THE INTERPLAY BETWEEN INFLAMMATION AND MICROBIAL ACTIVITIES IN COLORECTAL CANCER

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ABSTRACT

Sarah Emily Tomkovich: The interplay between inflammation and microbial activities in colorectal cancer
(Under the direction of Christian Jobin)

The microbiota affects host immune health by influencing immune system development and promoting tolerogenic immune responses, effects that have the potential to influence vaccine and cancer immunotherapy efficacy. Disruption of the delicate homeostatic balance between the host and microbiota can lead to intestinal diseases such as inflammatory bowel diseases (IBD) and colorectal cancer (CRC) and also extra-intestinal pathologies such as metabolic syndrome and autoimmune diseases.

This dissertation focuses on the impact of the microbiota on host intestinal immune responses in relation to inflammation and carcinogenesis. The aim of the first project was to evaluate the role of the microbiota in modulating systemic neutrophil numbers and function in the developing zebrafish. Using a gnotobiotic approach we demonstrated colonization of germ-free (GF) zebrafish with a conventional microbiota increased neutrophil numbers and myeloperoxidase expression, altered neutrophil localization and migratory behavior and improved neutrophil recruitment to extra-intestinal injury. We showed that neutrophil migratory behavior was mediated through the acute phase response protein serum amyloid A (SAA), which was also induced by the microbiota. *In vitro* experiments revealed SAA

exposure activated nuclear factor (NF)-κB in zebrafish cells, and NF-κB was also required within neutrophils for SAA-dependent migration.

The goal of the second project was to evaluate the ability of CRC-associated microbes to induce inflammation and CRC in genetically susceptible gnotobiotic mice. *Fusobacterium nucleatum* and *Escherichia coli* that contain the genotoxic island, polyketide synthase (*pks*) are part of the altered microbiota that is associated with human CRC. We mono-associated $Apc^{Min/+}$;$Il10^{-/-}$ mice with either *F. nucleatum* or *E. coli* and found only *pks*+ *E. coli* had the capacity to induce inflammation and tumorigenesis. Next, we examined the functional role of human biofilm associated microbes in CRC development using $Apc^{Min/+}$;$Il10^{-/-}$ mice. We found that biofilm forming microbes promoted tumorigenesis, suggesting bacterial organization also plays a role in CRC pathogenesis. Taken together these studies stress the importance of balance in host-microbiota interactions. Elucidating host and microbial factors that contribute to disease states has the potential to transform how diseases are prevented and treated.

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AB-GZM, gnotobiotic zebrafish medium with antibiotics

AHR, aryl hydrocarbon receptor

AIEC, adherent invasive *Escherichia coli*

AIM2, absent in melanoma 2

AMPs, antimicrobial peptides

AOM, azoxymethane

APC, adenomatous polyposis coli

APR, acute phase response

APRIL, a proliferation-inducing ligand

ATG16L1, autophagy related 16-like 1

BAFF, B cell-activating factor

BAY, Bay 11-7082

BF, biofilm negative

Bx, colonoscopy biopsy

B. theta, *Bacteroides thetaiotaomicron*

C5aR, complement component C5a receptor

CAC, colitis-associated cancer

ccf, commensal colonization factors

CD, Crohn's disease

CFU, colony-forming unit

CHT, caudal hematopoietic tissue

CHX, cycloheximide

CIMP, CpG island methylator phenotype

CONVD, conventionalized

CRC, colorectal cancer

CTL, cytotoxic lymphocytes

CTNNB1, Catenin beta 1

CXCL, C-X-C motif ligand

DAMPs, damage-associated molecular patterns

DC, distal colon

dpf, days post fertilization

DSS, dextran sulfate sodium

ECM, extracellular matrix

EGFR, epidermal growth factor

EHEC, enterohemorrhagic *E. coli*

ERK1/2, extracellular signal-regulated kinases 1 and 2

ETBF, enterotoxigenic *Bacteroides fragilis*

ETDA, ethylene diamine-tetraacetic acid

FAP, familial adenomatous polyposis

FBS, fetal bovine serum

FISH, fluorescence *in situ* hybridization

FMT, fecal microbiota transplantation

Fpr2/Fprl1, formyl peptide receptor 2

FPS, frameshift peptide

GF, germ-free

GI, gastrointestinal

GPRs, G protein-coupled receptors

GWAS, genome-wide association studies

GZM, gnotobiotic zebrafish

H_2S, hydrogen sulfide

HBUS, mouse that express the epidermal growth factor ligand, HB-EGF

HDAC, histone deacetylase

HDL, high-density lipoprotein

HFD, high-fat diet

HIF, hypoxia-inducible factor

I, indole-3-aldehyde

IBD, inflammatory bowel diseases

IECs, intestinal epithelial cells

IELs, intraepithelial lymphocytes

IFN, interferon

IgA, immunoglobulin A

IL, interleukin

ILCs, innate lymphoid cells

ILC2, group 2 innate lymphoid cell

ILC3, group 3 innate lymphoid cell

iNKT, invariant natural killer T cell

JNK, c-Jun N-terminal kinase

KEGG, Kyoto Encyclopedia of Genes and Genomes

LPS, lipopolysaccharide

MAMPs, microbial-associated molecular patterns

MAP, *MutYH*-associated polyposis

M cells, microfold cells

MDP, muramyl dipeptide

MHCII, major histocompatibility complex class II

Min, multiple intestinal neoplasia

MMP9, matrix metallopeptidase 9

MMR mismatch repair genes

MO, morpholino

mpo/mpx, myeloperoxidase

MyD88, myeloid differentiation primary response protein 88

NF, normal flanking

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NLR, nucleotide-binding domain, leucine-rich-repeat-containing receptors

