive exclusion of NTBF, limiting any defense against ETBF disease. In contrast to the results of Lee et al., we found that *B. fragilis* competitive exclusion was not absolute[138] and, in fact, 7-21% of NTBF→ETBF mice, 20% of ETBF→NTBF mice, and 27-44% of NTBF+ETBF mice displayed high level ($\geq 10^5$ CFU/100 ng) fecal co-

colonization (**Tables 1 and 2**). Persistent colonization (up to 4 months) with a *B. fragilis* challenge strain was consistently observed in our mice, with even a few instances of complete NTBF displacement observed in NTBF→ETBF mice. In our experiments, we found that NTBF and ETBF both localize to the mucosal surface, with patchy bacterial mucus penetration observed. Contrasting with previous data, we did not observe any colonization defects or alternative localization patterns of the mutant PSA NTBF strain, suggesting that PSA plays a negligible role in the bacterium's colonization of the gut.

Lending credence to the microbiota's role in protective barrier formation, one can also look to recent developments in the field of glycobiology. Johansson et al. have documented the inadequate properties of the germ free mouse colon mucus layer, characterized by inner mucus permeability, mucin oligossacharide alteration, and Muc2 deficiency[152]. Only after the conventionalization of GF mice with colonized cecal contents from mice with well-developed mucus layers, does the mucus fully develop its proper abundance and spatial organization of gel-forming mucins along with its characteristic inner layer impenetrability at 6 weeks[152]. Interestingly, Johansson et al. found shifts in the microbial communities of the mucus during mucus layer development, most notably a peak in the Firmicutes at 2 weeks followed by a transient shift to the Bacteroidetes (including *Bacteroides* genera) at 4 weeks[152]. This finding is suggestive of an integral role for *Bacteroides* in mucus maturation in the gut. The mucin attachment and degradation capabilities of some bacteria (including *Bacteroides fragilis*), which allow for close mucosal association, may have developed as a host-selective measure to retain beneficial microbes at specific locales. It will be fascinating to uncover the bacteria-driven mechanisms of host mucus maturation and development.

B. fragilis Fitness

Bacterial fitness factors may be possible mechanisms contributing to the establishment of strain dominance. Monitoring of individual NTBF and ETBF strain growth did not suggest any differences in nutrient utilization strategies and differential expression of colonization factor genes was not observed. However, it has been shown that type VI secretion (T6S), the direct injection of effector proteins into the membrane of a target organism, is a way that bacteria can maintain competitiveness, with a widely conserved T6SS (T6S system) locus observed in the Bacteroidetes phylum. Indeed, the T6SS of *B. fragilis* has been shown to be effective against the closely related *B. thetaiotaomicron* and against other *B. fragilis* strains[153,154]. Hecht, et al. recently identified a differentially encoded effector-immunity region of this T6S locus that determines this intraspecies competition[155]. In a SPF co-infection mouse model of ETBF (ATCC43858) DSS colitis, NTBF protected against disease, whereas an NTBF mutant in a crucial T6S component (Δ*tssC*), did not. NTBF T6S was able to abrogate disease by diminishing the colonization of pathogenic ETBF, and thus diminishing the enterotoxigenic burden in the host. In our model, T6S may be another pivotal means by which NTBF precludes ETBF disease.

We did observe the ETBF-specific secretion of a bacteroicin-like molecule with inhibitory activities on NTBF and other closely related strains, in a toxin-independent fashion. Easily harvested from culture supernatants, it appears that ETBF naturally produces this molecule in an unprovoked manner. Due to evidence of high *BSAP-1* expression levels in ETBF *in vitro*, we suggest that BSAP-1, a membrane attack complex/perforin, is a likely candidate in ETBF's broad inhibitory actions on other

strains contributing to its own survival. We hypothesize that both timing and order, in addition to the secretion of inhibitory molecules in a non-discriminate fashion, contributes to *B. fragilis* competition dynamics.

Clinical Framework for Therapeutic Treatment

ETBF carriage in healthy, asymptomatic people is quite common with a recent study showing that 40% of individuals (6 out of 15 subjects) in Boston, MA, carried ETBF in their feces[63]. Yet clinically, ETBF is known as an etiologic agent of acute diarrhea in children and adults and is also highly associated with active IBD[62,133,156]. Recently, our group and others have shown that ETBF detection is highly associated with CRC cases, suggesting that ETBF colonization is a risk factor for CRC development[133,157]. Boleij et al. compared *bft* gene detection in CRC patient mucosa (n=49) with healthy control mucosa (n=49) undergoing routine outpatient colonoscopies, showing left colon detection rates 85.7% and 53.1% (p=0.03) and right colon detection rates 91.7% and 55.5% (p=0.04), respectively[133]. Moreover, *bft* detection showed an increasing trend moving from early to late stage CRC, 72.7% and 100% detection, respectively (p=0.09)[133].

Thus, we evaluated the possible remedial effect of NTBF on pre-existing ETBF disease. Therapeutic treatments of stably colonized ETBF mice with NTBF (ETBF→NTBF^R), failed to abrogate disease severity. However, we did show evidence of introduction of this non-pathogenic NTBF strain into a subset of mice (3 out of 5). These findings are mimicked in the fecal microbial transplantation (FMT) literature in humans, showing that durable strain recipient replacement or steady coexistence (in $50.7 \pm 10.1\%$

of shared species) (up to 3 months) of both donor and recipient strains can be achieved, even without antibiotics prior to or during treatment[158]. However, microbiome resistance does occur. In 2 out of 5 ETBF→NTBF^R mice, NTBF could not establish within the host. Similarly, in the clinical setting, in 1 of the 3 recipients of the same donor at 3 months post-FMT, only 12% of donor strain populations were detected, versus 46.1% and 56.6% in the remaining patients[158]. Such findings stress the importance of longitudinal clinical translational studies coupled with bioinformatics approaches as probiotic-recipient or donor-recipient compatibility and pre-existing disease states are likely to become salient in the field of bacterial biotherapeutics.

We demonstrate a method by which non-toxigenic *Bacteroides fragilis* offers a preventative health benefit to the host that is not reliant on polysaccharide A or on direct host immune system modulation. Interestingly, the culturing of mucosal biopsies revealed that PSA-harboring human isolates of *B. fragilis* are not found more frequently at non-inflamed sites versus inflamed sites in IBD patients (CD, p=0.68; UC, p=1.0), nor are PSA strains more often detected in healthy controls versus IBD patients (p=0.73)[159]. Thus, it seems unlikely that this polysaccharide will be the foremost determining factor of patient health. In terms of real world therapeutic applications, it appears that the order in which the host sees either NTBF or ETBF, the specific *B. fragilis* strains being introduced to and already existing in the recipient, and further, yet uncharacterized bacterial fitness factors are likely crucial to determining the overall disease outcome. Further research must be done to determine the proportions, diversity, and impact, down to the strain level, of the ideal gut microbiome for individual patients. Numerous clinical isolates of *Bacteroides* species have documented bacteriocin production and

sensitivity[96,97]. The diversity of these responses points to the complexity of microbial niche establishment and also lays the basis for the engineering of probiotic organisms to exploit advantageous fitness factors. For instance, Hillman et al. have developed a patented replacement therapy for *Streptococcus mutans* that induces dental caries via lactic acid production. *S. mutans* recombinant strain BSCE-LI lacks the *Idh* gene (lactate dehydrogenase) and secretes a potent bacteriocin, mutacin 1140, effective at actively displacing naturally occurring *S. mutans* in the oral cavity, increasing the pH and diminishing the development of tooth decay[160]. It can be envisioned that such bioengineering of beneficial gut microbes can be achieved, as more genetic tools become available. In diseases with protracted etiology, such as CRC, it is possible that the mere introduction of beneficial or non-pathogenic bacterial species or strains could work to improve clinical outcomes over the long term.

Main and Supplemental Figures/Tables (in order of first reference)

Supplemental Table 1: Primers and probes used in this study.

Primers and Probes	Sequence 5' to 3'	Target	Reference
1	ACACATATCACTTCCGATGCC GTTGACGGAAATGATCGGTATAG	*orf1* and *upaZ* (NTBF PSA flanking)	131
2	TAACACGATAGGAGTTGCATGG ACATTGAGAAATACTCGTCCACC	*wzx* and *wcfN* (NTBF PSA)	131
3	CGACCCTTTCCTCATAATCCTTTCT ATGCTATTCACATTTGCCGCTTG	*B. fragilis 638R_1646* (*BSAP-1*)	This study
4	GGAATTTGCATGACACTTAT CTGAGAGGTTTCATCTTCTG	*B. fragilis ccfA*	138
5	AGTGTCCCCACTTCATCGTC TGAAACTTTTGCCGGAGAAT	*B. fragilis ccfB*	138
6	CTCGGTATGGAGTATGCTCCAG GTATTCCACTGCCCAATATGCCGC Probe: CTACAACAGGAACGGGAGCGACAC	B. fragilis CTn9343 (*bexA*)	132; This study
7	GCGAACTCGGTTTATGCAGT GTTGTAGACATCCCACTGGC Probe: AGCAGAAGGTTATGACGA	*B. fragilis bft*	133; This study
8	TCRGGAAGAAAGCTTGCT CATCCTTTACCGGAATCCT Probe: ACACGTATCCAACCTGCCCTTTACTCG	*B. fragilis* 16S	134
9	FISH Probe: \5Cy3\GTTTCCACATCATTCCACTG	*B. fragilis* 16S rRNA	135

Supplemental Figure 1: Schematic of SPF mouse co-infection experiments. Mice received peroral antibiotic water ad lib for 5 to 7 days prior to initial bacterial infection. Sequentially infected mice received secondary strains on day 3. Simultaneously infected mice received both strains on day 0. Fecal colonization of the initial strain (day 0) was confirmed for all sequentially treated mice prior to administration of the challenge strain. For colitis experiments, mice were sacrificed 2 weeks post initial strain infection (WT), microadenoma experiments at 2 to 4 weeks (Min), and macroadenoma experiments at 12 weeks (Min). For 'probiotic' treatment experiments, mice were colonized with single strains for 2 weeks (NTBF or ETBF). At 2 weeks, mice were gavaged with $NTBF^R$ (pre-colonized with ETBF) or $ETBF^R$ (pre-colonized with NTBF) daily, for nine days. Control NTBF or ETBF only mice were gavaged with 1XPBS (PBS^R). Mice were rested for two days after consecutive probiotic treatments and then sacrificed (24 days after initial strain gavage). Superscript ^{R} denotes repeated treatments.
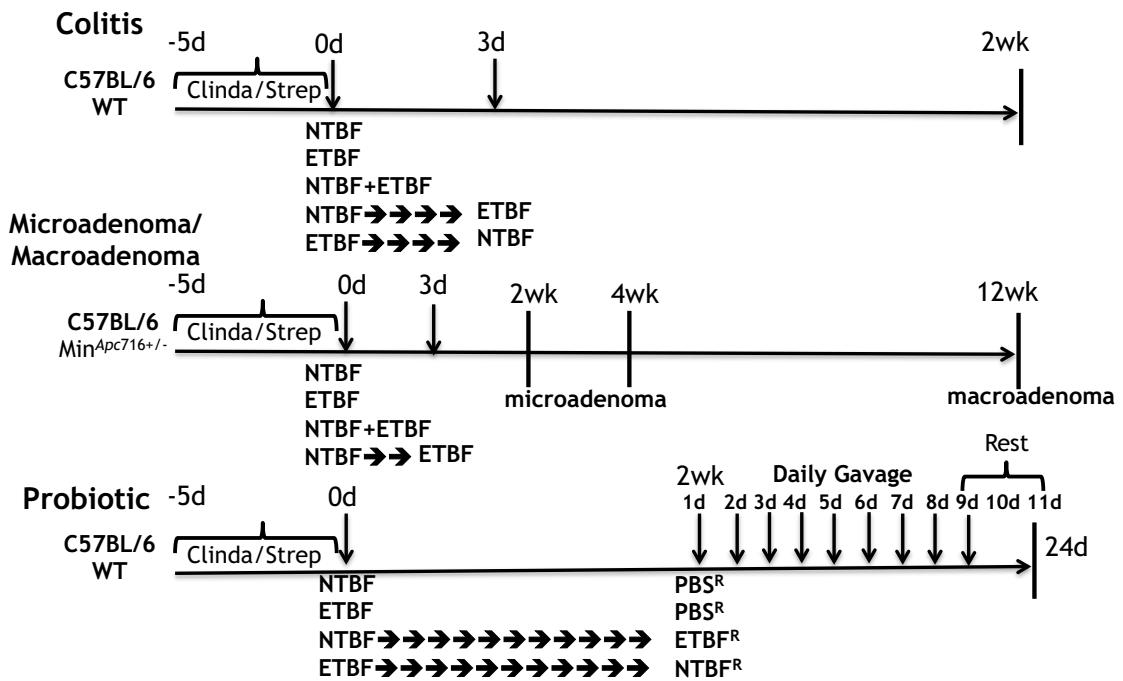
Supplemental Figure 2: Confirmation of PSA-competent and PSA-deficient NTBF strains. gDNA was extracted from NTBF and ΔPSA NTBF grown from single colony. Primer sets 1 (PSA flanking) and 2 (PSA) were used to confirm the PSA-mutant strain. ΔPSA NTBF was not amplified with primer set 2. NTC = no template control. Primer sets are found in **Supp. Table 1**.
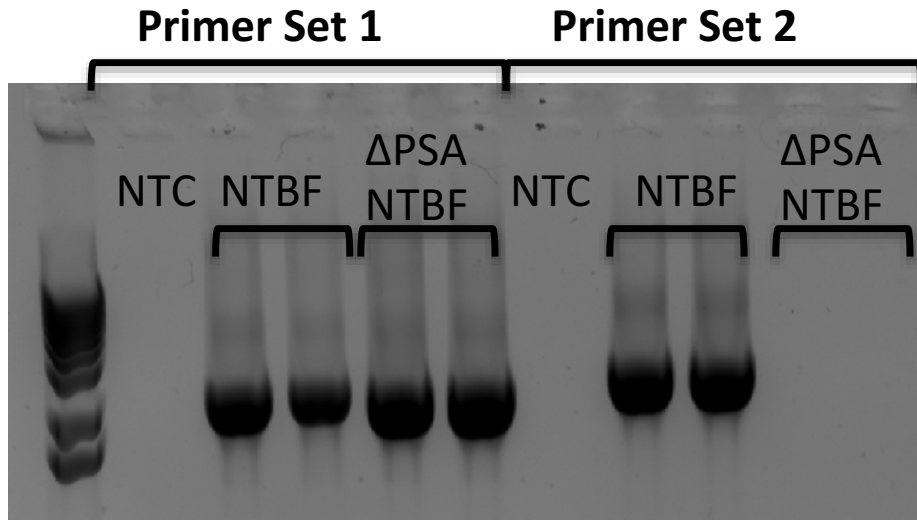
Figure 1. PSA-competent and PSA-deficient NTBF incompletely reduce inflammatory responses. A-C SPF WT mice were inoculated sequentially (NTBF→ETBF) (ETBF→NTBF) or simultaneously (NTBF+ETBF) and sacrificed 2 weeks later. Distal colon tissue was harvested for whole tissue qRT-PCR. The remaining colon was reserved for FFPE. Bars indicate median and interquartile range. Data are from 3 separate experiments with 3 to 7 mice per test group. **A** WT and ΔPSA NTBF→ETBF mice exhibit significant blunting of *IL17A* expression in comparison with NTBF+ETBF, ETBF→NTBF, and ETBF controls, although *IL17A* expression remains significantly elevated in NTBF→ETBF vs NTBF alone mice. *IL10* and *IFNG* expression was similar between WT and ΔPSA NTBF co-infected treatment groups. An increase in *IL10* and *IFNG* was observed in ΔPSA NTBF singly colonized mice, compared to PSA-sufficient NTBF, but remained close to baseline (< 2 fold increase). **B** Disease scores (additive hyperplasia and inflammation) of FFPE H&E sections. WT and ΔPSA NTBF→ETBF mice exhibit a lower disease score compared with ETBF controls. **C** Representative H&E histology depicting pronounced colon hyperplasia in NTBF+ETBF and ETBF only treated mice.
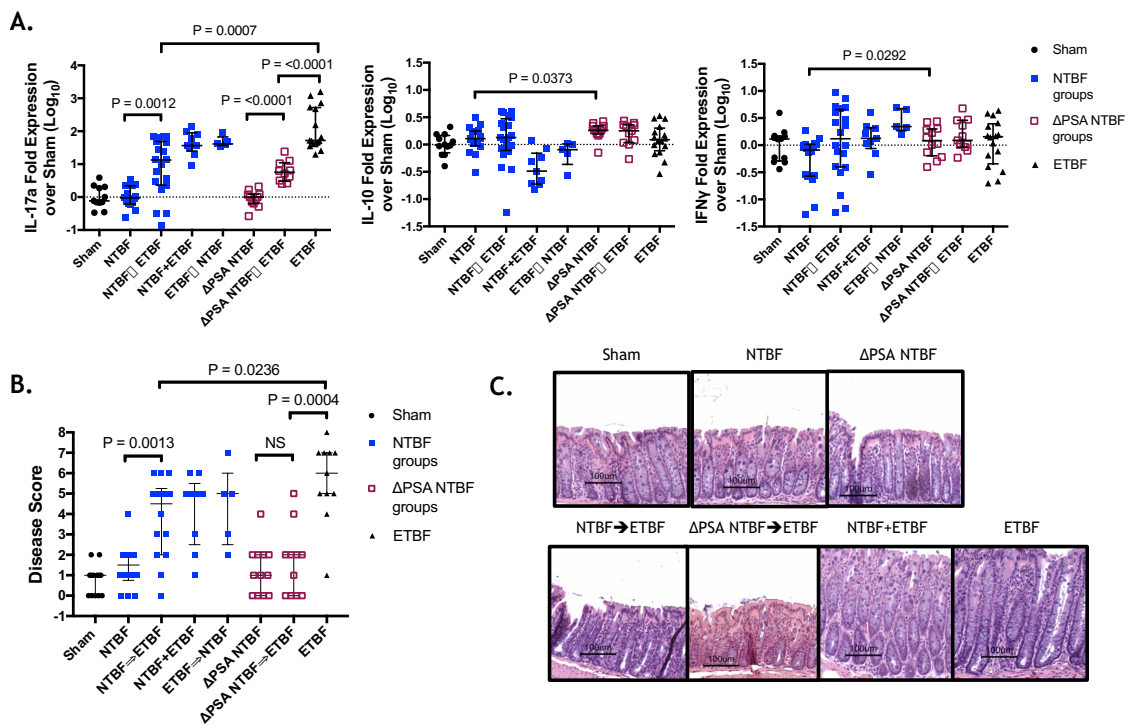
Supplemental Figure 3: *IL17A*, *IL10*, *IFNG*, and *IL4* expression determined by colon whole tissue qRT-PCR at 17, 24, and 45 days after NTBF infection. NTBF-colonized SPF mice exhibit no changes in total *IL17A*, *IL10*, *IFNG*, and *IL4* expression at early or late time points. Bars indicate median and interquartile range. Data shown are from 1 independent experiment with 4 to 5 mice per time point. Mice were compared to their sham control littermates.
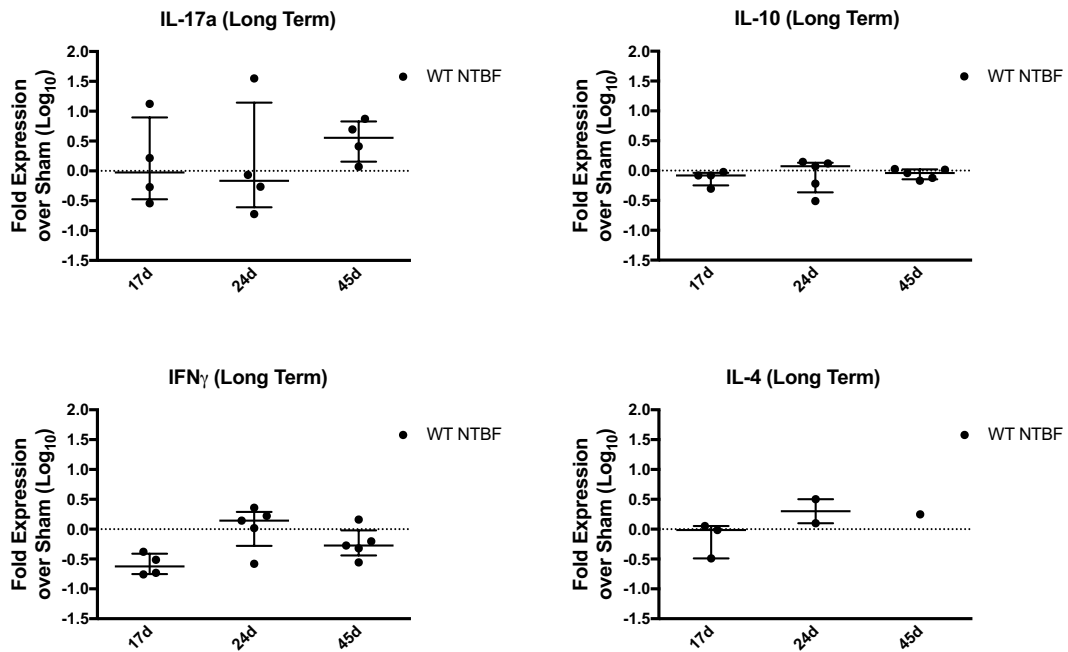
Figure 2. PSA-competent and PSA-deficient NTBF similarly blunt tumorigenesis. A-D SPF Min mice were inoculated sequentially (NTBF→ETBF) or simultaneously (NTBF+ETBF). Mice were sacrificed at 12 weeks (macroadenomas) or 2 to 4 weeks (microadenomas). Bars indicate median and interquartile range. Data shown are from 3 separate experiments with 3 to 4 mice per test group. **A** Images of representative methylene blue-stained distal colons. NTBF→ETBF and ΔPSA NTBF→ETBF mice display decreased tumor development compared with NTBF+ETBF and ETBF control mice. **B** Quantification of tumors from individual mice. Macroadenoma counts are recorded for the entire colon. Both WT and ΔPSA NTBF→ETBF mice exhibit significant blunting of tumorigenesis in comparison with NTBF+ETBF and ETBF controls. Inset shows colon images of NTBF→ETBF high tumor producers: mouse #312 and mouse #238. Macroadenoma numbers were similar between WT and ΔPSA NTBF treatment groups. **C** H&E colon sections displaying microadenoma histopathology of NTBF→ETBF and ETBF only treated mice. **D** Quantification of microadenomas from individual mice. NTBF→ETBF mice do not display a significantly lower microadenoma burden compared with ETBF control mice.

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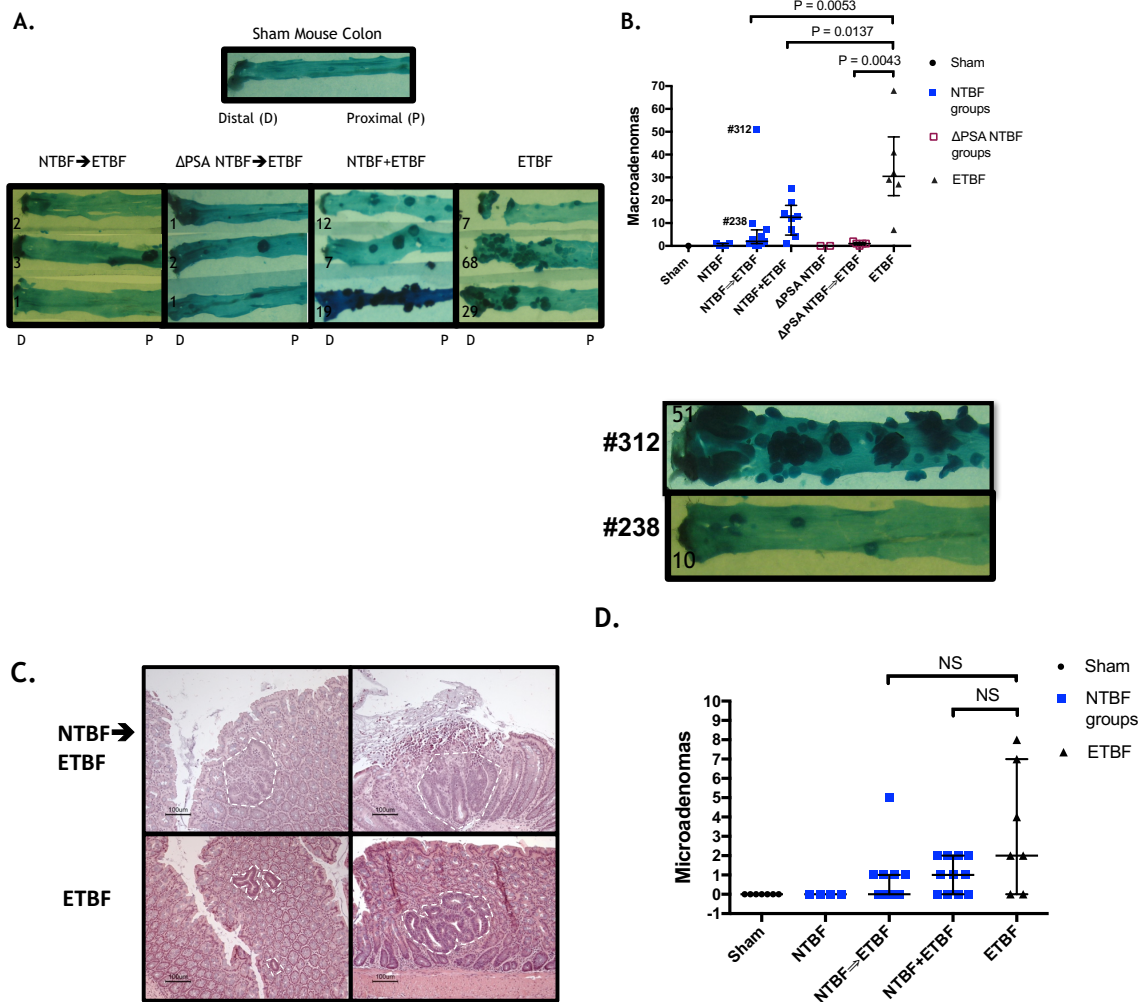
Supplemental Figure 4: After strain establishment, NTBF protection against macroadenoma initiation is durable. Formalin-fixed, methylene blue-stained colons of NTBF→ETBF mice and ETBF only control mice were counted for macroadenomas at 8, 12, and 16 weeks after initial infection. Data shown at 8 and 16 weeks are from 1 independent experiment (2 to 7 mice per treatment group). Data shown at 12 weeks represents a compilation of 3 independent experiments as shown in Figure 2B (3 to 4 mice per treatment group, per replicate). Bars indicate median and interquartile range.
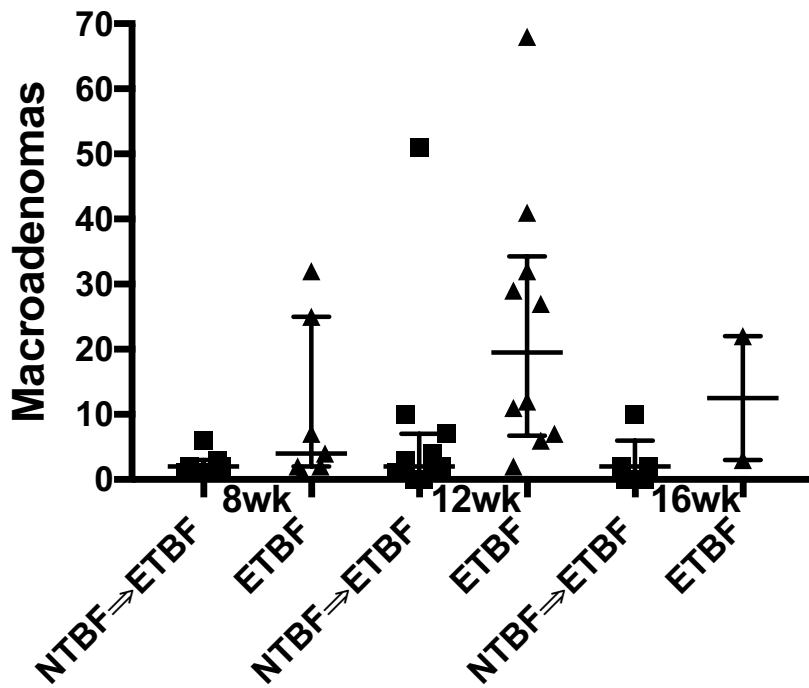
Figure 3. PSA does not impact *B. fragilis* niche occupation. A-B SPF and GF mice were monocolonized with WT or ΔPSA NTBF for 2 or 8 weeks. Mouse colons were unflushed, fixed in formalin, and FISH-stained: DAPI=blue, *Bacteroides fragilis* 16S probe=red. **A** Representative FISH-stained mouse colons (2wk p.i.); images taken at 40X. Insets show images taken at 63X. WT and ΔPSA NTBF colonize both the lumen and at the mucosal surface with patchy mucus penetration (examples are shown with arrows). The presence or absence of PSA does not affect the colonic niche of NTBF. **B** Stool and mucosal colonization of mice inoculated with WT or ΔPSA NTBF. PSA does not impact fecal or mucosal NTBF colonization. Bars indicate median and interquartile range. Data shown are from 2 to 3 separate experiments.
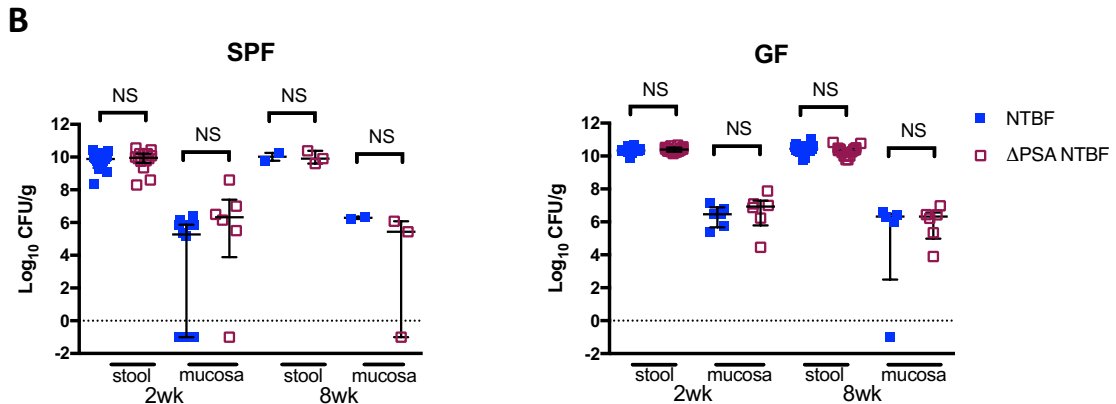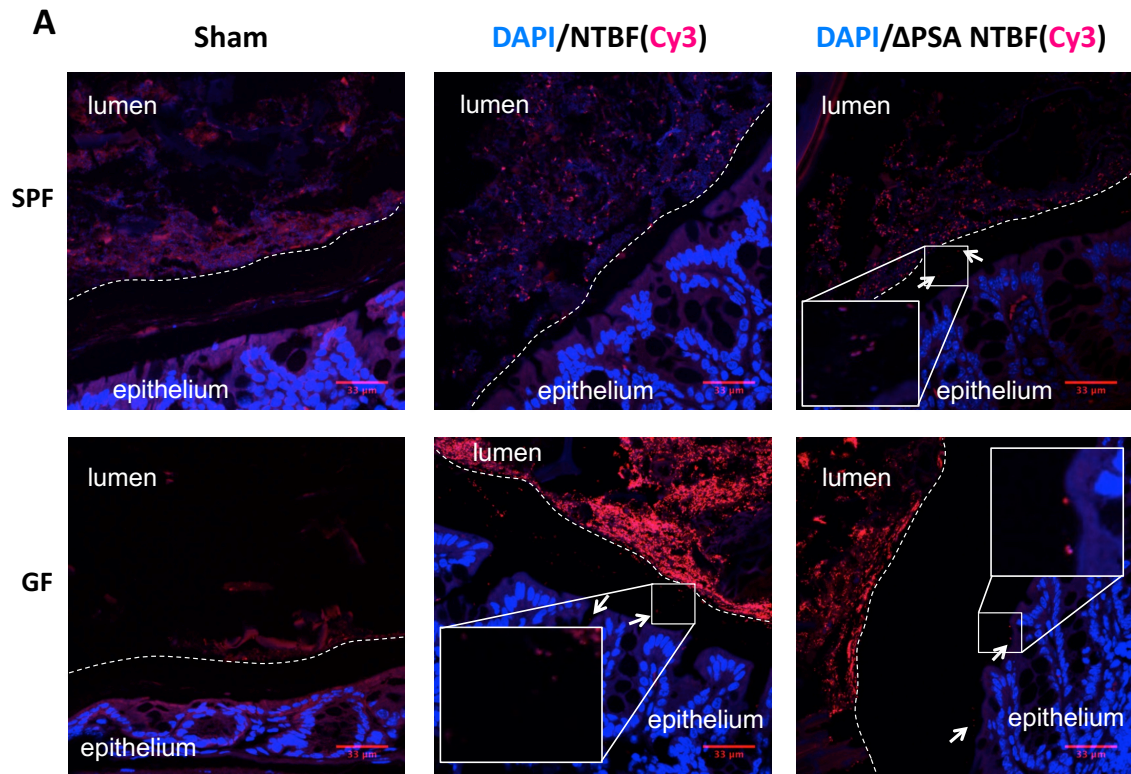
Supplemental Figure 5: NTBF and ETBF exhibit similar fecal and mucosal colonization burden in mice. SPF WT and SPF Min mice were colonized with various *B. fragilis* strains: NTBF, ΔPSA NTBF, and ETBF. In WT mice, stool colonization was evaluated at 3 days and 2 weeks p.i. and mucosal colonization at 2 weeks terminal sacrifice. In Min mice, stool colonization was evaluated at 3 days (NTBF and ΔPSA NTBF only) and 12 weeks p.i.. *B. fragilis* colonization burden was similar among all treatment groups.
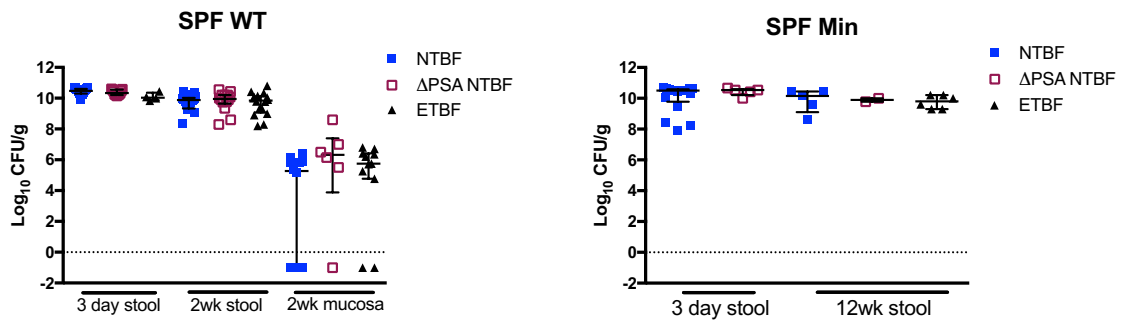
Figure 4. Bacterial strain dominance determines inflammatory and tumor responses. A Viable colony forming unit (CFU) percentages of NTBF and ETBF strain detected in stools harvested 1 week p.i. in SPF WT mice. Isolated *B. fragilis* colonies (up to 30) cultured from stool homogenates were randomly selected and screened by strain-specific PCR. In 9 of 10 NTBF→ETBF mice, only NTBF was detected; in 1 of 10, both NTBF and ETBF were detected. In 3 of 3 NTBF+ETBF mice, only ETBF was detected. Strain dominance is established early. **B-E** Strain quantification as measured by fecal qPCR at 2 weeks (SPF WT) or 12-16 weeks (SPF Min) and presented in the order of NT/ET: lo/hi, hi/hi, or hi/lo. Low = $10^1 < 10^5$ copies. High = $\geq 10^5$ - 10^8. **Circles** represent individual mice; **triangles** represent median *IL17A* expression or median tumor counts (see right vertical axis). ***** = mice with hi/hi co-colonization levels. **B** Strain quantification in WT co-infected mice. **C** Correlation of *IL17A* gene expression with ETBF copy number in WT mice. **D** Strain quantification in Min co-infected mice. **E** Correlation of tumor number with ETBF copy number in Min mice. **F** Representative IF staining in SPF (WT or Min) mice (2-4wk p.i.); images taken at 40X. Insets show images taken at 63X. Top row: Both NTBF and ETBF colonize the lumen and at the mucosal surface with patchy mucus penetration (examples are shown with arrows). Bottom row: Co-colonized mice, either NTBF-dominant (NT^{dom}) or ETBF-dominant (ET^{dom}).

Table 1. Stool copy numbers of NTBF and ETBF detected in SPF WT mice by treatment group at 2 weeks. Protocol described in Methods. Results are depicted as percent total (%) of colonized mice out of the total treatment group. Classification: Low ($10^1 < 10^5$ copies) or High ($\geq 10^5 - 10^8$).

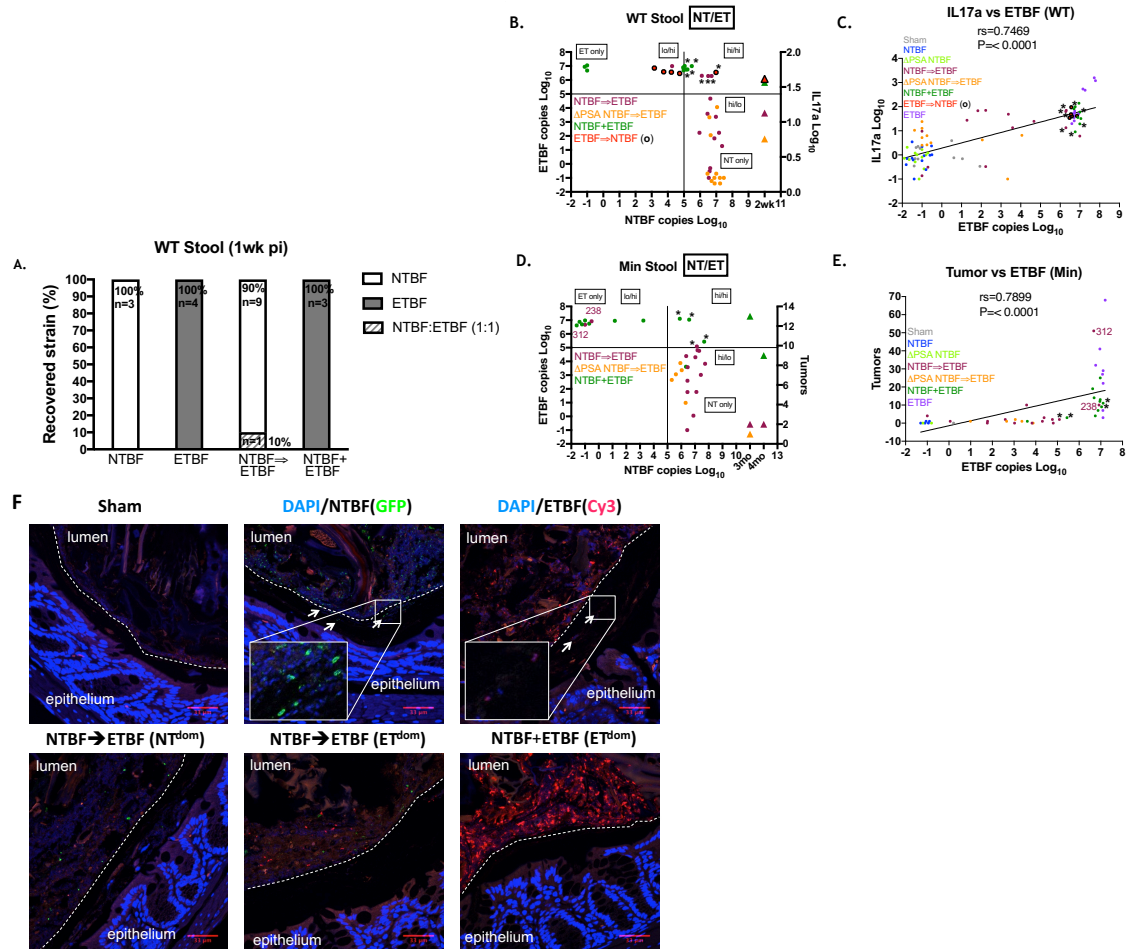| TABLE 1. NTBF and ETBF strain quantification (qPCR) in stool of SPF WT mice persistently infected with *B. fragilis* by different inoculation protocols at 2 weeks					
Percent total (# colonized/total mice)					
Type of Infection	**NTBF / ETBF Co-Colonization**			**NTBF Only**	**ETBF Only**
	Low / High $(10^1 - <10^5) / (\geq 10^5 - 10^8)$	High / Low $(\geq 10^5 - 10^8) / (10^1 - <10^5)$	High / High $(\geq 10^5 - 10^8) / (\geq 10^5 - 10^8)$	High $(\geq 10^5 - 10^8)$	High $(\geq 10^5 - 10^8)$
NTBF	0.0%(0/13)	0.0%(0/13)	0.0%(0/13)	100%(13/13)	0.0%(0/13)
NTBF➜ETBF	7.1%(1/14)	50.0%(7/14)	21.4%(3/14)	21.4%(3/14)	0.0%(0/14)
ΔPSA NTBF➜ETBF	0.0%(0/11)	27.3%(3/11)	0.0%(0/11)	72.7%(8/11)	0.0%(0/11)
ETBF➜NTBF	80.0%(4/5)	0.0%(0/5)	20.0%(1/5)	0.0%(0/5)	0.0%(0/5)
NTBF+ETBF	22.2%(2/9)	0.0%(0/9)	44.4%(4/9)	0.0%(0/9)	33.3%(3/9)
ETBF	0.0%(0/12)	0.0%(0/12)	0.0%(0/12)	0.0%(0/12)	100%(12/12)

Table 2. Stool copy numbers of NTBF and ETBF detected in SPF Min mice by treatment group at 12 to 16 weeks. Protocol described in Methods. Results are depicted as the percent total (%) of colonized mice out of the total treatment group. Classification: Low ($10^1 < 10^5$ copies) or High ($\geq 10^5$ - 10^8).