Nlrp3, NLR-pyrin domain containing 3

NOD, nucleotide-binding domain

NSAIDs, nonsteroidal anti-inflammatory drugs

OAS, oligoadenylate synthase

OMVs, outer membrane vesicles

pDCs, plasmacytoid dendritic cells

pks, polyketide synthase

PRR, pattern recognition receptor

PSA, polysaccharide A from *B. fragilis*

RAGE, receptor for advanced glycosylation end-products

RegIIIγ, regenerating islet-derived protein IIIγ

RIG-I, retinoic acid-inducible gene I

rRNA, ribosomal ribonucleic acid

SAA, serum amyloid A

Scarb1/CLA-1, scavenger receptor class B type 1

SCFA, short chain fatty acid

SFB, segmented filamentous bacteria

SHMT, serine hydroxymethyltransferase

SIgA, secretory IgA

SPF, specific-pathogen-free

T, tumor tissue

T3SS, type III secretion system

TGFβ, transforming growth factor-β

Th, T helper lymphocyte

TIGIT, T cell immunoglobulin and ITIM domain

TLR, Toll-like receptor

TMAO, trimethylamine-*N*-oxide

T. mu, *Tritrichomonas musculis*

TNBS, 2,4,6-trinitrobenzene sulfonic acid

TNF-α, tumor necrosis factor-α

TRAF6, tumor necrosis factor receptor associated factor 6

Treg, T regulatory lymphocytes

TRIF, Toll-interleukin receptor domain-containing adaptor protein interferon-β

TSLP, thymic stromal lymphopoietin

UC, ulcerative colitis

WT, wild-type

CHAPTER 1 : INTRODUCTION

1.1. Microbiota

The human microbiota consists of viruses, archaea, protists, fungi, helminths, and bacteria, although the vast majority of microbiota studies focus on the bacterial component (Chudnovskiy et al., 2016; Filyk and Osborne, 2016). The relationship between humans and the microbiota has evolved over millions of years, with evidence that bacteria from the Bacteroidaceae and Bifidobacteriaceae families mirror cospeciation between humans and the African Apes (Moeller et al., 2016). Given such a long-standing relationship, it is no surprise that the microbiota has been implicated in multiple aspects of human health and disease (Hooper et al., 2012; Rooks and Garrett, 2016). The microbiota impacts components of both innate and adaptive immunity, a relationship that begins at birth and continues throughout life (Honda and Littman, 2016; Tamburini et al., 2016; Thaiss et al., 2016a). Multiple diseases have been associated with the microbiota, including inflammatory bowel diseases (IBD), colorectal cancer (CRC), obesity, type 2 diabetes, cardiovascular disease, allergic asthma, rheumatoid arthritis, major depression, Parkinson's disease, and autism spectrum disorder (Gilbert et al., 2016; Rooks and Garrett, 2016; Schroeder and Bäckhed, 2016; Schwabe and Jobin, 2013; de Souza and

Fiocchi, 2016). However, the majority of the mechanisms by which members of the microbiota mediate this diverse range of effects on their hosts remain unclear.

Profiling the microbiota

The microbiota is found throughout the body, including the skin, nose, mouth, gastrointestinal (GI) tract, etc. (Human Microbiome Project Consortium, 2012). However, the majority of the microbiota, is concentrated in the gut, with an estimated ~10^{13} total bacteria (Sender et al., 2016). The microbiota refers to the collection of microbes associated with an organism, while the microbiome refers to the gene content within the microbiota (Kuczynski et al., 2011). Two main types of technologies have been employed for microbiota characterization: 16S ribosomal ribonucleic acid (rRNA) sequencing and shotgun metagenomics (Kuczynski et al., 2011). 16S rRNA sequencing is a targeted approach that amplifies the variable region of the 16S rRNA gene to infer bacteria composition, while metagenomics identifies the genes present within the microbiota (Kuczynski et al., 2011). Both approaches provide compositional information about the ecosystem, with the metagenomics studies adding functional information through pathway organization. Other functional approaches profile the metatranscriptome to examine actively expressed microbial genes (Franzosa et al., 2015) or characterize the metabolites of the microbiota through mass spectrometry (Vernocchi et al., 2016). Microbiota characterization studies have revealed an individual's microbiota is shaped by diet (Sonnenburg and Bäckhed, 2016), lifestyle (O'Sullivan et al., 2015), and genetics

(Blekhman et al., 2015; Goodrich et al., 2014, 2016). Interestingly, multiple immune genes correlate with microbiota composition, including chemokine signaling, barrier function and pattern recognition receptors (Blekhman et al., 2015; Goodrich et al., 2016).

Animal models for microbiota research

Mice and zebrafish are two of the common animal models used in microbiota research, and are important tools for establishing the mechanisms behind host-microbiota interactions. Zebrafish are inexpensive, develop quickly and are transparent through the larval stage, facilitating the use of powerful imaging techniques (Yang et al., 2014b). These advantages make zebrafish an ideal model for genetic and chemical screening experiments (Pham et al., 2008). Similar to mammals, the zebrafish intestine is composed of enterocytes, goblet cells, and enteroendocrine cells, with monocytes, macrophages and neutrophils in the lamina propria (Yang et al., 2014b). However, the zebrafish lacks a separate small and large intestine, crypts, Paneth cells, organized lymphoid structures, microfold cells (M cells) and adaptive immunity until 4 weeks of age (Brugman, 2016). The mouse intestinal tract is more similar to humans, but important differences include a large cecum, no appendix, no compartmentalization in the colon and differences in goblet and Paneth cells distributions (Nguyen et al., 2015).

Given the multiple anatomical differences that exist, it is not surprising that microbiota composition is species specific, with the zebrafish microbiota dominated

by members of the Proteobacteria phylum, while Firmicutes and Bacteroidetes are the 2 dominant phyla in mice and humans (Rawls et al., 2006). A meta-analysis of several human and mouse fecal 16S rRNA sequencing datasets, suggests only 79 genera occur in both species and there are also species-specific relative abundance differences within these genera (Nguyen et al., 2015). Although there are clear microbiota compositional differences between humans and mice, a comparison of gut metagenomes suggests there is a high degree of similarity at the functional level, with 95.2% of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologous groups shared between the 2 species (Xiao et al., 2015). The majority of shared KEGG orthologous groups are related to metabolism, environmental information processing, and genetic information processing (Xiao et al., 2015).

Importantly, techniques have been established to derive both mice and zebrafish into germ-free (GF) conditions, facilitating gnotobiotic experiments, where a defined microbe or microbiota is introduced (Martín et al., 2016; Pham et al., 2008). Compared to mice, zebrafish are relatively easy and inexpensive to derive GF, only requiring antibiotics, povidone-iodine, bleach and sterile tissue culture techniques (Pham et al., 2008). The two main disadvantages of GF zebrafish are their slower growth rate and the epidermal tail degeneration that develops from feeding the zebrafish sterile food, limiting early experiments to under 8 days post fertilization (dpf) (Pham et al., 2008). However, a new feeding technique using GF *Tetrahymena thermophile* (a ciliate) and GF *Artemia salina nauplii* (brine shrimp) suggests GF zebrafish can be maintained for at least a month (Rendueles et al., 2012).

Deriving and maintaining GF mice is more expensive, requiring surgery for the initial derivation and then continued maintenance in gnotobiotic isolators that require sterilization of all materials, special handling and entry protocols, and frequent monitoring for potential contamination (Martín et al., 2016; Packey et al., 2013). Studies with GF mice have revealed the microbiota affects the host's immune system, metabolism, behavior and other physiological aspects (Martín et al., 2016). For example, immune abnormalities in GF mice include germinal center deficiencies in lymphoid tissue, reduced and less diverse antibodies (immunoglobulin A-IgA), and decreased expression for a subset of antimicrobial peptides (regenerating islet-derived protein IIIγ-RegIIIγ, angiogenin 4) (Bevins and Salzman, 2011; Round and Mazmanian, 2009). Gnotobiotic models are also a powerful approach for studying microbe-microbe interactions, by colonizing GF animals with specific combinations of microbes (Pham et al., 2008). Importantly, both zebrafish and mice are capable of being colonized by microbiota from multiple animals including humans, although community members from a different host species undergo selection over time and do not fully recapitulate immune maturation (Chung et al., 2012; Rawls et al., 2006; Seedorf et al., 2014; Toh et al., 2013).

Microbiota colonization factors in the intestine

Environmental conditions change over the course of the intestinal tract with oxygen and antimicrobial peptides (AMPs) concentrations decreasing along the length of the intestinal tract while pH increases along the small and large intestine

(Wlodarska et al., 2015). Mucus structure also differs, with a single adherent layer in the small intestine and 2 layers, an adherent inner layer and a loose outer layer, in the colon (Wlodarska et al., 2015). These environmental conditions influence bacterial abundance and composition along the GI tract; from 10^3-10^4 bacteria/mL in the upper small intestine to 10^{11} bacteria/mL content in the colon (Sender et al., 2016; Wlodarska et al., 2015). Compositional differences along the mouse intestinal tract include more aerobic bacteria (Lactobacillaceae) in the stomach and small intestine and more anaerobes in the colon, there's also more inter-mouse variation in the colon (Gu et al., 2013). An oxygen gradient also exists along the crypt axis, due to the secretion of oxygen by the epithelium (Donaldson et al., 2016). Both aerobic *Acinetobacter* spp. and anaerobic *Bacteroides fragilis* have been found residing in the intestinal crypts in mice (Lee et al., 2013; Pédron et al., 2012). There are also nutrient distribution differences, with fatty acids and simple carbohydrates absorbed and depleted along the small intestine, while complex polysaccharides pass through to the colon, where they are broken down by *Bacteroides* spp. and other bacteria (Donaldson et al., 2016).

Diet, is another factor modulating bacterial colonization by selecting for bacteria with the metabolic capacities to utilize the dominant nutrients in its environment as well as the host's diet (e.g. protein-based versus animal-based) (David et al., 2014). For example, mucus degrading bacteria such as *B. acidifaciens*, *B. fragilis* and *Akkermansia muciniphila* tend to localize to the colonic mucus, indicating a microbe's niche is influenced by it's metabolic capacities (Donaldson et al., 2016).