Table 2. NTBF and ETBF strain quantification (qPCR) in stool of SPF Min mice persistently infected with *B. fragilis* by different inoculation protocols at 12 to 16 weeks					
Percent total (# colonized/total mice)					
Type of Infection	NTBF / ETBF Co-Colonization			NTBF Only	ETBF Only
	Low / High (10^1 - $<10^5$) / ($\geq 10^5$-10^8)	High / Low ($\geq 10^5$-10^8) / (10^1 - $<10^5$)	High / High ($\geq 10^5$-10^8) / ($\geq 10^5$-10^8)	High ($\geq 10^5$-10^8)	High ($\geq 10^5$-10^8)
NTBF	0.0%(0/5)	0.0%(0/5)	0.0%(0/5)	100%(5/5)	0.0%(0/5)
NTBF➔ETBF	0.0%(0/15)	66.7%(10/15)	6.7%(1/15)	13.3%(2/15)	13.3%(2/15)
ΔPSA NTBF➔ETBF	0.0%(0/5)	80.0%(4/5)	0.0%(0/5)	20.0%(1/5)	0.0%(0/5)
NTBF+ETBF	18.2%(2/11)	9.1%(1/11)	27.3%(3/11)	0.0%(0/11)	45.5%(5/11)
ETBF	0.0%(0/7)	0.0%(0/7)	0.0%(0/7)	0.0%(0/7)	100%(0/7)

Supplemental Figure 6: *B. fragilis* strain-specific immunofluorescence. Optimization of IF staining was done on formalin-fixed *B. fragilis* NTBF and ETBF culture pellets. Image shows IF staining on *B. fragilis* NTBF/ETBF mixed culture at 40X.
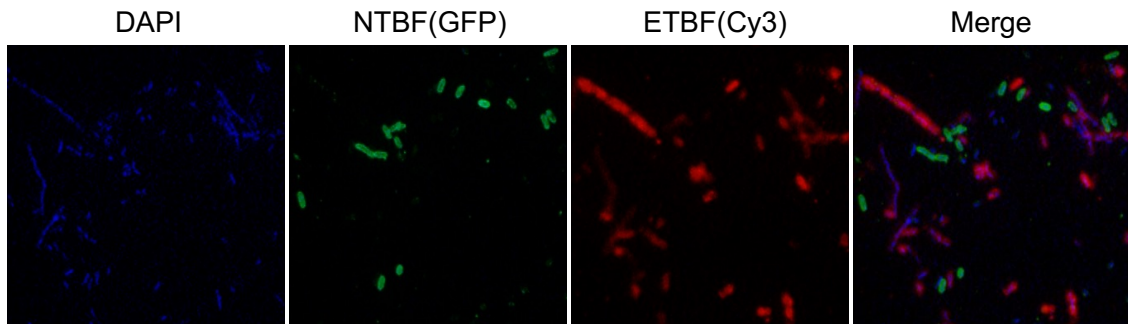
Supplemental Figure 7: Periodic acid-Schiff (PAS) stains matched to IF images. PAS stains for carbohydrate macromolecules, showing the level of mucus preservation retained in presented images. **A.** PAS images parallel to **Fig. 3A B.** PAS images parallel to **Fig. 4F**.
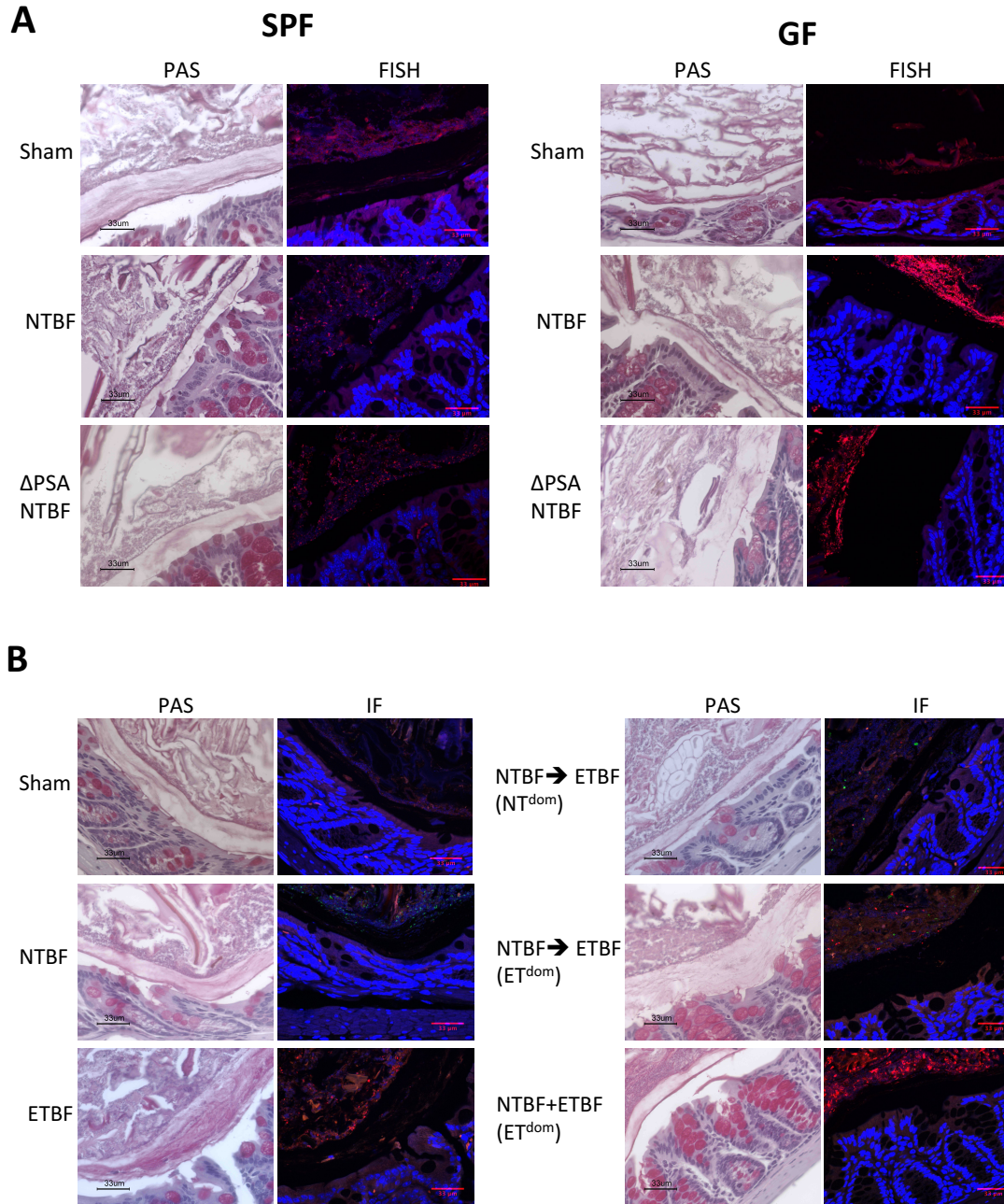
Figure 5. Therapeutic treatment with NTBF fails to disrupt ETBF strain dominance and disease burden. A-D SPF WT mice were stably infected with ETBF or NTBF for 2 weeks and then treated with NTBF or ETBF, respectively (ETBF→NTBF^R and NTBF→ETBF^R). Superscript ^{R} denotes repeated treatments for 9 days. Mice were rested for 2 days before sacrifice. Symbols represent individual mice. Data shown include a single experiment with 4 to 5 mice per treatment group. Bars indicate median and interquartile range. **A** Strain quantification as measured by stool qPCR at sacrifice. **Circles** represent individual mice. Red: 3 of 5 ETBF→NTBF^R mice establish detectable levels (10^2 - 10^6 copies) of treatment strain NTBF (mouse ID: 62, 61, 59). Purple: 0 of 5 NTBF→ETBF^R mice exhibit detectable levels of challenge strain ETBF. **B** Representative images of cecum and spleen: ETBF→PBS^R control and ETBF→NTBF^R ceca are small, pale, and contracted; spleens are enlarged. NTBF→PBS^R control and NTBF→ETBF^R ceca are comparable to Sham→PBS^R mice (not shown) in size, brown color, and content; spleens are not enlarged. **C** Mouse cecum weights normalized to total body weight. ETBF→NTBF^R cecum weights are comparable to ETBF→PBS^R controls, indicative of disease. NTBF→ETBF^R cecum weights are significantly higher when compared to ETBF→PBS^R control and ETBF→NTBF^R mice, indicative of protection. **D** Mouse spleen weights normalized to total body weight. ETBF→NTBF^R spleen weights are significantly higher when compared to NTBF→ETBF^R mice but comparable to ETBF→PBS^R controls, indicative of disease. NTBF→ETBF^R spleen weights are comparable to NTBF→PBS^R controls, indicative of protection.
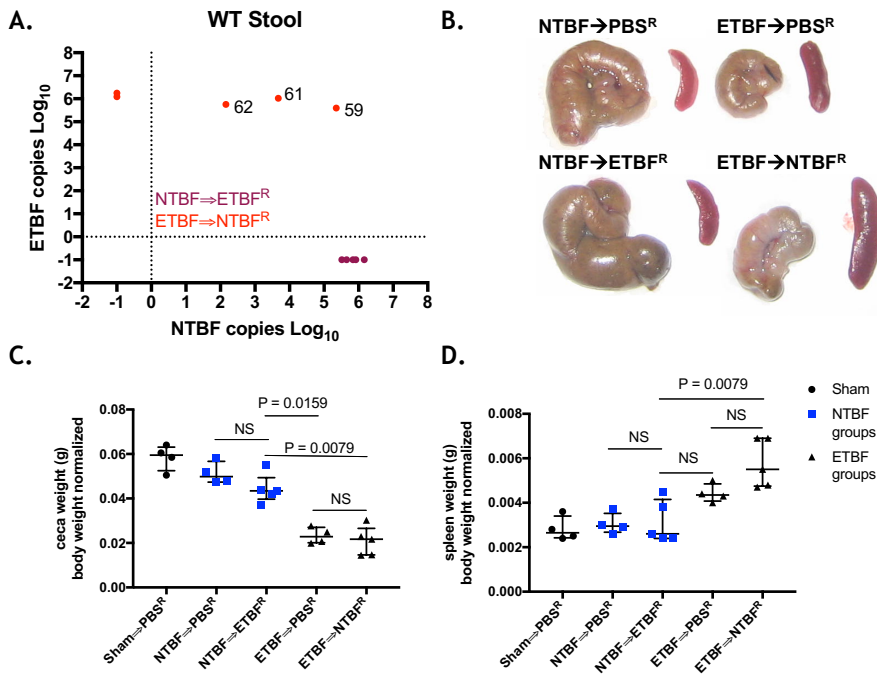
Figure 6. PSA-competent and PSA-deficient NTBF do not alter IL-17A$^+$ T cell or Treg responses in SPF mice. LPLs were isolated from distal colons of SPF WT and SPF Min mice 2 weeks after Sham, NTBF, or ΔPSA NTBF treatment. LPLs were subjected to 4 hour incubation with cell stimulation cocktail and subsequent ICS for IL-17A and Foxp3. Data are presented as percent IL-17A$^+$ of live CD3$^+$ lymphocytes and percent Foxp3$^+$ of live CD3$^+$ CD4$^+$ lymphocytes (top). Live CD3$^+$ IL-17A$^+$ and live CD3$^+$ CD4$^+$ Foxp3$^+$ cell numbers were also normalized by the mass of colon tissue (bottom). Symbols represent individual mice. Bars indicate mean $^\pm$1 SD. Data shown include two separate experiments with 4 to 5 mice per treatment group. No significant differences were noted among WT and ΔPSA NTBF treatment groups in any parameters tested in SPF WT or SPF Min mice.
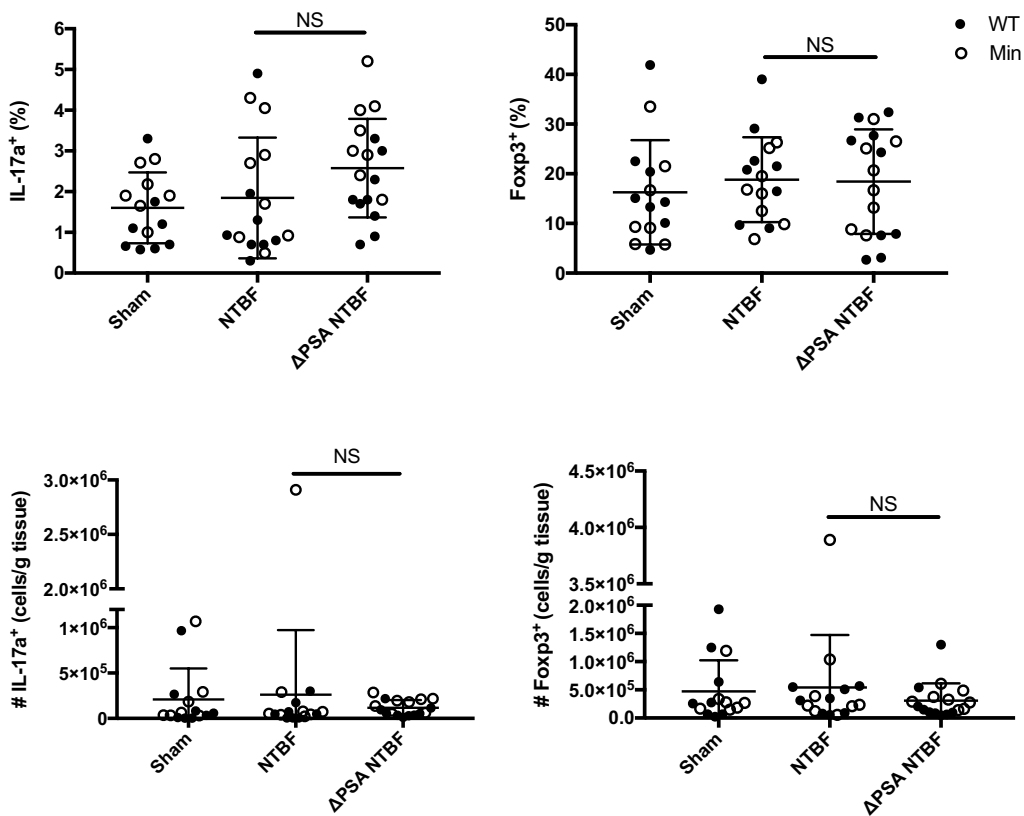
Supplemental Figure 8: IFNγ[+] and IL-17A^{+} Foxp3^{+} T cell responses in the colon LP of SPF WT and Min mice colonized with NTBF or ΔPSA NTBF. Individual colons were harvested from WT and Min mice of 2 replicate experiments, 4 or 5 mice per group (shown in **Fig. 6**). Data are presented as percentage, IFNγ^{+} of live CD3^{+} lymphocytes and Foxp3^{+} of live CD3^{+} IL-17A^{+} lymphocytes (top), and as T cell density (# of cells per gram of colon tissue) (bottom).
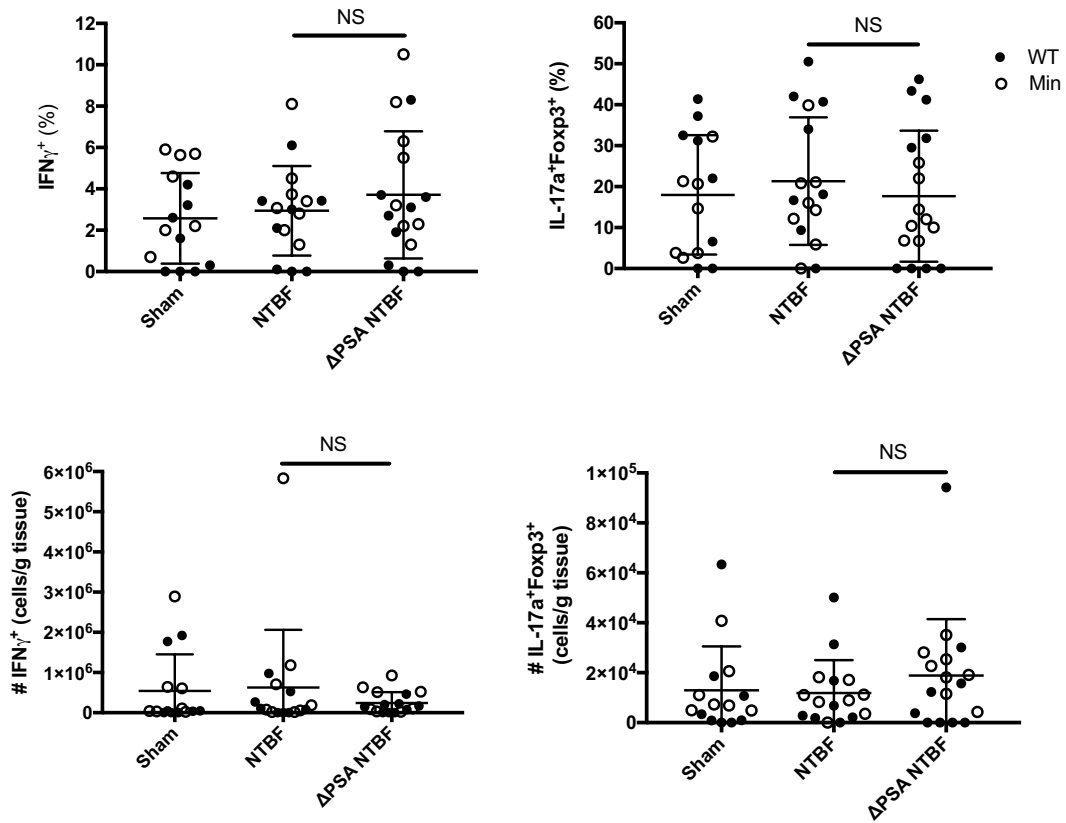
Supplemental Figure 9: T cell response in the MLNs and spleens of SPF mice colonized with NTBF or ΔPSA NTBF. MLNs and spleens were pooled from WT (closed circles) and Min (open circles) mice of 2 replicate experiments (shown in **Fig. 6**), 4 or 5 mice pooled per experiment. Data are presented as percentage (top row) and T cell density (# of cells per gram of colon tissue) (bottom row). No differences between sham, NTBF, or ΔPSA NTBF-colonized mice were identified.
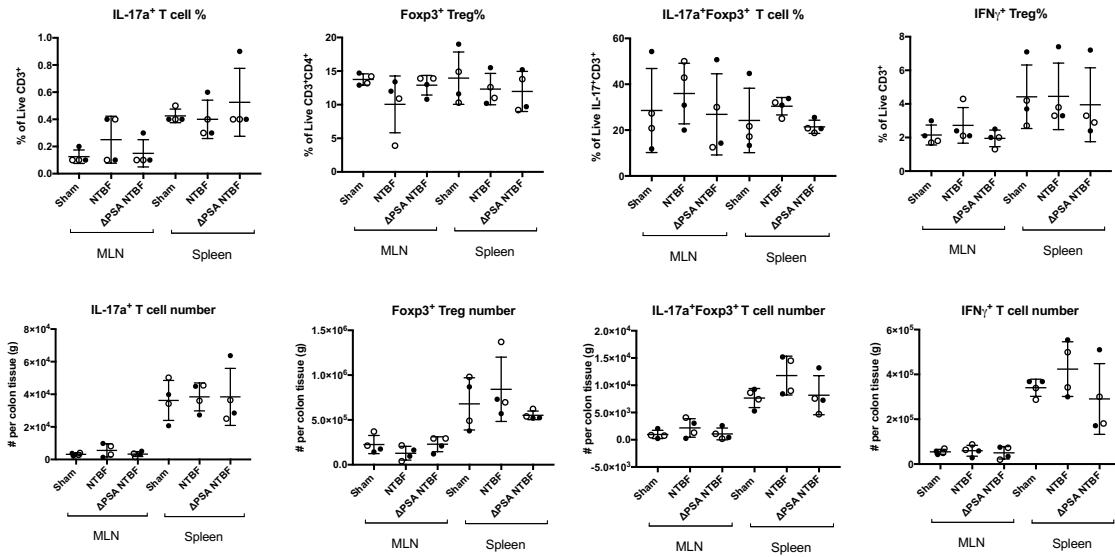
Supplemental Figure 10: Splenic morphology of SPF and GF monocolonized mice. **A.** Representative H&E stains of formalin-fixed spleens of SPF and GF sham and monocolonized mice 8wk post-infection. **B.** After formalin-fixation, spleens from GF mice were weighed. SPF C57BL/6 control mouse spleen weights consistently range from 0.05g-0.1g. All spleens from monocolonized GF mice (2 to 8wk p.i.) fell within this normal range with no differences seen among treatment groups. Bars indicate median and interquartile range. Data are representative of 2 independent GF experiments.

A.