In addition to differential metabolic capabilities, other microbial factors are also involved in modulating intestinal colonization. Bacteria from the Bacteroidetes, Firmicutes, and Actinobacteria phylums, which are the most dominant members of the mammalian intestine are more resistant to cationic AMPs produced during inflammation compared to Proteobacteria like *Escherichia coli* and *Campylobacter jejuni* (Cullen et al., 2015). Resistance was due to a protein, LpxF, that modulates the bacteria's lipopolysaccharide (LPS) (Cullen et al., 2015). There are also factors within bacteria that influence interspecies or interbacterial competition for colonization. For example, *Bacteroides* spp. contain a specific genetic locus, commensal colonization factors (ccf), that inhibits colonization by members of the same species and also contributes to stable colonization of the colon (Lee et al., 2013). Similarly, *Clostridium scindens* can prevent colonization by *Clostridium difficile* in mice, through metabolites such as secondary bile acids, which inhibit *C. difficile* growth *in vitro* (Buffie et al., 2015). In mice, *Lactobacillus* adhere to the proximal region and form biofilms in the mouse stomach, abilities that are modulated by *L. reuteri* genes (Fap1-like protein, SecA2) involved in adhesion and protein secretion (Frese et al., 2013). The composition and location of the microbiota are both significant factors in mediating interactions with the immune system. Whether microbial localization defines spatial development of intestinal pathologies is unclear.

1.2. The relationship between the immune system and the microbiota

Early life

The relationship between the microbiota and the immune system begins at birth, and continues as the microbiota assembles into a stable community during the first 2 years of life (Jain and Walker, 2015). Factors that modulate the infant microbiota include mode of delivery, diet (breast milk vs formula), and antibiotic treatment (Tamburini et al., 2016). Secretory, immunoglobulin A (IgA) is one of the most important early immune components, shaping the initial microbiota through maternal IgA in the breast milk until the baby starts producing their own after several months (Pabst et al., 2016). Twin studies that sequenced IgA-coated versus non-coated bacteria throughout the 2 year developmental period, suggest the proportion of IgA-targeted bacteria correlates with age (Planer et al., 2016). These findings were recapitulated in gnotobiotic mice fed diets mirroring the transition in foods that infants undergo (Planer et al., 2016). Alterations in early microbiota composition have so far been associated with Crohn's disease, asthma, and milk allergy persistence; suggesting this early microbiota developmental period has important implications for host health (Tamburini et al., 2016).

Innate intestinal immunity

Once the microbiota stabilizes, IgA continues to shape the microbiota in adults and conversely the microbiota itself is capable of modulating secretory IgA levels

(Fig. 1.4.1) (Macpherson et al., 2015). A study that isolated IgAs from the small intestine of wild-type (WT) mice, found IgAs had differential binding abilities (Okai et al., 2016). One IgA in particular, W27, bound and suppressed *E. coli* growth but did not affect 2 *Lactobacillus spp.* (Okai et al., 2016). Epitope mapping revealed W27 binds to a four amino acid (EEHI) motif in serine hydroxymethyltransferase (SHMT), a sequence mostly found in Gamma- and Betaproteobacteria, 2 classes that include multiple pathogenic bacteria (Okai et al., 2016). Interestingly, *E. coli* appears to be one of the bacteria capable of inducing an IgA response, as demonstrated with an auxotrophic *E. coli* strain in GF mice (Hapfelmeier et al., 2010). Segmented filamentous bacteria (SFB) are also capable of inducing IgA in mice (Macpherson et al., 2015), while other bacteria such as *Sutterella* spp. appear to degrade IgA (Moon et al., 2015).

AMPs are primarily produced by Paneth cells, and include defensins, C-type lectins, phospholipases and lysozyme C (Bevins and Salzman, 2011). Paneth cell produced AMPs have key roles in maintaining intestinal homeostasis by preventing bacterial translocation and modulating microbiota composition in the small intestine of mice (Bevins and Salzman, 2011). Enterocytes produce less AMPs compared to Paneth cells, but do produce the C-type lectin, RegIIIγ (Peterson and Artis, 2014). RegIIIγ maintains the zone of separation between the microbiota and small intestine epithelial cells, in a myeloid differentiation primary response protein 88 (MyD88)-dependent manner, suggesting AMPs also control bacterial distribution in the intestine (Vaishnava et al., 2011). Goblet cells are responsible for maintaining the mucus layer along the small and large intestine (Fig. 1.4.1) (Peterson and Artis,

2014). Recent work suggests the microbiota, itself, plays a role in modulating the initial mucus production and function in the small and large intestine of mice, with simultaneous alterations in bacteria distribution and composition occurring until stabilization of the mucus layer and microbiota after 8 weeks (Johansson et al., 2015).

Macrophages and dendritic cells are found within the intestinal lamina propria and play important roles in antigen uptake and presentation (Fig. 1.4.1) (Mowat and Agace, 2014). Although the exact mechanisms of antigen uptake are still unclear, M cells in lymphoid tissue (Peyer's patches isolated lymphoid follicles), small intestinal goblet cells and transepithelial dendrites extending between epithelial cells appear to be involved (Mowat and Agace, 2014; Peterson and Artis, 2014). Eosinophils and mast cells are also present in the intestinal mucosa and appear to be important for tissue repair and regulation of barrier integrity, respectively (Mowat and Agace, 2014). Neutrophils are not typically present in the intestine unless recruited by chemokines, such as C-X-C motif ligand 1 (CXCL1) and CXCL8 (interleukin (IL)-8) (Fig. 1.4.1), which are typically released by epithelial cells in response to pattern recognition receptor (PRR) signaling (Szabady and McCormick, 2013). Under homeostatic conditions neutrophils prevent bacterial translocation across the epithelial barrier (Fournier and Parkos, 2012). However, increased neutrophils are also associated with IBD (Fig. 1.4.2), suggesting resolution of inflammation is an additional important factor for maintaining homeostasis (Fournier and Parkos, 2012). Neutrophils utilize AMPs (α–defensins and lysozyme C), myeloperoxidase, hydrolytic enzymes, proteases, and metal chelators as defense mechanisms against microbes,

but many of these factors can also cause damage to the host if not appropriately controlled (Bevins and Salzman, 2011; Fournier and Parkos, 2012; Kolaczkowska and Kubes, 2013)

PRRs expressed by epithelial and immune cells, play a key role in maintaining intestinal homeostasis by controlling the microbiota, as well as sensing and mounting an appropriate response to pathogens (Chu and Mazmanian, 2013; Thaiss et al., 2016b). There are 6 main families of PRRs: Toll-like receptors (TLRS), C-type lectins, nucleotide-binding domain (NOD), leucine-rich-repeat-containing receptors (NLRS), retinoic acid-inducible gene I (RIG-I)-like receptors, absent in melanoma 2 (AIM2)-like receptors, and oligoadenylate synthase (OAS)-like receptors (Thaiss et al., 2016b). These PRRs recognize specific microbial-associated molecular patterns (MAMPs) from viruses, fungi, and bacteria, as well as host damage-associated molecular patterns (DAMPs) (Chu and Mazmanian, 2013; Thaiss et al., 2016b). Upon ligand stimulation, the majority of TLRs signal through MyD88 which leads to nuclear factor-κB (NF-κB) activation (Tomkovich and Jobin, 2016). Many of the inflammatory responses generated by MAMPs, fall into the DAMP category (defensins, heat-shock proteins, cytokines and lipoproteins such as serum amyloid A-SAA); thus resolution of PRR signaling is key to maintaining homeostasis and preventing a chronic feedback cycle of inflammation (de Souza and Fiocchi, 2016).

PRR signaling often leads to the induction of either anti- or pro-inflammatory cytokines (depending on the PRR, the MAMP, and the location). Thymic stromal lymphopoietin (TSLP) and transforming growth factor-β (TGFβ), which are produced by intestinal epithelial cells (IECs) in response to microbiota signaling, promote

tolerogenic dendritic cells and macrophages (Fig. 1.4.1) (Peterson and Artis, 2014). The acute-phase response (APR), is a systemic inflammatory reaction, initiated in the liver through the production of acute-phase proteins: serum amyloid A (SAA), C-reactive protein, and complement (Ye and Sun, 2015). However, *in vitro* studies, suggest IECs are also capable of producing SAA in response to a specific combination of inflammatory cytokines (tumor-necrosis factor (TNF)-α, IL-6, and IL-1β) or LPS (Eckhardt et al., 2010; Molmenti et al., 1993; Vreugdenhil et al., 1999). Interestingly, studies in zebrafish suggest the microbiota induces *saa* in the liver, swim bladder and intestine (Kanther et al., 2011). SAA has cytokine-like properties, can act as a chemoattractant, and interacts with multiple receptors including TLR2 and TLR4, so it's unclear whether microbiota-induced SAA is pro- or anti-inflammatory (Eklund et al., 2012; Ye and Sun, 2015).

Innate lymphoid cells (ILCs), particularly ILC3s act as mediators by coordinating signals between the microbiota and other facets of innate and adaptive immunity, while also controlling microbiota composition (Fig. 1.4.1) (Tait Wojno and Artis, 2016). ILC2 proliferation and activation is promoted by IL-25, IL-33, and TSLP from epithelial cells (Peterson and Artis, 2014). Members of the Alpha- and Betaproteobacteria classes were recently found within intestinal lymphoid tissue in mice, and shown to modulate DC functions and promote ILC3 and T helper lymphocyte type 17 (Th17) responses, although it is still unclear how the bacteria are mediating these effects (Fung et al., 2016). IL-22 from ILC3s also enhanced colonization of the lymphoid tissue-resident bacteria by inhibiting colonization of other intestinal bacteria (Fung et al., 2016). Microbial metabolites may play a role in

mediating ILC3 interactions through the aryl hydrocarbon receptor (AHR), as indole-3-aldehyde, a metabolite produced when *Lactobacillus* convert tryptophan, is an AHR ligand (Zelante et al., 2013). In addition to ILC3s, AHR is also highly expressed by the adaptive immune cells (Th17 and Th22) and is required for IL-22 production (Zhou, 2016).