B.
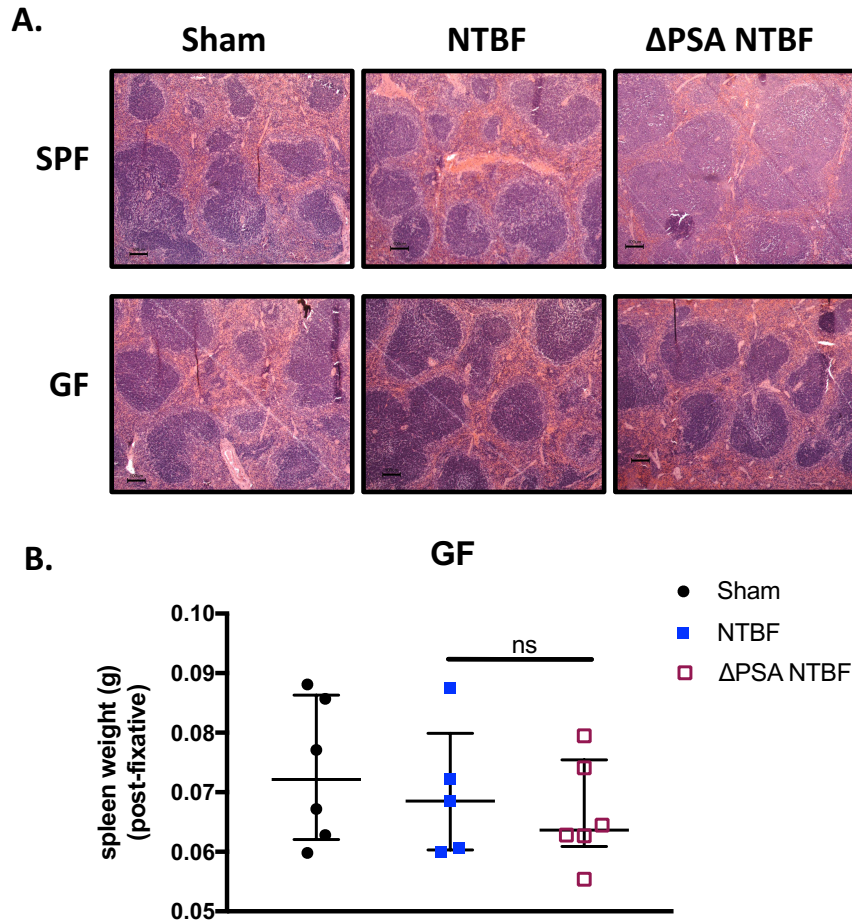
Figure 7. PSA-competent NTBF does not alter Th17, Treg, or Th1 responses in GF mice. A LPLs were isolated from distal colons of GF WT mice 2 and 8 weeks after Sham, NTBF, or ΔPSA NTBF treatment. Data represent absolute cell number normalized to colon tissue mass. Symbols represent individual mice. Bars indicate mean $^{\pm}$1 SD. Data shown include two separate experiments with 4 to 5 mice per treatment group. Only total IFNγ results differed between NTBF and ΔPSA NTBF mice. **B** In parallel, GF mouse distal colon tissue was harvested for whole tissue qRT-PCR. No differences were observed among treatment groups in total *IL10*, *IFNG*, and *IL4* expression, although monocolonized mice exhibited increased *IL17A* expression compared with uninfected GF controls at 2 weeks but not at 8 weeks. Bars indicate median and interquartile range. Data shown include 2 separate experiments with 2 to 3 mice per group per experiment.
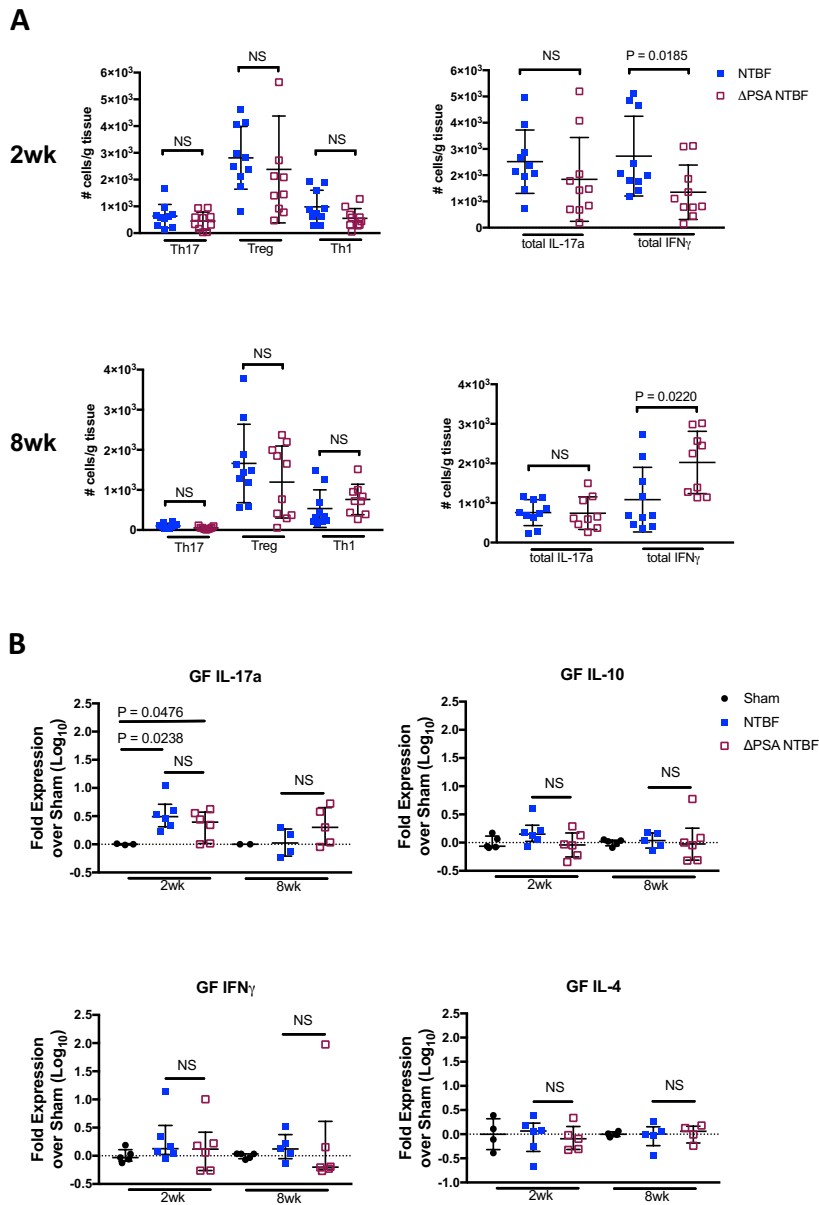
Supplemental Figure 11: IL-17A^{+} and IFNγ^{+} γδ T cell responses in the colon lamina propria of GF mice colonized with NTBF or ΔPSA NTBF. Individual distal colons were harvested from GF mice of 2 replicate experiments, 4 or 5 mice per group (shown in **Fig. 7**). Data are presented as T cell density (# of cells per gram of colon tissue) 2 weeks (top) or 8 weeks (bottom) post-infection.
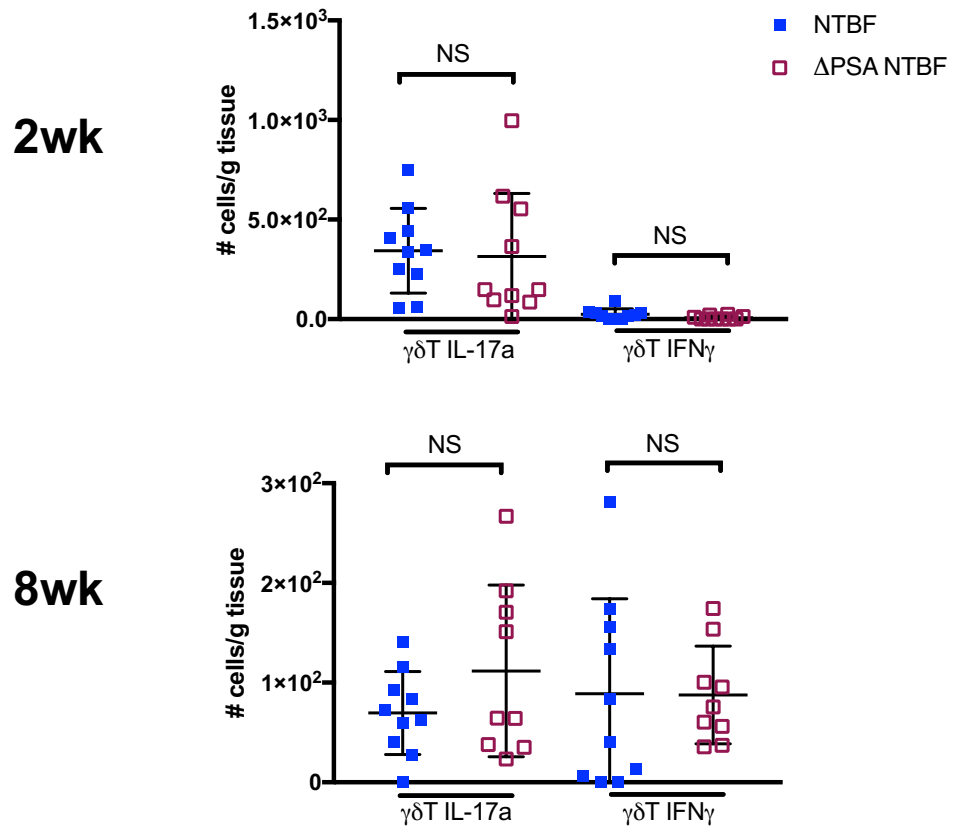
Figure 8. ETBF secretes an intraspecies inhibitory molecule. A Overlay of individual NTBF and ETBF growth curves. There are no growth differences observed between strains. Curves represent the average of the triplicate values. **B** Co-inoculation assay of NTBF with ETBF. Isolated *B. fragilis* colonies (10) plated from co-cultures were randomly selected and screened by strain-specific PCR. After equal parts inoculation, NTBF is no longer detected at 24 and 48 hours. **C** Co-inoculation assay of NTBF with ETBF. After differential inoculation, NTBF is only detected at an input ratio of 90:10 (NTBF:ETBF) at 24 and 48 hours. **D** Agar diffusion assay of NTBF indicator strain (grown on the agar) with ETBF cell-free supernatant (in wells). A zone of clearing (stippled line) is induced by the ETBF stationary phase supernatant. Protease treatment with trypsin and proteinase-K and heat treatment at $100^{o}C$ destroys ETBF supernatant activity. Top row: ETBF WT supernatant; bottom row: ETBF BFT mutant supernatant.
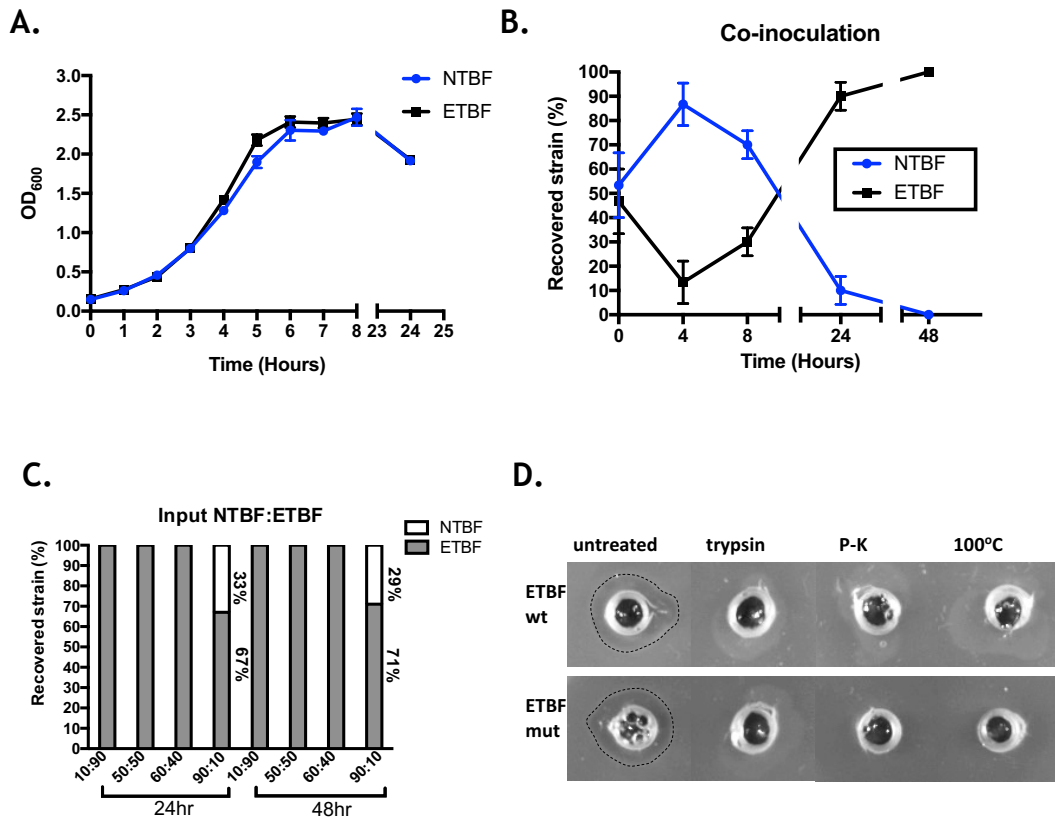
Supplemental Figure 12: ETBF secretes an intraspecies inhibitory molecule, active on ΔPSA NTBF, K570 ETBF (BFT-3 isolate), and VPI ETBF (BFT-1 isolate). Agar diffusion assay of various *B. fragilis* indicator strains plated on the agar with ETBF WT supernatant in wells (See Methods).

SUP	untreated	trypsin	P-K	100ºC	indicator
ETBF wt					ΔPSA NTBF
ETBF wt					K570 ETBF
ETBF wt					VPI ETBF
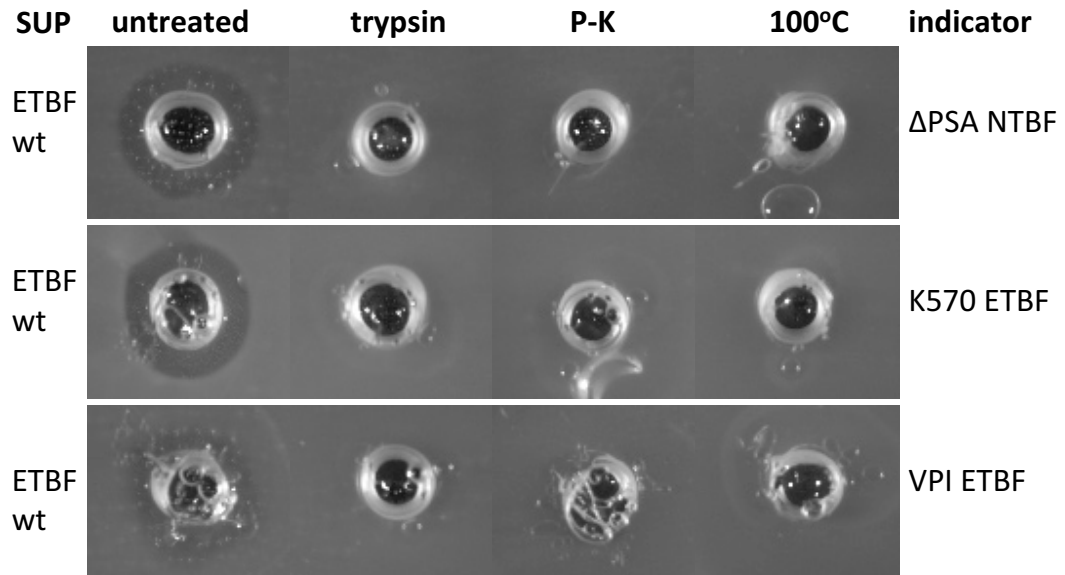
Supplemental Figure 13: *B. fragilis BSAP-1* and *ccf* gene expression. **A.** RNA was extracted from stationary phase cultures of NTBF and ETBF. cDNA was synthesized and SYBR Green PCR was run with *BSAP-1* (primer set 3) and *B. fragilis 16S* (primer set 8) primers (**Supp. Table 1**). Raw Ct values are presented. **B.** Distal colon whole tissue RNA was extracted from SPF Sham, NTBF, or ETBF colonized mice (same mice presented in **Figure 1**). cDNA was synthesized and *ccfA* (sigma factor) and *ccfB* (anti-sigma factor) gene expression was determined via SYBR Green PCR with primer sets 4 and 5 (**Supp. Table 1**). Samples were normalized to *B. fragilis 16S* and presented as fold expression over sham ($2^{\wedge \Delta \Delta Ct}$).

A.
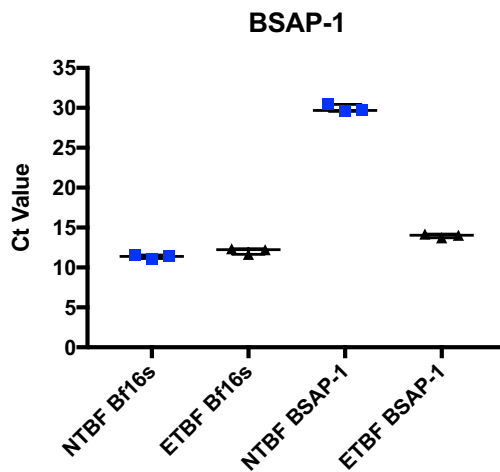
B.
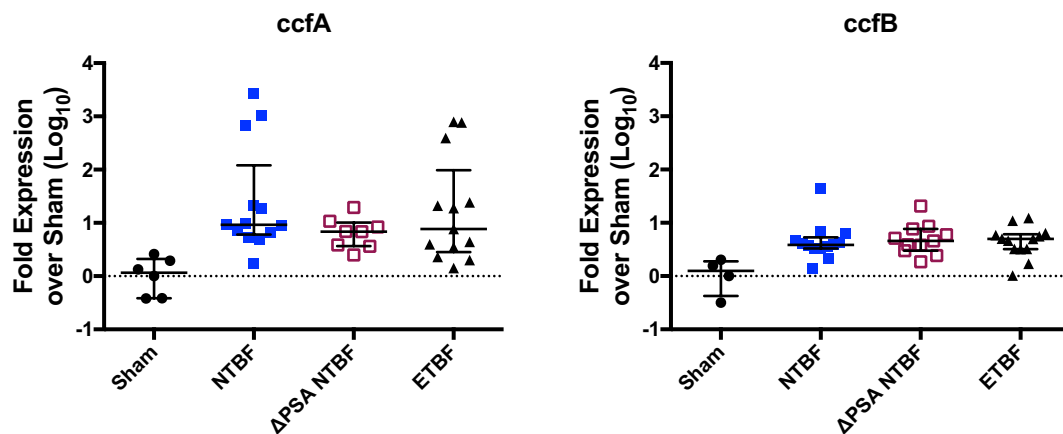
CHAPTER 3 - IL-22 in enterotoxigenic *B. fragilis* disease

<u>Background</u>

In an effort to understand the molecular mechanisms underlying CRC development, gut immune signaling pathways are currently being studied. For instance, signaling by interleukin-22 (IL-22), a potent cytokine effector molecule and STAT-3 activator, is under intense investigation. Clinically, in patients with sporadic CRC, IL-22-producing cells and *IL22* gene expression levels were enriched in the tumor versus normal mucosa or paratumor sites[161,162]. Similarly, IL-22 was found to be more highly expressed in tumor-infiltrating leukocytes (TILs) isolated from fresh colon tumors versus the peripheral blood or normal controls. IL-22-related proteins IL-23, IL-22RA1, and the phosphorylated active form of STAT-3 (pSTAT-3) were overexpressed in colon cancer tissues, appearing to localize in tumor and intestinal epithelial cells as measured and detected by IHC[163]. Co-transplantation of patient IL-22-expressing TILs or IL-22 directly, along with a colon cancer cell line, induced tumor growth and metastasis in subcutaneous transplantation mouse models[162,163]. Notably, Andoh et al., Jiang et al., and others showed that individuals with active IBD maintained increased levels of IL-22 expression in their inflamed colonic mucosa[163–165]. Additional microarray and functional analyses with human sub-epithelial myofibroblasts (SEMFs) showed an IL-22-induced upregulation of inflammatory cytokines (IL-6, IL-8, IL-11, LIF) and matrix-degrading molecules (MMP-1, MMP-3), alluding to the possible role of IL-22 in IBD inflammation and tissue remodeling[164]. Furthermore, a commonly inherited IL-22 genetic polymorphism, the rs1179251 G variant, has been positively associated with colon cancer with haplotype analysis suggesting a 52% increased risk for those carrying at least one

copy[166]. Collectively, examples from both the IBD and CRC literature suggest that IL-22 may be a critical component in intestinal inflammation and the transition from colitis to cancer.

A member of the IL-10 cytokine family, IL-22 is known to be expressed by CD4$^+$ T cells of the T_H0, T_H17, and T_H22 subsets, as well as by some members of the more recently described ROR-γt$^+$ innate lymphoid cell (ILC) family, including Nkp46$^+$ NK-22 cells, CD4$^+$ LTi cells, and colonic ILCs[161,167]. IL-22 interacts with a heterodimeric receptor, comprised of IL-10R2 (shared by IL-10) and IL-22RA1 (specific to IL-22)[167]. As IL-10R is expressed ubiquitously, IL-22RA1 is the likely determining factor of the cellular targets of IL-22 and is typically found expressed on non-hematopoietic tissue, including the epithelial cells and SEMFs of the small and large intestines[167]. As such, in the context of the colon, reports indicate that IL-22 signaling is critical to the maintenance of epithelial cell innate immunity, protection, and repair. As very little is known about the role of IL-22 in microbial-induced colitis and CRC, we aimed to study IL-22 in mouse models of ETBF infection.

Materials and Methods

ETBF Mouse Models

C57BL/6 *IL-22*^{-/-} mice were obtained from Genentech and crossed into the C57BL/6 Min$^{Apc716+/-}$ background. WT (non-Min) *IL-22*^{-/-} mice were used for colitis experiments and Min *IL-22*^{-/-} mice were used for tumor experiments. In the main text, Min = Min$^{Apc716+/-}$ and IL-22KO = *IL-22*^{-/-} mice. Mice were housed in specific pathogen free conditions according to the Association for the Assessment and Accreditation of Laboratory Animal Care International. All protocols were approved by the Johns Hopkins University Animal Care and Use Committee. Three to four-week old mice were placed on antibiotic water containing clindamycin (0.1 g/L) and streptomycin (5 g/L) for 5 to 7 days prior to infection. Enterotoxigenic *B. fragilis* strain 86-5443-2-2 was used for all mouse experiments[74,75]. Bacteria were grown anaerobically at 37°C with brain-heart infusion (37 g/L) (BD Bacto) culture media supplemented with yeast extract (5 g/L) (BD Bacto), L-cysteine (50 mg/L) (Sigma), hemin (0.5 mg/L) (Sigma), vitamin K (0.1 mg/L) (Sigma), and clindamycin (10 μg/ml) (Research Products International). In preparation for orogastric administration, bacterial pellets were washed twice and suspended with sterile 1X Dulbecco's phosphate-buffered saline (1X PBS free of calcium chloride & magnesium chloride). All mice received 100 μl of the inoculum, corresponding to ~10^8 ETBF CFU per mouse. The 86-5443-2-2 strain is innately resistant to both clindamycin and streptomycin.

Intestinal Permeability

Intestinal permeability was analyzed by a method adapted from Wang et al.[168]. Four hours prior to sacrifice, food was removed and mice received an orogastric administration (20 ml/kg body weight) of a 22 mg/ml fluorescein isothiocyanate-dextran stock (FITC-dextran, avg. molecular weight 4,000) (Sigma 46944). At sacrifice, blood was harvested by cardiac puncture and allowed to clot at room temperature. Blood samples were centrifuged at 3,000 rpm for 20 minutes at 4^oC, and serum was harvested. Serum samples were diluted 1:2 with sterile 1X PBS (pH 7.4) and aliquoted to a clear-bottom, black 96-well plate (Corning 3603) in triplicate wells. Fluorescein concentrations were determined by spectrofluorometric plate reader (excitation 485 nm and emission 530 nm), using serially diluted samples of the FITC-dextran marker as the standard.

Immunohistochemistry

pSTAT-3 and BrdU staining was done in accordance with standard immunohistochemistry protocols. FFPE tissues were deparaffinized, rehydrated, and subjected to antigen retrieval by steaming with citrate buffer (1.8 mM citric acid and 8.2 mM sodium citrate) for 30 minutes. Endogenous peroxidase was quenched by treatment with 3% H_2O_2 for 10 minutes. After blocking, tissue sections were incubated with either rabbit anti-pSTAT3 primary or biotin-conjugated anti-BrdU primary overnight at 4^oC followed by the secondary antibodies Powervision Poly-HRP anti-rabbit (Leica PV6119) or streptavidin-HRP, respectively, for 1 hour at RT. DAB development was done for 20 minutes. Tissues were counterstained with hematoxylin.

Statistical Analysis

Unless otherwise indicated, the nonparametric two-tailed Mann-Whitney U test was used to compare means of all treatment groups. A p value of <0.05 was considered significant.

Fecal Colonization and Mucosal Adherence

Assays were carried out according to previously described methods (Ch. 2, pg. 27).

Quantitative RT-PCR

Assays were carried out according to previously described methods (Ch. 2, pg. 27-28).

Histology, Microadenoma, and Macroadenoma Counts

Assays were carried out according to previously described methods (Ch. 2, pg. 28).

LPL Isolations

Assays were carried out according to previously described methods (Ch. 2, pg. 30).

Flow Cytometry and FACS

Assays were carried out according to previously described methods (Ch. 2, pg. 30). In some experiments, FACS was performed on the FACSAria II (BD Bioscience).

Results

IL-22 Protects Against Epithelial Cell Shedding and Intestinal Permeability

In the absence of IL-22, we sought to evaluate the impact on ETBF colitis. C57BL/6 WT and IL-22KO mice were infected with ETBF and analyzed at day 1, day 3, and day 7 post-infection. Fecal and mucosal ETBF colonization was similar between IL-22KO mice and parental controls (**Supp. Fig. 1**). During the acute stage of infection, IL-22KO mice exhibited striking colon epithelial cell shedding (anoikis), indicative of cellular apoptosis (**Fig. 1A**). At 3 days, IL-22KO mice displayed the highest anoikis disease scores in comparison with WT mice (p=0.009) (**Fig. 1B**). Suspecting that these marked tissue disruptions may impact barrier function, we analyzed intestinal permeability by means of fluorescein isothiocyanate (FITC) detection in the blood. In mice receiving FITC-dextran by oral gavage, we discovered that ETBF-infected IL-22KO mice displayed the highest concentrations of FITC in the serum compared to WT controls (p=0.003), coinciding with the time point at which severe epithelial cell shedding occurred (**Fig. 1C**). Other characteristic histopathological features of ETBF colitis, hyperplasia and inflammation, were comparable between WT controls and IL-22-deficient mice (**Fig. 1D**). Both treatment groups displayed a synchronous increase in these histopathologic parameters over the course of the infection. As IL-22 has a purported role in wound healing and tissue repair, we sought to evaluate proliferation in IL-22KO mice. Twenty-four hours prior to terminal sacrifice, mice were given bromodeoxyuridine (BrdU) by intraperitoneal injection; mice colonized with ETBF for 3 days were assessed. Epithelial cell proliferation in mouse colons was evaluated by

immunohistochemistry. Stained slides were observed for the number of BrdU-positive cells and the migration distance of BrdU-positive cells along the crypt-villus axis. No differences were observed in this proliferative marker between groups (**Supp. Fig. 2**). These data point towards the vital role of interleukin-22 in colon epithelium protection and intestinal barrier maintenance.