Adaptive intestinal immunity

Sensing of microbes by innate immune cells (typically dendritic cells) leads to the production of cytokines that are MAMP specific and guide the adaptive immune response (Iwasaki and Medzhitov, 2015). Type 1 immunity includes cytotoxic lymphocytes (CTLs), Th1 cells and Th17 cells, which mediate defense responses to viruses, intracellular bacteria/protozoa, and extracellular bacteria/fungi, respectively (Iwasaki and Medzhitov, 2015). Tolerance to the microbiota is mediated by T regulatory lymphocytes (Tregs) which produce the anti-inflammatory cytokine, IL-10 (Fig. 1.4.1) (Maynard et al., 2012). Although, it is unclear whether MAMPs and PRRs play a direct role in induction of type 2 immunity to helminths and allergens, dendritic cells are still required to promote differentiation of Th2 cells (Iwasaki and Medzhitov, 2015). T and B cells are found in the lamina propria, and within the intestinal epithelium (intraepithelial lymphocytes-IELs), and the concentration of IELs and Th17 cells decreases along the intestinal tract while Treg concentrations increase (Mowat and Agace, 2014). B cell concentrations are highest in the proximal and

distal regions of the intestinal tract, and are primarily devoted to IgA production (Mowat and Agace, 2014).

The microbiota influences multiple aspects of adaptive intestinal immunity. B cells are stimulated by a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) from IECs after induction of PRR signaling from the microbiota (Fig. 1.4.1) (Peterson and Artis, 2014). Bacterial adhesion to epithelial cells appears to be one of the cues promoting Th17 cell induction in the small intestine and colon by SFB and *Citrobacter rodentium* an enterohemorrhagic *E. coli* (EHEC), respectively (Atarashi et al., 2015). Although SFB have only been isolated from mice, GF mice associated with a set of 20 human bacterial strains including *Clostridium* (Clusters IV, XIVa, XVIII), *Bifidobacterium*, *Ruminococcus*, and *Bacteroides* also had Th17 cell induction associated with bacterial adherence to colon epithelial cells (Atarashi et al., 2015). *Clostridium* IV and XIVa induce colonic Tregs in mice (Atarashi et al., 2011). Additional studies revealed that Treg induction was mediated by the bacteria metabolites: short chain fatty acids (SCFAs), particularly butyrate, through histone deacetylase (HDAC) inhibition (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). *Bacteroides fragilis* polysaccharide A (PSA) signals through TLR2 on Tregs to suppress Th17 cell responses and facilitate its colonization within the colonic crypts (Round et al., 2011).

Systemic Immunity

While the microbiota modulates multiple aspects of immunity in the intestine, a growing number of studies demonstrate the intestinal microbiota and their metabolites also promote systemic immune function. Interestingly, the effects of SCFAs on Tregs extend to the periphery where both butyrate and propionate promote Treg differentiation (Arpaia et al., 2013). Additionally, *B. fragilis* PSA also promotes the suppressive function of peripheral Tregs (Johnson et al., 2015b; Telesford et al., 2015). SCFAs also improve antibody responses by regulating both intestinal and systemic B cell metabolism and gene expression (Kim et al., 2016). The systemic immunomodulatory effects also extend to innate immune cells, with GF mice exhibiting microglial (brain tissue macrophages) defects that were ameliorated with a mixture of SCFAs in drinking water (Erny et al., 2015). Another bacterial metabolite: trimethylamine-*N*-oxide (TMAO), produced from a component of red meat and associated with luminal *Prevotella* abundance, modulates cholesterol in macrophages, promoting atherosclerosis (Koeth et al., 2013). Microbes have also been implicated in allergen-induced Th2 responses, increasing the peripheral basophils via MyD88-dependent signaling in B cells (Hill et al., 2012). It is unclear whether the gut microbiota modulates extra-intestinal innate immune cells in addition to basophils and macrophages. Understanding the factors governing interactions between the microbiota and the immune system have important implications for vaccines (Oh et al., 2014; Valdez et al., 2014) and anticancer

immunotherapies efficacy (Pope et al., 2016), which both rely on a functional immune system.

1.3. The interplay between inflammation, colorectal cancer, and the microbiota

IBD and the microbiota

There are two main forms of inflammatory bowel diseases (IBD): Crohn's disease (CD) and ulcerative colitis (UC) (de Souza and Fiocchi, 2016). Both are characterized by intestinal inflammation, however CD typically affects the ileum and the colon, but is capable of affecting any region of the GI tract, while UC is restricted to the rectum and colon (Abraham and Cho, 2009). IBD is a chronic disease and a significant health-care burden with costs likely to grow due to a high prevalence in the western world and a rising rate of incidence in newly industrialized countries (Kaplan, 2015). The established epidemiological risk factors of IBD include: genetics, environmental and lifestyle factors, and the microbiota (Ananthakrishnan, 2015). Environmental risks have been attributed to smoking, appendectomy, infections, antibiotics, medications and diet while lifestyle factors relate to stress, sleep, and exercise (Ananthakrishnan, 2015). Dietary risk factors specific for IBD include fiber, fat, vitamin D, zinc and iron (Ananthakrishnan, 2015). Almost all IBD risk factors are able to directly modulate and interact with the microbiota, making it difficult to pinpoint an exact cause of IBD (Ananthakrishnan, 2015; Lozupone et al., 2012; O'Sullivan et al., 2015). Interestingly, some environmental risk factors such as smoking and appendectomy have opposite associations with UC and CD, both being

associated with increased risk of CD (Ananthakrishnan, 2015). Insight into the genetic components of IBD have been revealed with genome-wide association studies (GWAS), which identified 163 loci, 66 of which overlap with loci previously implicated in other immune-mediated diseases (Jostins et al., 2012).