IL-22 Deficiency Initiates a Pro-Inflammatory Microenvironment

For all nine select inflammatory and anti-microbial genes analyzed, baseline differences between un-infected WT parental and IL-22KO mice were not observed (**Supp. Fig. 3**). Our data show that IL-22 is indeed an important innate immune cytokine, with brisk upregulation in *IL22* expression seen only one day after ETBF exposure in WT mice (**Fig. 2A**). As described above, the early damage sustained by the epithelium in the absence of IL-22 may promote increased mucosal exposure to luminal contents. We hypothesized that a transient or persistent interaction of this nature could initiate a more severe inflammatory response. Consistent with this hypothesis, we observed that ETBF-infected IL-22KO mice had increased expression levels of the genes encoding the pro-inflammatory cytokines IL-11 (day 3 and day 7) (p=0.03 and p=0.02, respectively) and IL-17A (day 7) (p=0.01), as determine by whole tissue qRT-PCR (**Fig. 2B and D**). After ETBF colonization, IL-22KO mice appeared to express normal levels of the antimicrobial genes, *RegIIIβ* and *RegIIIγ*, and the nitric oxide synthase gene, *NOS2* (**Supp. Fig. 4**) Furthermore, although IL-22 is a potent STAT-3 activator, IL-22KO ETBF-colonized mice continue to exhibit potent STAT-3 activation as measured by immunohistochemical staining (**Fig. 2C**). In both WT and IL-22-deficient mice, pSTAT-3 was detected in both

the colon epithelial cell and lamina propria compartments. This finding is demonstrative of the host's redundant mechanisms that contribute to STAT-3 signal transduction. For instance, ETBF-colonized mice deficient in IL-6, another known STAT-3 activator, also exhibited sufficient pSTAT-3 staining (**Supp. Fig. 5**). In our model, the notable increase of *IL11* expression, an IL-6 cytokine family member, may be compensatory in response to ETBF infection.

IL-22 Deficiency Promotes Adenoma Formation

Although IL-17 acts as a potent anti-microbial component of host defense, IL-17 has also been shown to be pro-tumorigenic[169]. Thus, we next evaluated the impact of IL-22 deficiency on microadenoma and macroadenoma development in Min IL-22KO mice. Fecal ETBF colonization was comparable between Min IL-22KO mice and Min parental controls (**Supp. Fig. 1**). Overall, only a modest, non-significant increase in microadenoma formation was observed 2 to 3 weeks after ETBF colonization of Min IL-22KO mice compared to ETBF-colonized parental Min mice (**Fig. 3A**). Yet, intriguingly, ETBF-colonized Min IL-22KO mice developed significantly more macroscopic tumors compared with ETBF-colonized parental Min mice from 1 to 3 months post-infection (overall p-value=0.0001) (**Fig. 3B-C**). Together, these data strongly suggest that the brisk IL-22 response to ETBF infection (**Fig. 2A**) acts to inhibit tumor development in response to ETBF infection.

Early (1 week) evaluation of distal colon tissue by qPCR from ETBF-colonized Min IL-22KO mice and matched Min parental controls showed that IL-17A and IL-11 inflammatory responses were specifically induced by the *B. fragilis* toxin, BFT;

colonization with an ETBF bft-mutant (Δbft-2) yielded gene expression levels comparable to sham mice (**Supp. Fig. 6**). Although *IL11* expression levels were similar between ETBF-colonized Min IL-22KO mice and Min controls, *IL17A* expression was significantly higher in the Min IL-22KO treatment group (**Supp. Fig. 6**). To evaluate possible differences within the tumor microenvironment in the presence or absence of IL-22, tumors were harvested from Min parental and Min IL-22KO mice to analyze gene expression through qPCR. However, no differences were observed in total tumor expression of *IL17A*, potent STAT-3 activators *IL6* and *IL11*, and nitric oxide synthase encoded by the *NOS2* gene (**Supp. Fig. 7**).

IL-22 Deficiency Promotes a Specific Myeloid Expansion

Flow analysis of lamina propria isolates from parental Min and Min IL-22KO mice colonized with ETBF for 1 week showed an expansion of myeloid subsets characterized by low MHC Class II expression (MAC2) (**Fig. 4**). Additionally, we observed a trend for the expansion in Min IL-22KO mice of what we are defining as 'MAC1' cells, characterized by MHC Class II high expression. The myeloid cells of interest were gated as $CD45^+/CD11b^+$, $Ly6C^-/Ly6G^-$, and MHC Class II high or low. Concurrently, the number of $CD103^+$ dendritic cells was significantly lower in the Min IL-22KO deficient mice. The number of polymorphonuclear (PMN) cells remained similar between both parental Min and Min IL-22KO mice (**Fig. 4**). The literature has defined two polarized macrophage types: 1) classically activated (M1) induced by GM-CSF, TNFα, and IFNγ alone or together with LPS and 2) alternatively activated (M2) induced by IL-4 and IL-13. M1 macrophages are typically viewed as pro-inflammatory

with high production of IL-1, TNFα, and IL-6 along with nitric oxide and reactive oxygen species and high antigen presentation capacity, whereas M2 macrophages are typically viewed as anti-inflammatory, with high production of IL-10 and involvement in homeostasis and cellular repair[170]. Until the function and phenotype of the 'MAC1' and 'MAC2' cellular populations from our mice can be established, no similarities can be drawn with the M1/M2 macrophage paradigm. We have sorted these cells for transcriptional analysis.

Discussion and Future Steps

Reports indicate that IL-22 can have dual roles, dependent on model and context, acting in ways that can be either protective or harmful to the host. In experimental models, multiple studies have shown that IL-22 acts as a host-protective cytokine in the gut, promoting preservation of the mucosal barrier through goblet cell maintenance and mucin production, production of antimicrobial peptides and chemokines, and epithelial cell regeneration[171]. For instance, directed, localized microinjections of IL-22 into the colons of mice protected them from a Th2-induced form of colitis through the ability to retain goblet cells and induce mucus production[172]. Additionally, IL-22 protected mice from colitis induced by murine pathogen, *Citrobacter rodentium*, likely through induction of regenerating islet-derived protein 3 (RegIIIβ and RegIIIγ) antimicrobial production[173]; a microbe related to human attaching and effacing pathogens, e.g. enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *E. coli*, *C. rodentium* intimately attaches to and causes effacement of host intestinal epithelial cells. In contrast, IL-22 has a documented capacity to induce intestinal pathology in a cell-transfer colitis model. In this model, mice

that received cell transfer from IL-22KO mice had reduced disease severity with diminished body mass loss, reduced colitis, and increased colon length[174]. Additionally, IL-22 production by ILCs has been shown to be a driver of invasive colon cancer in Rag-deficient mice infected with *Helicobacter hepaticus* and treated with azoxymethane (AOM)[161]. Further, the direct action of IL-22 to activate STAT-3, a signaling molecule implicated in numerous colitis-associated-cancer animal models and in CRC itself, portrays IL-22 as a promoter of aberrant cellular growth, detrimental to the host. Further *in vitro* studies by Ziesche et al. are consistent with these data, showing that IL-22 together with IFNγ can induce the production of reactive oxygen species (e.g. iNOS) typically associated with cancer, in human colon carcinoma cell lines[175].

In our ETBF model of colitis and colon tumorigenesis, it appears that IL-22 plays a protective role, likely combating pathogenic ETBF in its early stages of infection and contributing to epithelial barrier maintenance. In contrast to previous studies, we did not observe any early deficiencies in antimicrobial peptide or nitric oxide synthase gene expression in WT (non-Min) IL-22-deficient mice infected with ETBF (**Supp. Fig. 4**), nor did we observe any loss of STAT-3 activation (**Fig. 2C**) (**Supp. Fig. 5**). Competent STAT-3 activation was also observed in ETBF-infected IL-6-deficient mice (**Supp. Fig. 5**) suggesting that many redundancies exist in this crucial signal transduction pathway in the ETBF model. We hypothesize that increased damage to the epithelium in the absence of IL-22 promotes increased activation of the mucosal immune system, resulting in increased IL-17 and IL-11 responses that contribute to tumor initiation. In spite of the increased intestinal permeability and inflammatory burden observed in IL-22-deficient mice, the level of ETBF fecal and mucosal colonization remained similar between IL-22

KO mice and parental controls (**Supp. Fig. 1**). Furthermore, un-defined MHC class II[low] macrophage subtypes also accrue in the lamina propria of ETBF-colonized Min IL-22-deficient mice that may be pro-carcinogenic. We would like to understand the phenotype and function of these novel myeloid cell populations as well as the role of IL-22 in the regulation of these macrophage populations; it is suspected that mediation is through colon epithelial cells expressing IL-22RA1, possibly through control of chemokine or barrier function effectors.

Questions remain regarding the specific mechanism underlying increased adenoma development in the absence of IL-22. For instance, in the M1/M2 paradigm, M1-like macrophages are thought to be anti-tumorigenic due to their cytotoxic capabilities and improved antigen presentation leading to better immune surveillance, whereas M2-like macrophages are thought to promote tumors due to their immunosuppressive nature and pro-angiogenicity. Some studies have shown that the re-education of tumor microenvironment (TME) M2-like macrophages into M1-like macrophages leads to improved anti-tumor immunity and tumor growth reduction[176]. One hypothesis is that IL-22 deficiency promotes the accumulation of specific macrophage populations whose primary function entails epithelial tissue remodeling and repair (M2-like); the presence of such cells could promote an increase in aberrant cellular growth. To address this hypothesis, myeloid-derived 'MAC1' and 'MAC2' cell subsets have been sorted from one week ETBF-infected Min IL-22KO and parental Min control mice. To characterize the phenotype and function of these populations, these cells will be analyzed for mRNA expression of key genes of interest: pro-inflammatory cytokines, chemokines, reactive oxygen species, and cell proliferative markers, using high-throughput qRT-PCR

array plates. Furthermore, it will be exciting to uncover any links existing between IL-11/IL-17 expression and recruitment of these macrophage subsets. Understanding the interplay between the enhanced epithelial cell shedding (**Fig.1A & B**) and permeability (**Fig. 1C**) with macrophage recruitment will also be crucial. In response to cell damage and altered intestinal barrier function, are specific macrophages called to repair the damage, and in the case of ETBF, is this a case of a good response gone bad?

To further probe this hypothesis of 'pathogenic' or tumor-promoting macrophages in ETBF disease, murine macrophage depletion experiments will also be conducted. Previous attempts at colonic macrophage depletion by administering commercially available chlodronate-containing liposomes were unsuccessful. CD11b-DTR mice (official designation: FVB-Tg(ITGAM-DTR/EGFP)34Lan/J) were recently obtained from Jackson Laboratories and crossed into the Min background. Future studies will utilize diphtheria toxin-mediated depletion of macrophages during ETBF infection, and the effect on adenoma formation in parental Min and Min IL22KO mice will be analyzed.

Main and Supplemental Figures/Tables (in order of first reference)

Supplemental Figure 1: IL-22 deficiency does not modify ETBF colonization.
After being placed on antibiotic water, ad libitum, for 5 to 7 days, WT and IL-22KO mice and parallel Min mice received orogastric treatment with ETBF. **A** At respective time points, feces were collected, homogenized, and cultured for determination of *B. fragilis* colony-forming units (CFU) per gram of stool. Colonization was similar between WT and IL-22KO mice. **B** At sacrifice, ranging from 7 to 28 days p.i., colon tissue from WT and IL-22KO mice was pulverized and cultured to quantify tightly adherent ETBF (CFU per gram of tissue). Aggregate data is presented. ETBF mucosal adherence was comparable between WT and IL-22KO mice.
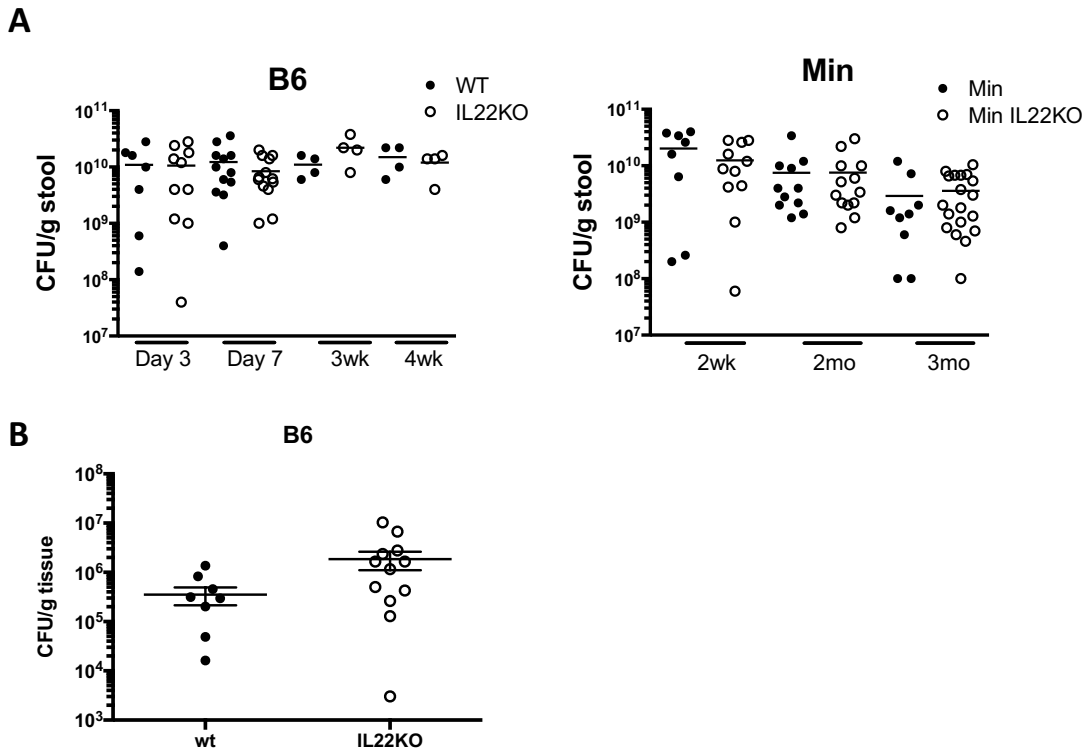
Figure 1: IL-22-deficient mice exhibit severe epithelial cell damage and intestinal permeability. WT and IL-22KO C57BL/6 mice were inoculated with ETBF and sacrificed 1, 3, and 7 days afterwards. H&E-stained FFPE colon tissues were analyzed for histopathology. Bars = Mean $\pm$ SEM. Data are from 2 replicate experiments. **A** Representative histopathology, displaying severe epithelial cell shedding (anoikis) in IL-22KO mice versus WT controls. Red arrow depicts characteristic cell rounding and detachment, indicative of apoptosis. **B** Disease scores of colon anoikis. At an early time-point (day 3), IL-22KO mice exhibit a more severe anoikis score than WT controls. **C** Intestinal permeability as measured by FITC-dextran concentrations in the serum. ETBF-infected IL-22KO mice exhibit increased intestinal permeability at day 3 p.i. **D** Disease scores of colon hyperplasia and inflammation are comparable between WT and IL-22KO mice, generally increasing over the course of infection.
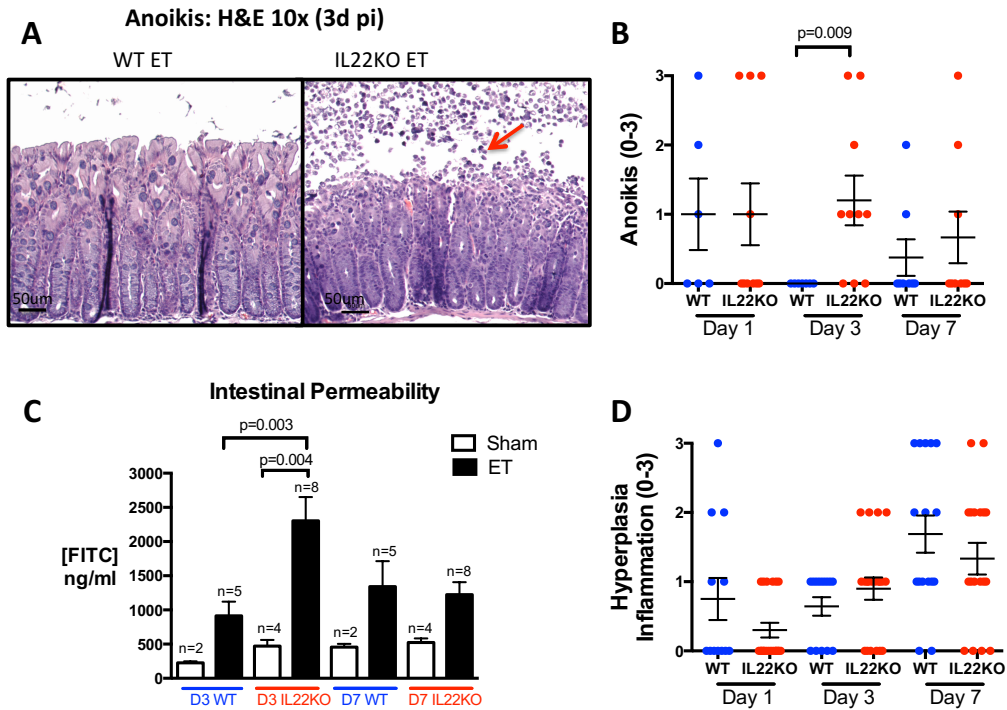
Supplemental Figure 2: Epithelial cell proliferation is comparable between WT and IL-22-deficient mice. Mice received bromodeoxyuridine (BrdU) by intraperitoneal injection 24 hours prior to sacrifice (3 days after ETBF inoculation). The number of BrdU-positive cells and migration distance along the crypt-villus axis are comparable between WT and IL-22-deficient mice.
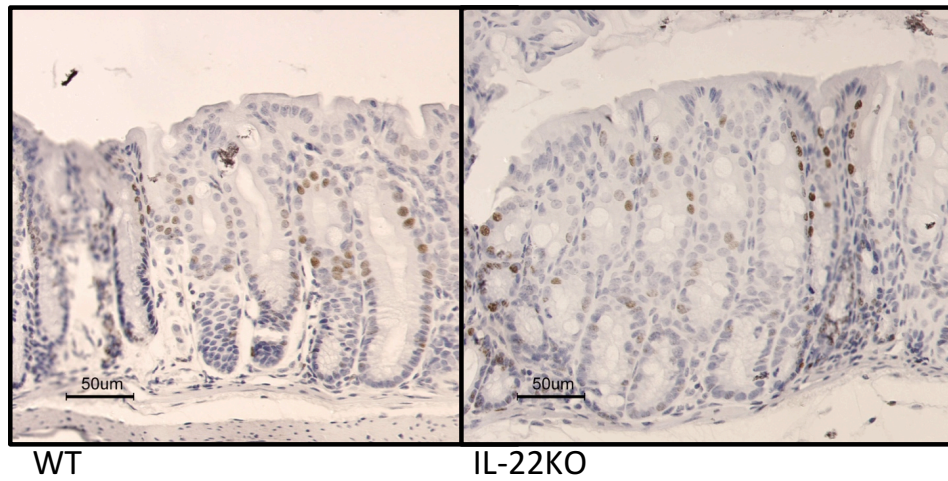
WT   IL-22KO