One of the most significantly enriched Gene Ontology categories associated with IBD loci was regulation of cytokines, including interferon (IFN)-γ, IL-12, TNF-α, and IL-10 (Jostins et al., 2012). Alterations in PRR and autophagy genes are also associated with IBD through GWAS, these include the PRR, *NOD2*, and the autophagy gene, autophagy related 16-like 1 (*ATG16L1*) (de Souza and Fiocchi, 2016). Changes in intestinal innate immunity associated with IBD, include increased chemokine expression, an influx of neutrophils, and more pro-inflammatory cytokine production (i.e. IFN-γ, IL-1β, IL-6, IL-18, IL-23, TNF-α, etc.) by macrophages and dendritic cells (Fig. 1.4.2) (de Souza and Fiocchi, 2016). Alterations in adaptive immunity include increased antibodies (against self or microbial antigens), pro-inflammatory cytokines from effector T cells (IL-6, IL-17, IL-22, TNF-α,), T cell resistance to apoptosis and Tregs with insufficient suppressive capacities (IL-10, TGFβ) (Fig. 1.4.2) (Neurath, 2014; de Souza and Fiocchi, 2016). The increased inflammation characteristic of IBD is thought to result from miscommunications between the host's immune system and the microbiota (Manichanh et al., 2012).

Dysbiosis, or an altered microbiota, has been associated with IBD, but the exact role of dysbiosis in disease pathogenesis is still unclear, with the stool microbiota from CD patients but not UC patients promoting colitis in gnotobiotic *IL10*$^{-}$

$^{-/-}$ mice (DeGruttola et al., 2016; Nagao-Kitamoto et al., 2016). General characteristics of IBD-associated dysbiosis include decreased overall bacterial diversity, an increase in Enterobacteriaceae (e.g. adherent invasive *E. coli*: AIEC) and sulphate-reducing bacteria (*Desulfovibrio*) and a decrease in *Faecalibacterium prausnitzii* (a butyrate producer) and obligate anaerobes (e.g. *Bifidobacterium* spp.) (Fig. 1.4.2) (DeGruttola et al., 2016; Rowan et al., 2010). An important consideration when evaluating dysbiosis is the location chosen for sampling. For example, there are clear differences between the luminal and mucosal associated microbiota in pediatric CD patients with the majority of the dysbiotic signature only observed in the tissue-associated communities from the ileum and rectum (Gevers et al., 2014). The fungal and viral microbiota also appear to be altered in IBD patients, with variations based on disease type (UC vs CD) as well as age (adult versus pediatric) (Lewis et al., 2015; Norman et al., 2015; Sokol et al., 2016). These studies suggest that fungal and viral dysbiosis may also accompany the bacterial dysbiosis observed in IBD patients. Likely, there exist transkingdom interactions among members of the microbiota that could influence the way the microbiota modulates host responses and IBD development.

Functional studies in mice suggest differences in bacterial metabolism may partially explain the increase in Enterobacteriaceae associated with dysbiosis in IBD patients. For example, *Il10*$^{-/-}$ mice, with spontaneous colitis exhibit an approximate 100 fold increase in luminal *E. coli* compared to WT mice (Arthur et al., 2012). Subsequent studies with nitric oxide synthase deficient mice, suggest the increased nitrate expressed during inflammation facilitates the growth of luminal *E. coli* by

providing electron acceptors for anaerobic respiration (Winter et al., 2013). Additionally, *C. rodentium*, an Enterobacteriaceae specific to mice, induced colonic crypt hyperplasia with its type III secretion system (T3SS), which increased oxygen levels at the mucosal surface and facilitated *C. rodentium* expansion (Lopez et al., 2016). Increased expression of a bacterial tricarboxylic acid cycle enzyme that is not required under anaerobic conditions (*sucA*) from mucus-associated bacteria compared to stool-associated bacteria suggests the mucosally attached *C. rodentium* are the beneficiaries of the increased oxygen levels (Lopez et al., 2016). These studies support the idea that oxygen levels altered by inflammation may be one of the mechanisms that promote dysbiosis (DeGruttola et al., 2016).

The spatial organization of the microbiota, in addition to its composition is another important consideration when studying the pathogenesis of intestinal diseases. A subset of mouse and human studies have examined intestinal biofilms with the use of Carnoy's fixative, which preserves the intestinal mucus layer, and fluorescence *in situ* hybridization (FISH), which identifies bacteria with specific fluorescent probes (Macfarlane and Dillon, 2007; Randal Bollinger et al., 2007; Swidsinski et al., 2005a, 2005b). These initial studies suggest biofilms are concentrated and close to the epithelial layer in the appendix of healthy humans and the cecum of WT mice (Palestrant et al., 2004; Swidsinski et al., 2005a, 2005b). Biofilms then progressively decrease along the rest of the intestinal tract until practically absent in the distal colon (Palestrant et al., 2004; Swidsinski et al., 2005a, 2005b). One theory suggests the biofilms within the appendix serve as a microbiota

reservoir, with both the bacterial organization and anatomical isolation of the appendix facilitating resiliency (Randal Bollinger et al., 2007).