Supplemental Figure 3: Baseline gene expression in un-infected WT and IL-22-deficient mice. Normalized Ct values were compared between Sham (PBS-treated) WT and IL-22KO mice. Bars = Mean. Differences in baseline gene expression were not detected.
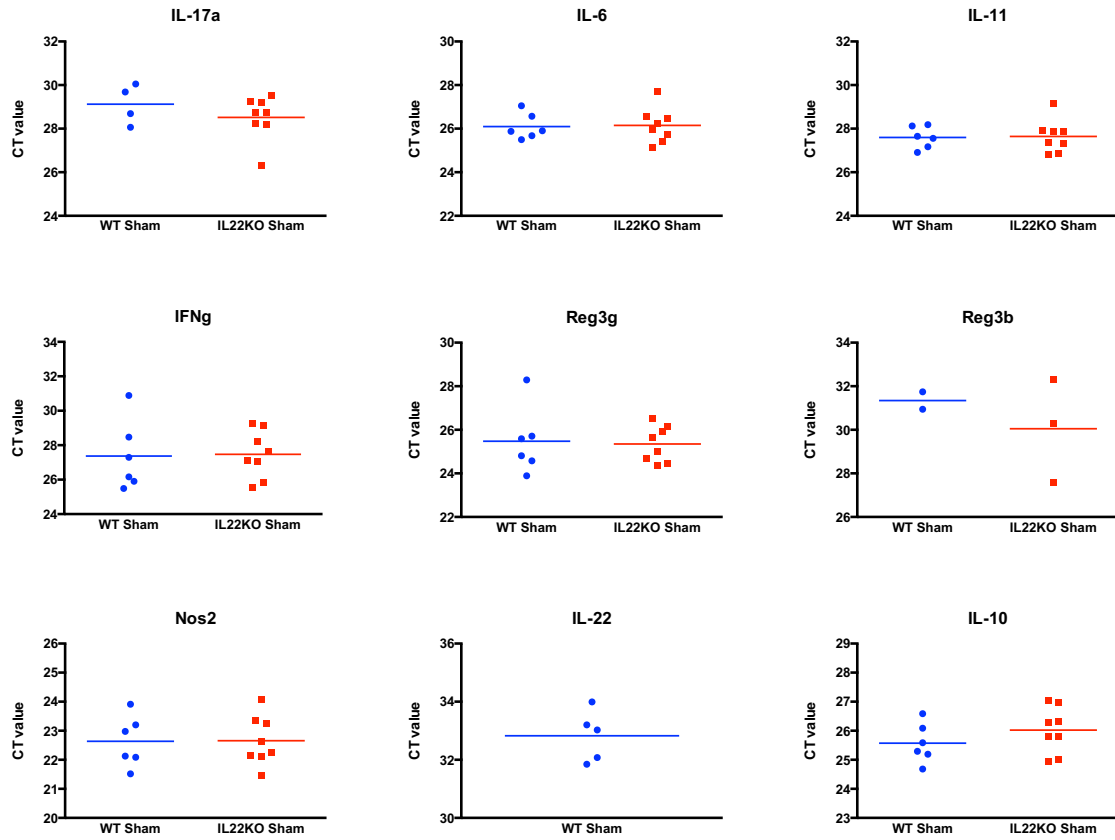
Figure 2: Early cytokine expression in IL-22-deficient mice. ETBF-inoculated WT and IL-22KO C57BL/6 mice, analyzed at days 1, 3, and 7 p.i.. Distal colon tissue (2nd cm piece in from the most distal colon region) was harvested for whole tissue qRT-PCR. Data are presented as fold expression over sham. Bars = Mean $\pm$ SEM. Data are from 2 replicate experiments. **A** In WT mice, *IL22* is most highly expressed immediately after infection. *IL22* expression was not detected (ND) in any IL-22-deficient mice. **B** *IL11* continues to be expressed more highly in IL-22KO mice at days 3 and 7 p.i.. **C** pSTAT-3 IHC staining shows comparable STAT-3 activation between IL-22KO mice and WT controls in both epithelial and lamina propria compartments. **D** *IL17A* expression increases over time in IL-22KO mice.
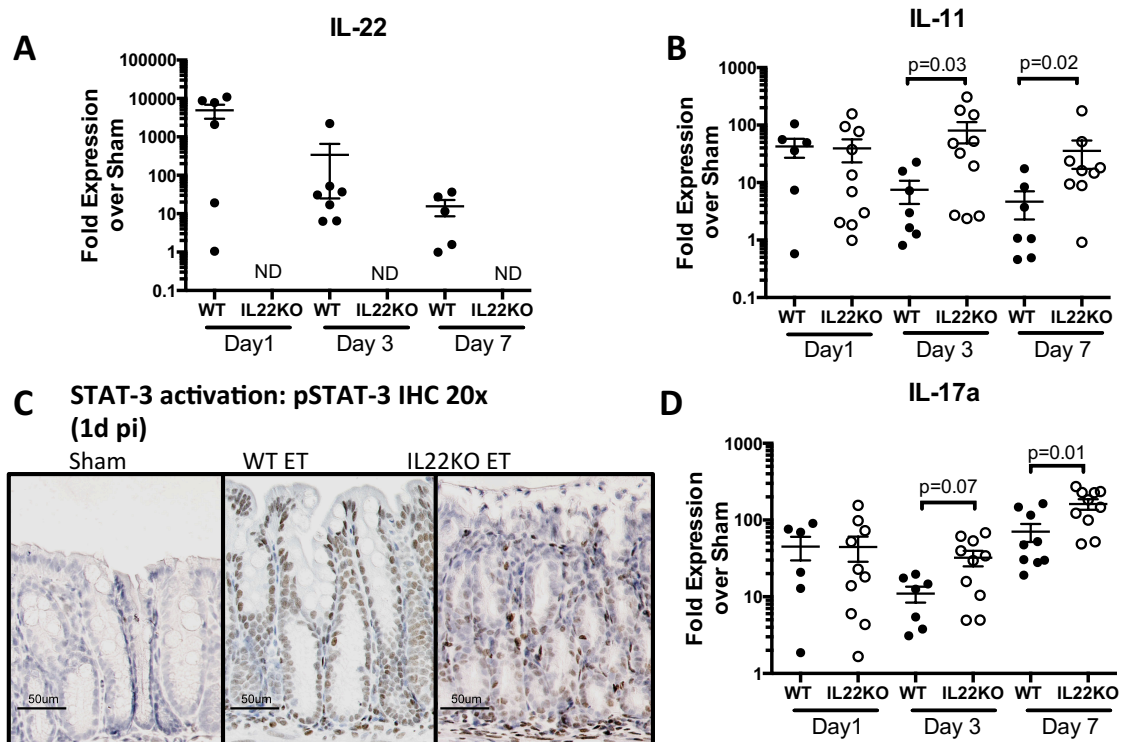
Supplemental Figure 4: Additional gene expression analysis of WT and IL-22-deficient mice. Baseline *RegIIIβ*, *RegIIIγ*, and *NOS2* expression was analyzed in ETBF-colonized WT and IL-22KO mice. Bars = Mean. Expression of these genes was comparable between groups across all time points.
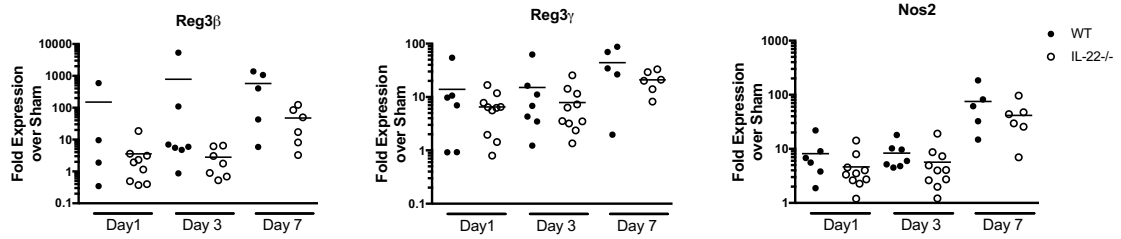
Supplemental Figure 5: pSTAT-3 detection is comparable among control, IL-6-deficient, and IL-22-deficient mice. ETBF-colonized mice were sacrificed at 1 week (WT mice) and 12 weeks (Min mice). FFPE tissue sections were stained for pSTAT-3 and representative images were taken with 10X objective lens. pSTAT-3 detection was similar among control, IL-6KO, and IL-22KO ETBF-infected mice.
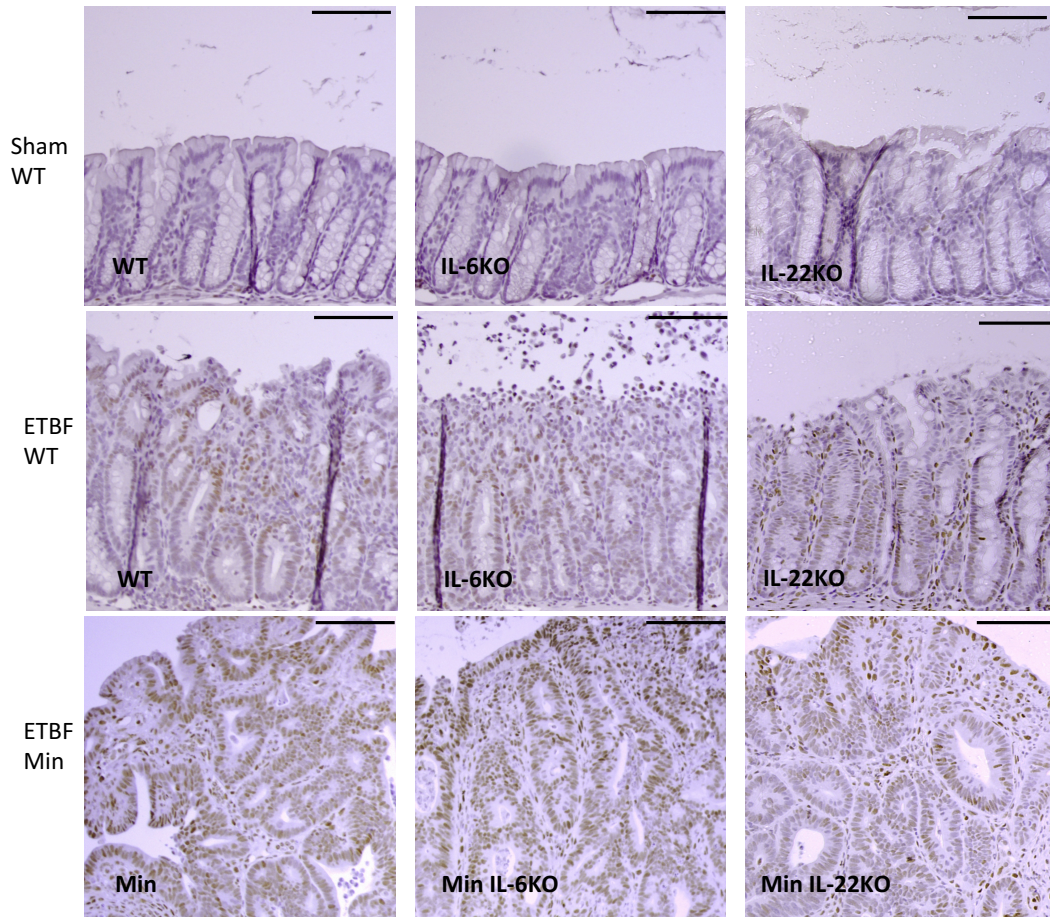
Figure 3: IL-22-deficient mice exhibit a higher adenoma burden. Adenomas were quantified in WT and IL-22KO C57BL/6 Min mice after ETBF inoculation. Bars = Mean $\pm$ SEM. Data are from 2 to 3 replicate experiments. **A** H&E colon sections displaying characteristic microadenoma morphology. Quantification [...] individual mice observed at 2 to 3 weeks p.i.. A significan[...] was not observed in ETBF-colonized Min IL-22KO mice [...] Images of representative methylene blue-stained distal co[...] IL-22KO mice display increased macroadenoma developm[...] Min mice. Tumor counts are recorded for the entire colon[...] macroadenomas from individual mice. Min IL-22KO mic[...] macroadenoma burden over the course of infection compa[...]

A

Min   Min IL-22KO

B
ETBF tumorigenesis after 3 months in Min mice
(distal colon)

Min   Min IL22KO

C   Tumor Counts

Overall:
p=0.0001

98
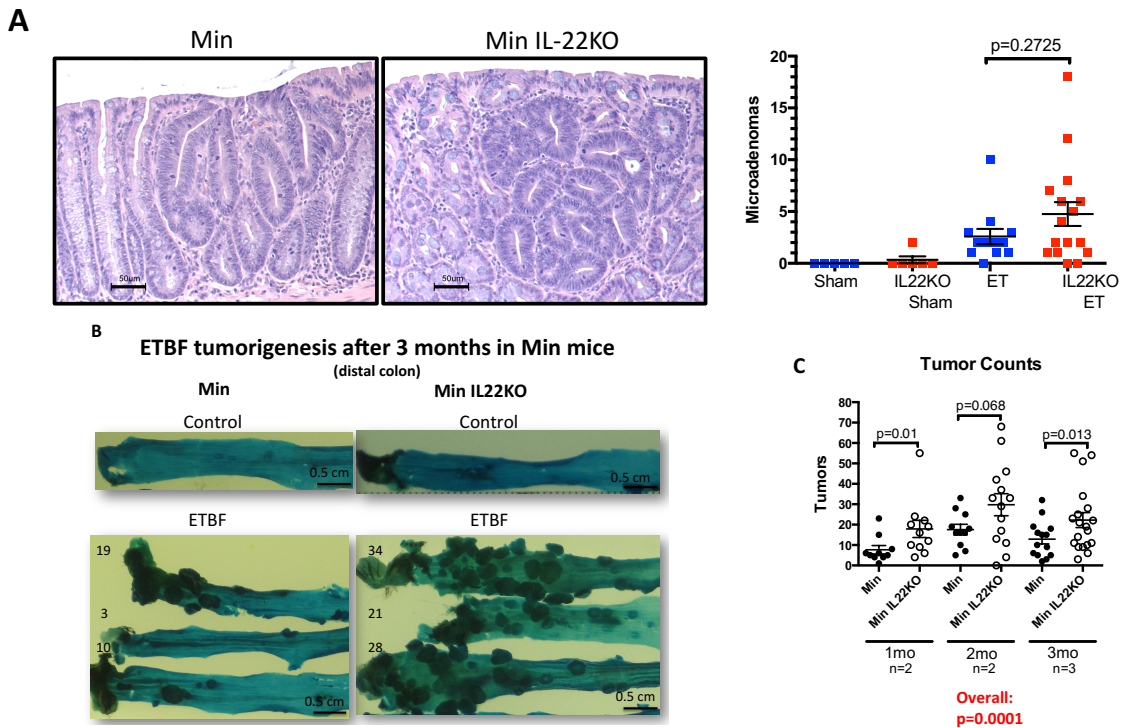
Supplemental Figure 6: BFT induces the inflammatory response in Min mice.
Parental Min and Min IL-22KO mice were colonized with either Δbft-2 ETBF mutant or
parental ETBF bacterial strains and sacrificed 1 week later. Distal colon tissue was
evaluated for cytokine expression by qPCR. *IL17A* and *IL11* gene expression was
increased in mice colonized with parental ETBF compared with Δbft-2 ETBF; BFT is
required for induction of the inflammatory response. ETBF-colonized Min IL-22KO
mice exhibit increased *IL17A*, but not *IL11*, expression compared with parental Min
controls.
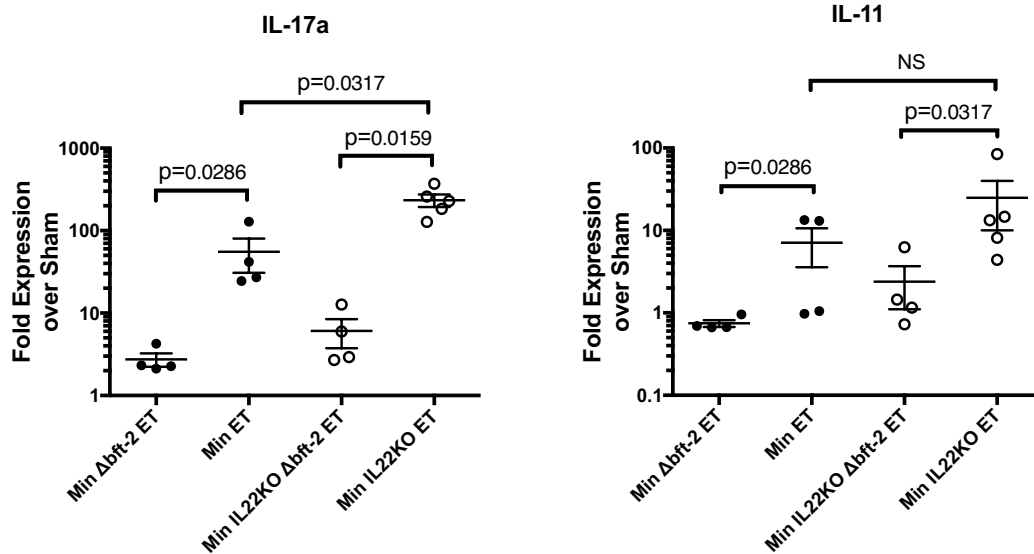
Supplemental Figure 7: Gene expression in IL-22-competent or IL-22-deficient tumors. Tumors were harvested from ETBF-infected Min parental and Min IL-22KO mice and gene expression assessed by qPCR. Data are presented as change in fold expression compared to ETBF-infected Min parental mice. Bars = Mean. Tumors from ETBF-colonized parental Min and Min IL-22KO mice display similar expression levels of *IL17A*, *IL6*, *IL11*, and *NOS2*.
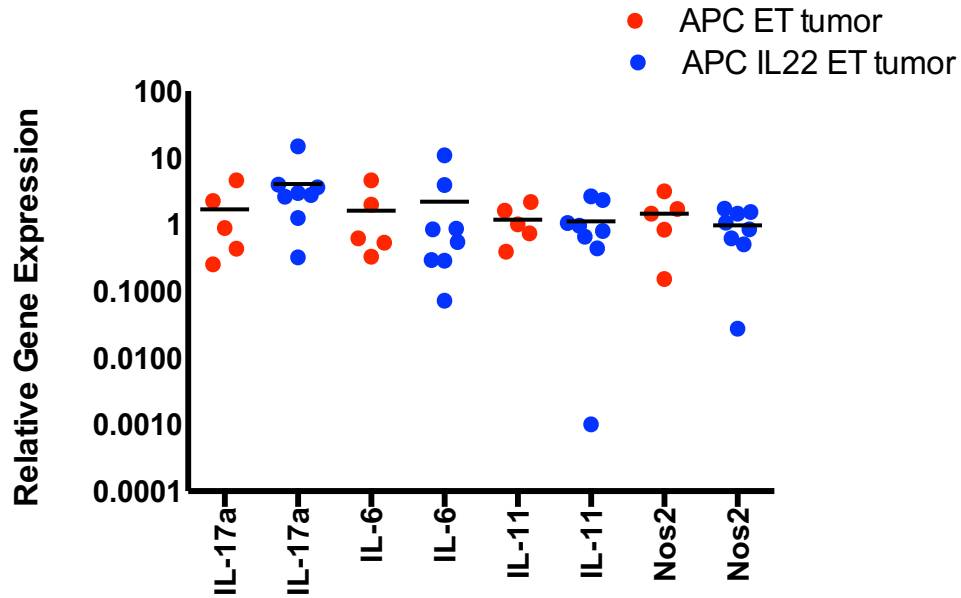
Figure 4: Expansion of specific myeloid subsets occurs in IL-22-deficient mice. The lamina propria immune cells of C57BL/6 parental Min and Min IL-22KO mice were analyzed by flow cytometry 1 week after ETBF inoculation. Data are presented as total cell number normalized by colon tissue mass from which the cells were isolated. Bars = Mean $\pm$ SEM. Data are from 2 replicate experiments. red = expt 1; black = expt 2. Live cells were gated as CD45$^+$/CD11b$^+$, Ly6C$^-$/Ly6G$^-$, and either MHC Class II high or low. Min IL-22KO mice show a trend of increased 'MAC1' (Class II^{high}) cell accumulation and a significantly increased 'MAC2' (Class II^{low}) cell accumulation in comparison with Min controls. The number of polymorphonuclear (PMN) cells is comparable between groups. Min IL-22KO mice show a significant decrease in the number of CD103$^+$ dendritic cells.
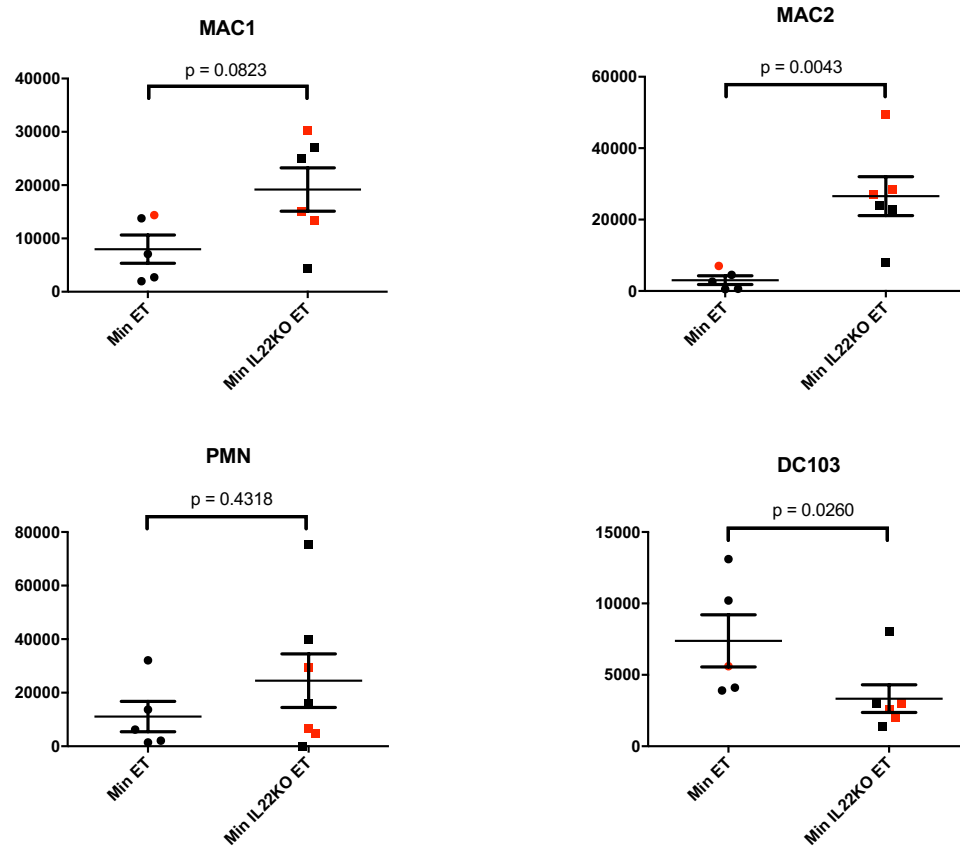
CHAPTER 4 - Bacterial induction of immune homeostasis: a clinical and historical context

The Clinical Picture

Studying the impact of the gut microbiota on the host immune response, and vice versa, has given us more insight into how intestinal homeostasis is established and how extensive co-evolution has shaped both systems. Understanding these interactions also has immense potential for clinical application. Within the last quarter century in the United States, the number of newly discovered or synthesized antibiotics has been steadily decreasing. A striking depiction of this can be seen through evaluating the number of "systemic antibacterial new molecular entities" approved by the U.S. Food and Drug Administration (FDA) in 5-year increments of this time period (**Fig. 1**)[177]. For instance, from 1983-1987, 16 new antibacterial agents were approved; from 1993-1997, 10 new agents were approved and from 2008-2012, only a mere 2 new agents had been approved. Numerous reasons are cited for the decrease in antibiotic development, mainly dependent on lack of financial incentive for drug companies. For one, antibiotics typically have less of a return on investment than do other drugs[178]; this is due to their relative success as a treatment, requiring only short-course administration while typically curing the disease. This is in contrast to medications used in the treatment and management of long-term, chronic conditions, which are typically used for a lifetime. Additionally, the general consensus by the scientific community to limit the use of broad-spectrum antimicrobials coupled with the lack of formal FDA guidelines for the testing of new antibiotic agents, further deters their development[177]. It is clear that antibiotic

alternatives should be developed. In the context of intestinal conditions and diseases, one avenue of treatment may be through manipulation of the gut microbiome. This could come through the form of prebiotics (food sources promoting beneficial organisms), probiotics (beneficial live organisms), or synbiotics (combination of pre- and probiotics).
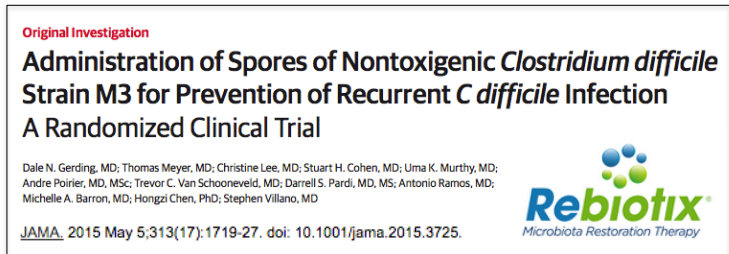
Figure 1: **Systemic antibacterial new molecular entities approved by the FDA**[177]

Total Number of New Antibacterial Agents
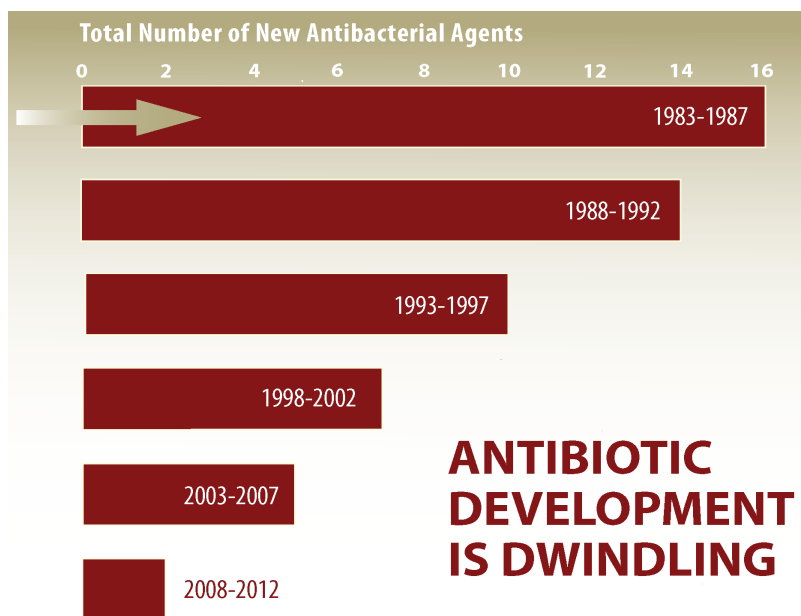
The utility of probiotics or live microorganisms in human intestinal health is currently being explored. Intriguingly, Dale Gerding and colleagues recently conducted a phase 2 randomized double-blinded, placebo-controlled (RDBPC) trial using the obligate anaerobe, nontoxigenic *Clostridium difficile* (NTCD), to prevent recurrent *C. difficile* infection (CDI) (**Fig. 2**). A major cause of nosocomial antibiotic-associated diarrhea, toxigenic *C. difficile* is a significant cause of morbidity and mortality in the United States, found to have caused ~ 453,000 infections and causing 29,000 deaths in 2011[179]. As a spore-former, resistant to conventional cleaners and alcohol-based hand washes, *C.*

difficile is highly contagious with high rates of recurrent infection. In the study, it was

found that oral administration of NTCD spores in humans was well-tolerated and safe

February 19, 2016
W
ORAGENICS

Oragenics Receives New Patent for Improved Replacement Therapy for Dental Caries

F ...eatment for recurrent CDI[180]

Original Investigation

Administration of Spores of Nontoxigenic *Clostridium difficile* Strain M3 for Prevention of Recurrent *C difficile* Infection
A Randomized Clinical Trial

Dale N. Gerding, MD; Thomas Meyer, MD; Christine Lee, MD; Stuart H. Cohen, MD; Uma K. Murthy, MD; Andre Poirier, MD, MSc; Trevor C. Van Schooneveld, MD; Darrell S. Pardi, MD, MS; Antonio Ramos, MD; Michelle A. Barron, MD; Hongzi Chen, PhD; Stephen Villano, MD

JAMA. 2015 May 5;313(17):1719-27. doi: 10.1001/jama.2015.3725.

Rebiotix®
Microbiota Restoration Therapy

- **Phase 2 RDBPC**
- Oral NTCD-M3 spores well-tolerated & safe
- Reduce CDI recurrence

Convergence of Colorectal Cancer Risk Factors

With notable differences (noted in Chapter 1) in the gut microbiota of CRC

patients compared with healthy individuals, this may suggest that directed microbiome

alteration may be beneficial in this disease. Numerous risk factors have been identified in

CRC development. Most CRC cases are sporadic, meaning non-hereditary (~75%), and

other cases are hereditary. Risk factors that may initiate and/or promote CRC include age

(typically >50 years old), physical inactivity, obesity, diet (increased consumption of red

and processed meats; diminished consumption of fruits, vegetables, and whole grain

fibers), smoking, heavy alcohol use, and history of IBD (**Fig. 3**)[181]. There may also be

underlying genetic factors affecting the development of certain CRC "environmental"

risk factors, e.g. obesity/BMI[182], diet[183], and physical activity/lifestyle[184,185]. In turn, these

combined factors, along with host-defined immune responses and gut architecture, impact

the microbiome composition of the gut[186]. Dictating microbial stability and dysbiosis, these elements may influence overall disease burden in patients. Furthermore, in CRC, it can be imagined that a host of acute triggering events such as antibiotic usage, foodborne illness, parasitic infection, etc., may also tip the microbiome balance in one direction or the other, influencing the burden and action of potentially oncogenic pathobionts.

Figure 3: Colorectal cancer risk factors
The interplay of host genetics with numerous factors, including diet, lifestyle, obesity, and history with IBD likely all converge to impact an individual's risk of colorectal cancer. Genetics also likely impact the composition of the gut microbiome, influenced by single microbes and the consortium of the microbial community. Gut microbiome composition/dysbiosis itself may also, in turn, impact diet, lifestyle, and the development of obesity and IBD. (Figure generated by June Chan)
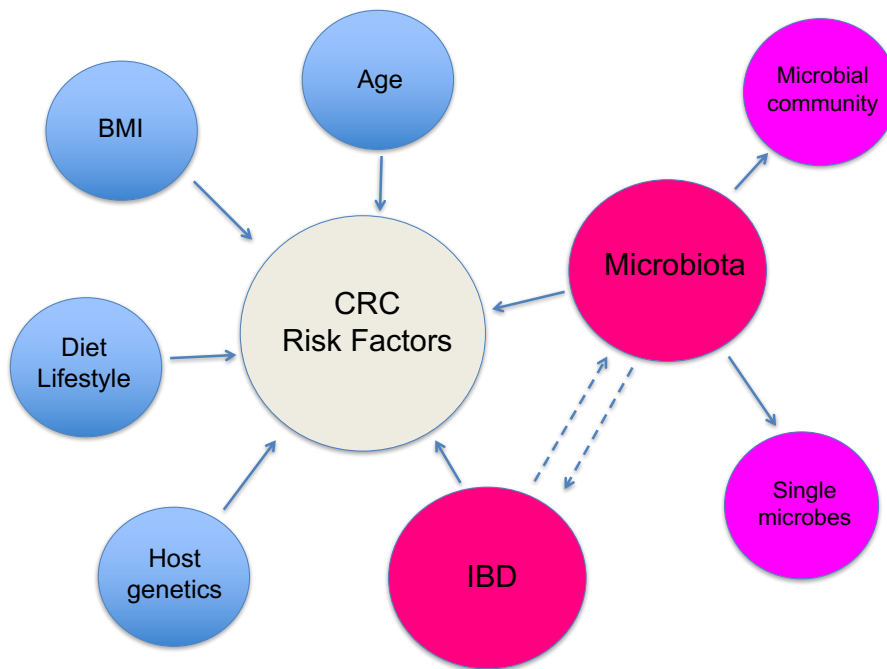
Historical model of PSA induction of immune tolerance

Providing the basis for the development of microbial-based treatments and therapies, commensal bacteria have been implicated in the induction of innate and adaptive mucosal immunity. Utilizing experiments with germ free mice, commensal gut bacteria have been reported to stimulate synthesis of angiogenin-4 (a potent bactericidal protein), promote B cell IgA production, intestinal epithelial lymphocyte recruitment, and lymphoid structural development, e.g. Peyer's patches[187]. Considering the complex interplay of risk for CRC and other inflammatory intestinal conditions, such as IBD, the use of probiotic microbes has been hypothesized as a means to disrupt disease progression or to induce disease mitigation.

In the example of anaerobic commensal bacterium, *Bacteoides fragilis*, covered extensively in chapter 2, NTBF has been reported to induce mucosal immune tolerance and to even reduce inflammation in various murine colitis models[136]. One way that the host immune response may contribute to IBD is through secreted inflammatory mediators by pathogenic Th17 cells, likely promoting ongoing mucosal inflammation in patients[188]. A culmination of studies since the early 2000s have been integrated into a model hypothesizing the ability of NTBF to disrupt the accumulation of pathogenic Th17 cells. This model poses that NTBF PSA, provided through outer membrane vesicles (OMVs), is taken up by dendritic cells (DCs) in a TLR-2 dependent manner. Further DC Gadd45α signaling and IL-10 production initiates responses from Tregs that may suppress pathogenic T cell responses, likely through IL-10 (**Fig. 4**). Shen et al. recently demonstrated that PSA OMVs administered orally or rectally could prevent experimental colitis in mice[145].

Figure 4: Polysaccharide A (PSA) is reported to promote tolerance and suppress inflammation (Figure adapted from MJ Kuehn[189])

Polysaccharide A (PSA) is reported to promote tolerance and suppress inflammation

Meta J. Kuehn
Cell Host & Microbe 2012
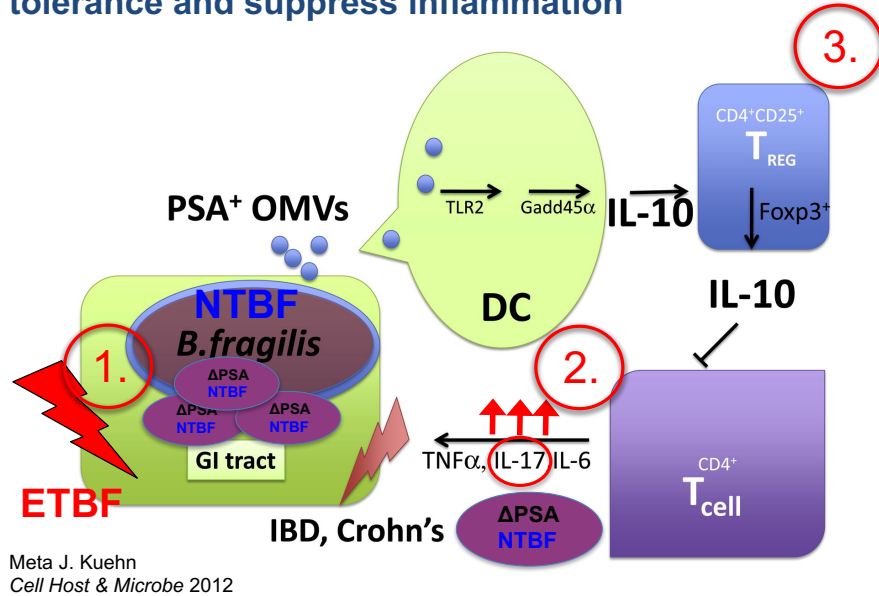
In summary, the previous literature reported several key findings that our study also addressed. First, it was reported that ΔPSA NTBF exhibited a deficiency in the mucosal colonization of GF mice compared with mice colonized with WT NTBF (**Fig. 4 #1**)[130]. Second, researchers reported that colonization of GF mice with ΔPSA NTBF induced a robust expansion of inflammatory Th17 cells, an outcome that was reversed when mice were colonized with WT NTBF or ΔPSA NTBF-colonized mice were treated with purified PSA (**Fig. 4 #2**)[130]. Third, the colonization of GF mice with WT NTBF or treated with purified PSA induced a significant expansion of IL-10 producing regulatory T cells (**Fig. 4 #3**)[129]. Fourth, it was reported that *B. fragilis* colonization resistance was absolute. For example, it was reported that if one NTBF strain had already colonized a

GF mouse, a secondary labeled challenge NTBF strain was unable to establish[138].

However, in our experiments, we demonstrated no difference in mucosal colonization

between WT and ΔPSA NTBF in both SPF and GF mice. We did not observe an

expansion in Th17 cells in the absence of PSA, nor did we observe an accumulation of

Tregs in the presence of PSA. However, there were some points of overlap with previous

studies. We found that NTBF could protect from experimental colitis induced by ETBF.

We also found that although *B. fragilis* species colonization resistance was quite robust,

nonetheless, the phenotype was more partial than previously reported (**Fig. 5**).

Figure 5: Key similarities and differences with previous literature reports

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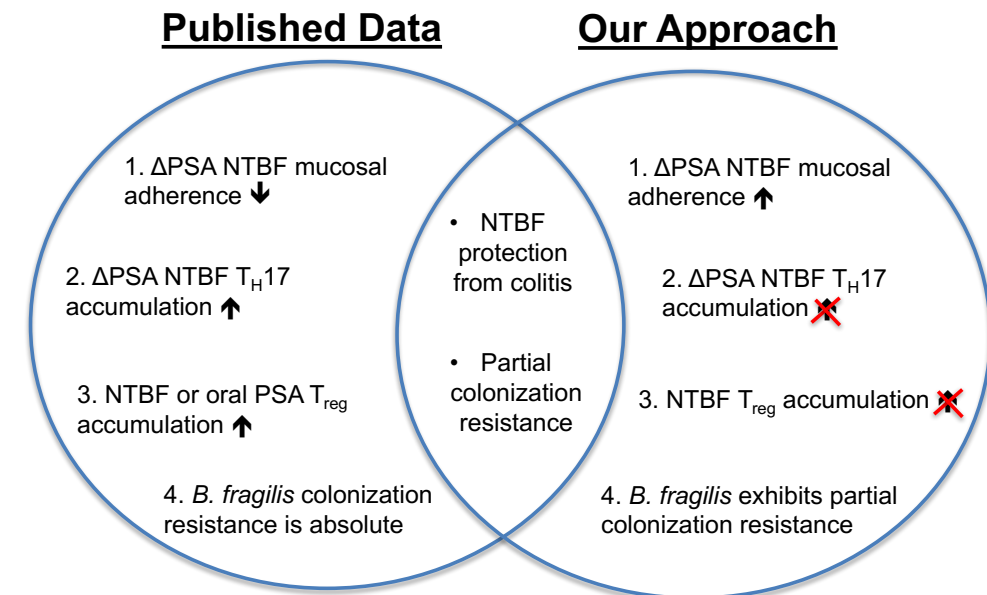
Microbiome-Host Interactions and Shifting Paradigms

The complex crosstalk between microbe and host is an important factor in determining the overall mucosal immune response. Historically, researchers have focused their efforts on identifying microbial species that have the ability to promote human health, i.e. emphasis placed on the effects of microbe-to-host. Although this type of focus has been incredibly instructive, this may only partially describe the complex nature of the microbiome. In contrast to the conventional view of microbe-to-host directed interactions, the surprising findings presented here, describing a conditional NTBF-mediated protection from ETBF pathogenesis independent of enhanced NTBF-induced immunosuppression (Chapter 2), suggest that more emphasis should be placed on understanding specific host-to-microbe or microbe-to-microbe directed interactions, i.e. emphasis placed on the evolutionary and ecological hurdles that microbes encounter[190]. These challenges could include selective factors coming from the host side, e.g. the immune system; it could also include the competition encountered with other microbes in the environment. Recent inferences made from applying evolutionary theory to mammalian microbiome data suggest that the microbiome may evolve as 'an ecosystem on a leash'[190]. In other words, while evolution of the microbiota is driven by the need to compete and persist within the host, the living host, itself, is under its own natural selective pressures to shape a beneficial microbiome (**Fig. 6**). Thus, the microbiome is a dynamic ecosystem of its own that is led by the host with a rapidly changing 'leash'.

Figure 6: Ecosystem on a leash[190]

When a host encounters diverse and beneficial microbes, evolutionary theory predicts that microbiome evolution is driven by persistence in the host, while the host attempts to control the microbiota. black arrows = interactions within the microbiota; red arrows = mechanisms of control.
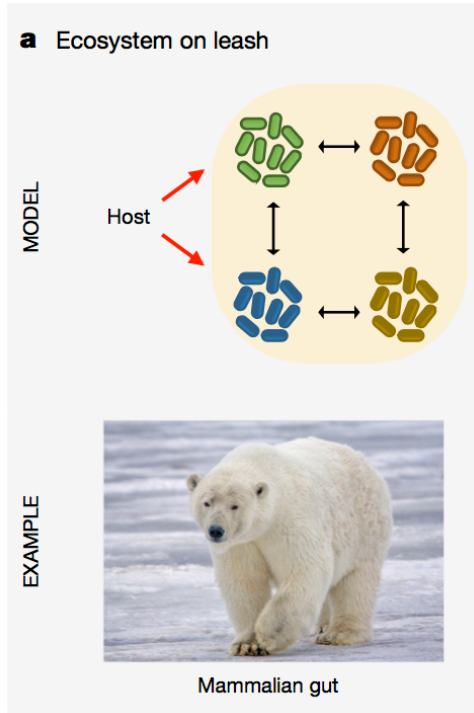
Consistent with this proposed model of microbiome development, our data seem to also prioritize the importance of microbe-to-microbe interactions in determining disease outcome. The ability of NTBF to interact with and establish on the colonic mucosa predisposed mice to protection from ETBF (NTBF→ETBF mice), likely due to the NTBF occupation of the *B. fragilis* ecological niche (colonization resistance). Yet, timing and competition interactions were also important as ETBF dominated the infection and disease trajectory in NTBF+ETBF mice.

In the broader context of microbiome harnessing as a potential treatment, it may be more advantageous to understand how microbes compete with one another through

metabolism, adhesion, or inhibitory compounds, than to focus solely on how symbionts directly affect us[190]. What would be the purpose of harnessing beneficial microbes if they cannot be integrated into the host? And can probiotics really work as one size fits all? Further understanding of what makes a stable microbiome susceptible to invasion would also be key. Additionally, even without direct impact on the host, there may be symbionts that lend benefit to the entire microbial community; it would be helpful to understand these interactions. On the one hand, the study of specific microbes in isolation, for instance in germ free animals, is essential to creating a simplified system from which molecular mechanisms can be identified. On the other hand, the results of isolation studies may not represent the overall nuanced outcome in a complex mammalian host. I propose that we begin to study the beneficial microbiome through both arms, in isolation and together as a microbial community.

CHAPTER 5 - Concluding Remarks

Results Summary

In this work, an in-depth study of the putative probiotic organism, NTBF, was undertaken. We showed that prophylactic treatment with NTBF could restrict the colonic establishment of pathogenic ETBF; this protection was independent of NTBF-encoded polysaccharide A (PSA). The levels of NTBF and ETBF in co-infected mice were quantified, and murine disease burden was analyzed. We found that sequentially treated mice (NTBF→ETBF) exhibited a pronounced reduction in ETBF colonization burden. The mice also displayed diminished colonic inflammation and tumor burden compared with ETBF-only controls. However, NTBF protection from ETBF was not absolute, as the *IL17A* expression in NTBF→ETBF mice remained significantly higher than NTBF-only controls, demonstrating the limitations of bacterial colonization resistance. Key findings of both ETBF dominance during simultaneous treatment (NTBF+ETBF) of mice and ETBF secretion of anti-bacterial inhibitory molecules allude to the complexity of gut microbial interactions and community development. Understanding these interactions better may further gut microbiome manipulation to promote health and wellness in the clinic.

Furthermore, we demonstrated that IL-22, an immune signaling molecule, plays a host-protective role during murine ETBF infection. Functional IL-22 in the mouse promoted intestinal barrier function and mucosal epithelium maintenance. IL-22 signaling also appeared to prevent a severe inflammatory response, characterized by diminished levels of *IL17A* and *IL11* gene expression. In the absence of IL-22 (IL-

22KO), ETBF-colonized mice developed a higher tumor burden compared with WT (IL-22-competent) controls. The mechanism by which IL-22 prevents adenoma formation is yet to be elucidated. We hypothesize that in the absence of IL-22, the accumulation of MHC Class II low macrophages drives epithelial cell proliferation, promoting tumor development.

The 'Good Bugs' and How to Study Them

Dating back to 1908, the idea that specific bacterial species or strains in their live form could be used to promote health and wellness started to become a burgeoning topic of interest. Nobel laureate, Élie Metchnikov, spent the latter years of his career studying specific long-lived human populations in Bulgaria that consumed large amounts of yogurt containing lactic acid-producing *Lactobacillus* bacteria[191]. In the 1930s, Minoru Shirota, a Japanese physician, successfully isolated and cultured the eponymous *Lactobacillus casei* strain Shirota, resistant to gastric and bile acids, and in 1935, developed the Yakult probiotic drink incorporating this strain that is still widely used today[191]. Since then, the probiotic market has exploded worldwide with $30 billion in annual sales. However, as Dr. Eric Pamer puts it, "…there is little solid evidence in humans of their effectiveness in enhancing health, promoting longevity, or reducing infections"[192]. For truly evidence-based approaches, the next-generation of probiotic organisms will require more rigorous study and analysis, culminating in the clinical evaluation of their safety and effectiveness.

The study of individual microbes has provided some insight into how commensals may promote intestinal health. For instance, one of the most widely studied and used probiotic strains, *Lactobacillus rhamnosus* GG (LGG), is thought to promote intestinal

homeostasis through its strong pilus-mediated intestinal adherence capabilities preventing pathogenic colonization; further production of immune effectors, anti-apoptotic proteins, and antimicrobials by LGG may also play a role[193]. Other probiotic formulations come as multi-species cocktails, such as VSL#3, thought to suppress intestinal inflammation through acetate production and tissue reductions in IFNγ, TNFα, and iNOS[194]. Pioneering research conducted by Smith et al. has shed light on the importance of short chain fatty acids (SCFAs) (e.g. acetate, butyrate, and propionate), bacterial fermentation products of dietary fiber, in gut health[195]. On a mechanistic level, the authors show that SCFAs have direct receptor-mediated interactions with colonic Tregs through GPCR43, inducing Treg expansion, Treg suppressive activity, and protection from colitis all likely mediated through histone deacetylase inhibition[195]. Furthermore, butyrate has been implicated in protection from CRC development, with one animal study showing that AOM/DSS-treated mice colonized with butyrate-producing bacteria and fed a high fiber diet exhibited diminished colon tumor development[196]; other studies suggest a role for butyrate in colon motility promotion, apoptosis induction, inflammation reduction, and inhibition of tumor cell progression[197]. In addition, obligate anaerobes noted to be in low abundance in dysbiotic states have also emerged as possible novel probiotics. For instance, *Faecalibacterium prausnitzii*, a dominant member of the healthy adult intestinal microbiota, has distinct low abundances in cases of irritable bowel syndrome (IBS), IBD, CRC, and celiac disease[198]. Preliminary data from *in vitro* and animal models suggest that treatment with *F. prausnitzii* bacteria or culture supernatants can diminish colitis through butyrate production and secretion of anti-inflammatory metabolites[198]. In another example, Buffie et al. showed through sequencing and mathematical modeling that the

presence of *C. scindens*, another *Clostridium* species, was correlated with strong *C. difficile* inhibition in both humans and mice[199]. As proof of concept, colonization by a human *C. scindens* isolate diminished *C. difficile* infection and disease severity in mice. The investigators showed that *C. scindens* was able to convert primary bile acids into secondary bile acids, deoxycholate and lithocholate, which are inhibitory to *C. difficile* vegetative cell growth, leading to infection resistance[199]. A simplified summary of several proposed beneficial bacteria under study is presented in **Table 1**. Furthermore, recent research indicates that destruction, specifically of the indigenous obligate anaerobes of the gut, contributes to the expansion of pathogenic bacteria, demonstrating their vital role in colonization resistance[200,201]. It is likely that researchers will begin to focus their efforts more specifically on these strict anaerobes.

Although technical challenges exist in studying interactions between bacteria requiring strict anaerobic conditions and human colonocytes requiring aerobic conditions, recent advancements in the development of dual-environment co-culture models will allow for better study of these mechanisms. For instance, the 'apical anaerobic co-culture model', developed by Ulluwishewa et al., allows for the study of anaerobic bacteria in direct contact with human cell lines for up to 12 hours[202]. Used in an anaerobic workstation, this device has an upper chamber containing anaerobic medium for obligate anaerobes and a lower chamber containing aerobic medium, separated by a microporous membrane. Human cell lines are seeded on top of the membrane with the diffusion of oxygen occurring from the aerobic medium underneath. Due to built-in electrodes, trans-epithelial resistance can be measured to assess intestinal barrier function and distinct compartment separation allows for sampling of the basal medium without system

disruption. For instance, paracellular tracers could be administered on the luminal side and tight junction integrity evaluated by collection on the basal side. To simulate a more physiological gastrointestinal tract, mucus-secreting cell lines, such as HT29-MTX, could be seeded to induce mucus layer separation of microbes from intestinal epithelial cells (IECs). One can even envision the study of more complex interactions of microbes and IECs along with immune cells of various types, currently under development. The further incorporation of intestinal enteroids, three-dimensional 'mini-intestines'[203], in combination with co-culture systems would provide an even more powerful tool to study these interactions.

I propose the rigorous study of probiotic organisms using a two-pronged approach, utilizing both germ free animals and dual-environment co-culture modeling to elucidate individual bacteria and community effects. For both experimental arms, first, individual bacterial species would be studied in isolation in monocolonized GF mice to establish effects on the host *in vivo* and in co-culture systems to establish initial host-microbe interactions *in vitro*. Second, after establishing the mechanistic effects of single species on the host or on cultured human cells, varied combinations of bacterial species would be introduced in both systems. These experiments would help determine whether specific bacteria have the capability to co-colonize in the host, and if so, to analyze any synergistic impact. For instance, is there combined host cell benefit of specific microbes together and to what extent? Can we customize the ideal composition of 'good' microbes? Beyond these experiments, meticulous study should take place in the clinic to examine the proportions of specific 'good microbes' in healthy individuals. We need to know whether 'adequate', durable colonization can be achieved in humans through

different approaches, such as oral treatment, microbial transplantation, and dietary changes.

The Bright Future of Microbial-Based Therapies

Excitingly, the next generation of microbial therapy is already moving towards potential applications in cancer treatment. In recent years, checkpoint blockade immunotherapy, aimed at impeding inhibitory immune cell checkpoints, has begun to revolutionize patient standard of care with, for example, demonstrated efficacy in cases of malignant melanoma[204], renal cell cancer[205], and non-small-cell lung cancer[206]. The current checkpoint blockades approved by the Food and Drug Administration target suppressive receptors on the surface of T cells, e.g. cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1), with the goal of unleashing the endogenous antitumor immune response. Nevertheless, it should be underscored that only a subset of patients responds favorably to such treatments with various aspects of the tumor microenvironment, the tumor itself, and circulating factors as contributors to response outcome. New research has now implicated the gut microbiome as another important modulating factor.

In a mouse melanoma model, Sivan et al. showed that mice housed in two different facilities (Jackson Laboratory (JAX) and Taconic Farms (TAC)) and known to have differential microbial makeups, exhibit different rates of tumor growth (slower in JAX mice); this difference disappeared after co-housing, with all co-housed mice seeming to display the JAX, slower-growing tumor phenotype[207]. The transfer of feces from JAX mice to TAC mice also resulted in slower-growing tumors and greater tumor

infiltration with CD8^{+} T cells in TAC mice. Interestingly, anti-PD-L1 antibody treatment was more effective in JAX mice than in TAC mice and more effective in TAC mouse recipients of JAX stool than either fecal transfer or anti-PD-L1 treatment alone. Further 16S rRNA analysis of mice that received JAX feces revealed an increased abundance in bacteria of the genus *Bifidobacterium* that associated with antitumor T cell responses. Furthermore, colonization by live *Bifidobacterium* spp. mimicked the results of the JAX stool transfer, implicating *Bifidobacterium* in antitumor immunity and improved anti-PD-L1 treatment efficacy.

In the same melanoma mouse model, Vétizou et al. showed that mice housed in specific pathogen free (SPF) conditions responded to anti-CTLA-4 treatment exhibiting diminished tumor growth, whereas mice housed in germ free (GF) conditions did not[208]. Additionally, the treatment of SPF mice with broad-spectrum antibiotics diminished anti-CTLA-4 therapeutic efficacy. Anti-CTLA-4 treatment altered the microbiome, resulting in the enrichment of *Bacteroide*s spp. (specifically *B. thetaiotaomicron* and *B. uniformis*) in the small intestine, contrasting with a fecal decrease of these bacteria. When antibiotic-treated SPF mice or GF mice were inoculated with different *Bacteroides* isolates, the checkpoint therapy regained effectiveness. Similarly, ipilimumab (anti-CTLA-4) treatment in metastatic melanoma patients initiated a fecal clustering of microbes more abundant in distinct *Bacteroides* spp.. The fecal microbial transfer (FMT) of these patient stools to GF mice resulted in improved anti-CTLA-4 efficacy and increased colonization levels of *B. fragilis* and *B. thetaiotaomicron*, demonstrating the compelling potential for human gut microbes as a tool to boost cancer immunotherapy responses. While many questions remain, these studies provide strong evidence that intestinal microbes can affect

responses to checkpoint blockade therapy and that intestinal microbes can be manipulated for a beneficial therapeutic outcome.

Study in and application of microbiome science to human clinical disease is not without significant challenges. Human patients live in distinct geographic locations and environments, exhibiting a vast array of dietary habits, social customs, and physical activity. In contrast to the convenience and simplicity of laboratory mice used in modeling studies, the complex variability in the human environment, behaviors, and exposures, likely contribute to vast inter-patient differences regarding the makeup of their gut microbiomes. For instance, preliminary data gathered by the Sears group has begun to identify key differences in the prevalence of colon tumor-associated and normal tissue-flanking invasive biofilms in CRC patients sampled from two distinct populations, Malaysia and the United States. The U.S. cohort exhibits an almost universal detection of invasive biofilms in the right (89%), defined as ascending colon to the hepatic flexure, versus the left colon (12%)[135]. And while invasive biofilms are primarily detected in the right colon, the gap between right and left colon detection appears to significantly narrow in the Malaysian cohort. In addition, the habitual exposure of cancer patients requiring chemotherapy to antibiotics and antibiotic-resistant bacteria also contributes to alterations in the development of their microbiomes[201]. Multifaceted research approaches will be needed to address these distinct variables.

Although complex, this endeavor is a worthwhile pursuit. Microbial manipulation holds promise, not just for the treatment of cancer, but also for other diseases such as microbial infection, inflammatory bowel disease, obesity, and arthritis. For example, colon microbial alteration by fecal microbiota transplantation (FMT) has shown

remarkable effectiveness in treating recurrent *Clostridium difficile* infection (CDI) in patients, with relapse-free cure rates of 80-90%[209]. And although not yet studied in randomized controlled trials (RCTs), there are early case reports of FMT or FMT-like therapies leading to clearance of other pathogenic or drug-resistant bacteria. A secondary analysis of patients in a CDI phase 2 trial with RBX2660, a microbiota-based drug derived from live human microbes, also showed evidence of effectiveness against vancomycin-resistant *Enterococcus* (VRE) colonization[210]. Bilinski and colleagues found that FMT treatment of an immunocompromised patient led to clearance of β–lactamase-positive *Klebsiella pneumoniae* and ESBL-positive *E. coli* colonization[211]. In addition, Crum-Cianflone and colleagues found that a single patient treated by FMT for recurrent CDI also had the added benefit of clearing carbapenem-resistant *Enterobacteriaceae*, MRSA, VRE, and multi-drug resistant *Acinetobacter baumannii*, lessening the burden of infection, sepsis, and antibiotic usage post-FMT[212].

As described in Chapter 1, IBD is associated with intestinal dysbiosis with noted decreased bacterial phyla diversity, namely in Firmicutes and Bacteroidetes. In functionality, the microbiome of IBD patients may also diverge, with changes observed in oxidative stress pathways and carbohydrate metabolism[213]. Still in the early stages of study, so far, the results of FMT efficacy in the treatment of IBD have been heterogeneous. In two RCTs of active ulcerative colitis, one yielded no significant difference in clinical remission but increased bacterial diversity in responsive subjects (n=50)[214], and another showed a greater proportion of FMT recipients versus placebo (24% vs 5%) in remission 7 weeks post-FMT (n=75)[215]. Interestingly, the FMT-recipients from a specific donor (donor B) appeared more responsive, suggestive of a donor-effect

in the context of IBD, a finding not observed in the recurrent *C. difficile* infection FMT literature[215]. The results of study in Crohn's disease patients have been similarly mixed. Thus, FMT therapy in IBD patients warrants further study. Although prospective data is lacking on pre- or probiotic treatments in obesity, a recent study evaluated three probiotic strains, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Bifidobacterium animalis* in mice fed a high fat diet for 12 weeks. Treatment by individual strains led to diminished weight gain and improved glucose-insulin homeostasis, while also shifting the gut microbiota to that of lean diet fed mice[216]. Furthermore, FMT treatment of germ free mice with stool from conventionally-raised mice with a genetic predisposition to obesity led to the development of phenotypically obese mice[217–219]. Similarly, a recent case report described a patient who developed new-onset obesity after a successful FMT procedure receiving feces from an overweight donor[220]. Collectively, these studies provide insight into the transmissibility of the 'obese' microbiota and suggest that microbial intervention may help combat this condition.

Arthritis, another immune-mediated disease, has also been associated with oral and intestinal dysbiosis; increased *Prevotella*, *Leptotrichia*, and *Lactobacillus* species have been observed in rheumatoid arthritis and overall decreased bacterial diversity has been observed in psoriatic arthritis[221]. And while probiotic treatment in arthritis patients has typically yielded negative results, dietary intervention with a vegan or Mediterranean-style diet has led to moderate improvements[221]. Clinical trials analyzing FMT therapies in rheumatic diseases have not been carried out but will likely soon be initiated.

In an age where antibiotic resistance is rapidly increasing while the discovery or synthesis of new antibiotics is waning, and diseases of the bowel and of an autoimmune

nature are significantly increasing, it is essential that the study and honing of microbiota-based methods progress. Unleashing the power of naturally occurring, symbiotic microbes may be a way to partly address these continuing challenges. At a basic level, the continued study of microbes in isolation, utilizing gnotobiotic mice, and the development of better methods to study complex communities of microbes together and with their host are key to moving this ambitious goal forward. At a translational level, the prospective analysis of the intestinal microbiota prior and in response to clinical treatments including cancer immunotherapy, antibiotics, and FMT, is essential to developing safe and effective future therapies. The future is fecal!

A Translational Approach to Studying IL-22

While animal models have yielded the functional dualities of IL-22, in that this cytokine appears to have the capacity for both preventative and permissive effects in both the development of chronic colitis and colon cancer development, much remains to be learned in its role in clinical disease. From seminal work carried out by Dejea et al., we learned that invasive polymicrobial biofilms were a key feature of right-sided colon tumors and matched normal tissues within the patient[135]. The presence of biofilms was also associated with diminished E-cadherin and enhanced IL-6 and STAT-3 activation in colon epithelial cells. It is likely that other cell-intrinsic or cell-extrinsic factors are influenced by the presence of these biofilms, and it will be exciting to uncover its possible effect on the IL-23/IL-22 axis. Furthermore, understanding how tumor mutational status and/or microsatellite status contributes to this complex web of interaction is of great interest. In collaboration with Fiona Powrie's laboratory at Oxford

University, who has begun to uncover some intriguing associations of tumor mutational status and IL-22 receptor expression profiles relating to CRC prognosis, we will begin to address some of these questions. Existing CRC clinical samples made available through the Johns Hopkins Hospital and UMMC in Kuala Lumpur, Malaysia have already been analyzed for invasive biofilm status. From a select pool, preliminary IL-22 and IL-22R expression will be analyzed by immunohistochemical means and confirmed with quantitative PCR where frozen tissues are available; in consort, genotyping of samples will be done to determine tumor mutational status. Understanding the impact of IL-22 signaling in CRC may yield important insights into CRC prognosis and treatment responsiveness. It may also provide the basis for the further development of anti-IL-22 cancer immunotherapies.

Table 1: Proposed beneficial bacteria under study

(Summary generated by June Chan)

Beneficial Bacteria	Anaerobe Type	Gut-Related Clinical Observations	Mechanism/Arsenal
Lactobacillus rhamnosus GG (LGG)	facultative	diarrheal incidence⬇, diarrheal duration⬇, GI nosocomial infection⬇, IBS pain frequency & intensity⬇, clearance of *Candida*, *C. difficile*, & VRE⬆, bifidobacteria⬆, species diversity⬆	Production of lactic acid; resistance to gastric & bile acids; strong pili-mediated adhesion to intestinal mucus glycoproteins & colonization persistence, lipotechoic acid & extracellular polysaccharide as survival & immune effectors, secretion of anti-apoptotic proteins & antimicrobials, induction of MUC2 & MUC3 expression
Lactococcus lactis	facultative	Antimicrobial activity against clinical *C. difficile* isolates & antibiotic-resistant *Enterococcus* species⬆, with oral administration VRE colonization in the gut (mouse)⬇	Production of lactic acid; Production of plasmid-encoded bacteriocins, nisin and lacticin, active on many Gram-positive bacteria including *C. difficile* isolates and various strains of VRE
VSL#3: *S. thermophilus, B. breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. paracasei, L. helveticus*	facultative & obligate	human meta-analysis: remission induction of IBD in single or combination forms; animal sudies: *C. jejuni* disease sequelae (mouse)⬇, immune signature recovery after antibiotic treatment (mouse)⬆, TNBS colitis severity, macrophage infiltration, serum cyotkine levels (rat)⬇, colitis in Muc2$^{-/-}$ mice⬇, intestinal barrier function in Muc2$^{-/-}$ mice⬆	Production of acetate; inflammation control through reduced tissue levels of TNFα, IFNγ, iNOS, MMP-2 & MMP-9
Bacteroides spp.: *B. thetaiotaomicron, B. fragilis*	obligate	*B. theta*: In pediatric and adult IBD, *B. theta* is⬇, nutrient provision to host (digests complex carbs)⬆, infectious diarrhea protection, e.g. rotavirus (preliminary)⬆, *B. theta/F. prausnitzii* synergy for mucosal homeostasis⬆; *B. fragilis*: protection from *H. hepaticus* and TNBS colitis (mouse)⬆	Polysaccharide utilization machinery to promote survival & use of complex polysaccharide substrates; *B. theta* (GF rat/mice): induction of expression in host nutrient absorption, mucosal barrier function, angiogenic & motility genes; increase in goblet cell differentiation & expression of mucus-related genes; *B. fragilis* (GF mice): Induction of Treg expansion & Treg-mediated IL-10 production
Faecalibacterium prausnitzii	obligate	In IBD, IBS, CRC, & celiac disease, *F. praus* is⬇, in children newly diagnosed with Crohn's disease, *F. praus* is⬆; treatment with whole bacteria or culture supernatants offers protection from TNBS colitis (mouse)⬆	Production of butyrate; inhibition of NF-κB activation & IL-8 secretion in Caco-2 cells; secretion of anti-inflammatory metabolites (unspecified); stimulation of PBMC IL-10 increased & IL-12 decreased production; increased Treg expansion; improved intestinal barrier integrity
Akkermansia muciniphila	obligate	In obese children, IBD, and body weight increase in pregnant women, *A. muc* is⬇, in ob/ob mice on high-fat diet, *A. muc* is⬇, In healthy mucosa, *A. muc* is⬆; protection from DSS colitis (mouse)⬆, prebiotic (oligofructose) or *A. muc* fed high-fat diet subjects normalize *A. muc* abundance and improve metabolic disorders (mouse)	Part of the mucosa-associated microbiota due to its ability to produce mucus-degrading enzymes and use of mucin as a sole carbon, energy, and nitrogen source; extracellular vesicles contribute to anti-inflammatory effects
Clostridium spp.: *C. scindens, C. difficile*	obligate	In humans & mice with *C. difficile* infection, *C. scindens* is⬇, *C. scindens* protects from *C. difficile* colonization and disease (mouse); Prophylactic treatment with modified *C. difficile* diffocins protects against pathogenic *C. difficile* strains (mouse)	*C. scindens*: conversion of $1°$ to $2°$ bile acids inhibits *C. difficile* growth; *C. difficile*: R-type diffocins (bacteriocins) can selectively kill other *C. difficile* strains, e.g. a genetically modified diffocin (Av-CD291.2) from *C. difficile* strain CD4 targets *C. difficile* epidemic lineage strains (BI/NAP1/027)
Bifidobacterium spp.: *B. longum, B. bifidum, B. breve, B. animalis*	obligate	In healthy infants, *Bifido* is⬆, in adulthood, *Bifido* is stable but⬇, in old age, *Bifido* is further⬇⬇; In obese children, IBS, elderly *C. difficile*-associated diarrhea, and body weight increase in pregnant women, *Bifido* is⬇; *B. longum* protects from enterohemorrhagic *E. coli* & there is a positive correlation between fecal acetate and resistance to infection (mouse)	Breakdown of human milk oligosaccharides, enhancing adhesion & anti-inflammatory cytokines; production of acetate stimulates anti-inflammatory/anti-apoptotic response & prevents an increase in epithelial barrier permeability, inhibiting translocation of *E. coli* O157:H7 Shiga toxin from gut lumen to blood; 'probiotic transporters' in specific *B. longum* subspecies promote efficient acetate production

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217.   Bäckhed et al. The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America 101, 15718–15723 (2004).

218.   Ridaura et al. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. Science 341, 1241214 (2013).

219.   Turnbaugh et al. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–131 (2006).

220.   Alang & Kelly. Weight Gain After Fecal Microbiota Transplantation. Open Forum Infectious Diseases 2, ofv004 (2015).

221.   Bravo-Blas, Wessel & Milling. Microbiota and arthritis: correlations or cause? Current Opinion in Rheumatology 28, 161 (2016).

CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University Bloomberg School of Public Health

June L. Chan   **September 2017**

Education

Ph.D.   Molecular Microbiology and Immunology,   Dec. 2017
             Johns Hopkins University   (Expected)
             Bloomberg School of Public Health
             Mentors: Cynthia L. Sears, M.D., Jay Bream, Ph.D.

B.S.   Microbiology,   May 2008
             University of Maryland at College Park
Minor   Asian American Studies

Professional Experience

Thesis Research, 2011-present   Lab of Cynthia Sears, JHU/SOM

Research Rotations, 2010-2011   Lab of Joseph Margolick, JHSPH
                                     Lab of Egbert Hoiczyk, JHSPH
                                     Lab of Isabelle Coppens, JHSPH
                                     Lab of David Sullivan, JHSPH

Research Assistant, 2008-2010   Lab of Michael Otto, NIH/NIAID
Post-baccalaureate IRTA Fellowship

Research Assistant, 2006-2008   Lab of Stephen Mount, Univ. of Maryland

Service and Leadership

Teaching Assistant, 2016-present   Microbiology with Lab, JHU/SON

Teaching Assistant, 2007   General Microbiology, Univ. of Maryland

Honors and Awards

2010-2011   Otis and Calista Causey Fellowship for Immunology, JHSPH

2008-2010   National Institutes of Health Intramural Research Training Award

2008   Sigma Alpha Omicron Award (Microbiology), Univ. of Maryland

Publications, peer reviewed

* Under Review

1. ***Chan JL**, Wu S, Geis AL, Chan GV, Gomes TAM, Beck SE, Wu X, Fan H, Tam AJ, Ding H, Pardoll DM, Housseau F, Sears CL. The probiotic potential of non-toxigenic Bacteroides fragilis in colitis and tumorigenesis. *Cell Host Microbe*. 2017 September.

2. *****Chung L, Thiele-Orberg E, Geis AL, **Chan J**, Fu K, DeStefano Shields C, Dejea CM, Fathi P, Chen J, Finard BB, Tam AJ, McAllister FM, Fan H, Wu X, Ganguly S, Metz P, Van Meerbeke SW, Huso DL, Wick EC, Pardoll DM, Wan F, Wu S, Sears CL, Housseau F. Bacteroides fragilis toxin coordinates a pro-carcinogenic inflammatory cascade via targeting of colonic epithelial cells. *Cell Host Microbe*. 2017 April.

3. Kamenyeva O[1], Boularan C[1], Kabat J[2], Cheung GY[3], Cicala C[4], Yeh AJ[3], **Chan JL**[3], Periasamy S[3], Otto M[3], Kehrl JH[1]. Neutrophil recruitment to lymph nodes limits local humoral response to Staphylococcus aureus. *PLoS Pathog*. 2015 Apr 17;11(4):e1004827. PMCID: PMC4401519

4. Wick Elizabeth C, Rabizadeh Shervin, Albesiano Emilia, Wu,XinQun, Wu Shaoguang, **Chan June**, Rhee Ki-Jong, Ortega, Guillermo, Huso David L., Pardoll Drew, Housseau Franck, Sears Cynthia L. Stat3 Activation in Murine Colitis Induced by Enterotoxigenic Bacteroides fragilis. *Inflammatory Bowel Diseases*. 2014 May: 20(5):821-834. PMCID: PMC4121853.

5. Joo HS, **Chan JL**, Cheung GY, Otto M. Subinhibitory concentrations of protein synthesis-inhibiting antibiotics promote increased expression of the agr virulence regulator and production of phenol-soluble modulin cytolysins in community-associated methicillin-resistant staphylococcus aureus. *Antimicrob Agents Chemother*. 2010 Nov; 54(11): 4942-4944. PMCID: PMC2976111.

Meetings Attended

1. JHMI Dept. of Medicine Retreat, Baltimore, MD (2016)
   - Poster Presentation: The probiotic potential of non-toxigenic *Bacteroides fragilis* (NTBF) in colitis and tumorigenesis
2. Digestive Disease Week, Chicago, IL (2014)
   - Research Symposium Oral Presenation: The contribution of interleukin 22 to the pathogenesis of enterotoxigenic *Bacteroides fragilis* murine colitis-associated cancer