However, microbial biofilms have also been identified in IBD patients (CD and UC) at a higher frequency (90-95%) than irritable bowel syndrome (IBS) (65%) or control patients (35%) (Swidsinski et al., 2005b). FISH staining, revealed IBD patients' biofilms displayed bacterial adhesion to the epithelial layer, crypt-associated bacteria, a high bacterial density (>10^9/ bacteria per mL) and a predominance of *B. fragilis* and Enterobacteriaceae (Fig. 1.4.2) (Swidsinski et al., 2005b, 2007). Similarly, dextran sulfate sodium (DSS)-treated and colitic *Il10*$^{-/-}$ mice also have more crypt associated bacteria and adhesion to the epithelial layer (Swidsinski et al., 2005a). Although, biofilms may be a feature of the healthy microbiota in the proximal GI tract, characteristics such as resistance to washing, bacterial density, adhesion to the epithelium, and invasion into crypts appear to be associated with inflammatory intestinal biofilms (Randal Bollinger et al., 2007).

While the exact host and bacterial factors driving biofilm formation are unknown, *in vitro* studies suggests an immune component, as both secretory IgA and intestinal mucus promote *E. coli* biofilm formation through interactions with the bacteria's type 1 pili (Bollinger et al., 2003, 2006). The host factor, hydrogen sulfide, may also play a role in biofilm formation as bacterial organization was disrupted in DNBS treated mice, which was further enhanced by treatment with a hydrogen sulfide inhibitor, resulting in a shift from linear biofilms separated from the epithelium to patchy biofilms that were in closer proximity to the epithelium (Motta et al., 2015).

The functional effects of biofilm organized bacteria on the host remain to be evaluated.

Types of CRC

Human colorectal cancer (CRC) can be categorized as either hereditary (predisposing mutations, family history, or IBD) or sporadic (Carethers and Jung, 2015). Tumorigenesis follows a stepwise progression going from an initiating step that leads to adenoma formation and typically takes 30-60 years, followed by tumor progression, where mutations continue to accrue over the next 1-20 years (Carethers and Jung, 2015). The final step is transformation into a malignant carcinoma, capable of metastasis, and is typically the fastest, taking between 0 and 5 years (Carethers and Jung, 2015). Many of the risk factors for IBD apply to CRC; these include genetics, diet, lifestyle, and environmental factors (Pope et al., 2016). 82.9% of the CRC mutation signatures are extrinsic, suggesting genetics and other intrinsic factors such as stem cell division rates play a minor role (Wu et al., 2016). The microbiota, which is also affected by the IBD and CRC risk factors, may be a key contributor to CRC pathogenesis

Sporadic CRCs account for approximately two-thirds of CRCs and fall into 2 main categories: hypermutated and nonhypermutated (Carethers and Jung, 2015). Hypermutated CRCs are characterized by microsatellite instability (MSI) or CpG island methylator phenotype (CIMP), due to defects in DNA mismatch repair (MMR) genes (Carethers and Jung, 2015). Epigenetic changes are associated with

hypermutated CRCs, with MSI resulting from hypermethylation of the DNA MMR gene (Carethers and Jung, 2015). CIMP is the result of excessive methylation of CpG islands within genetic loci and can be categorized into either high or low forms based on the amount of positive methylation markers and associated mutations (Carethers and Jung, 2015). CIMP-high overlaps with MSI CRCs while CIMP-low overlaps with the chromosomal instabilities associated with nonhypermutated CRCs (Carethers and Jung, 2015). Of note, both MSI and CIMP-high are associated with cancers located in the proximal colon (Fig. 1.4.3) (Carethers and Jung, 2015).

Nonhypermutated CRCs are more likely to have chromosomal and subchromosomal changes, originate in the distal part of the colon (Fig. 1.4.3) and have mutations in genes classically associated with CRC (*TP53*, *KRAS*, *PIK3CA*, *SMAD4*) (Benedix et al., 2010; Carethers and Jung, 2015; Kloor and Knebel Doeberitz, 2016). Although the pattern of gene mutations are drastically different between hypermutated and nonhypermutated sporadic CRCs, both share a high mutation frequency in the adenomatous polyposis coli (*APC*) gene (~60 and 81%, respectively) and consistent activation of Wnt signaling (Carethers and Jung, 2015). Loss of the tumor suppressor, APC, stabilizes β-catenin, resulting in nuclear translocation and activation of cellular proliferation genes (Clevers, 2006).

Approximately one-third of CRCs are hereditary and classified as either non-polyposis or polyposis, with the latter being less common (~1% of CRCs) (Stoffel and Boland, 2015). Polyposis syndromes include familial adenomatous polyposis (FAP) and *MutYH*-associated polyposis (MAP), both of which are characterized by

adenomatous polyposis with hundreds to thousands of colonic adenomas in the classic cases (Stoffel and Boland, 2015). Mutations in *APC* represent ~90% of FAP cases, while MAP is the result of mutations in a base excision repair gene. (Stoffel and Boland, 2015). Serrated polyposis is characterized by proximally located serrated polyps (Fig. 1.4.3) and may include CIMP as well as MSI (Stoffel and Boland, 2015). Lynch syndrome, a member the non-polyposis category, represents ~3% of CRCs, displays MSI due to germline mutations in MMR genes, and similar to sporadic MSI CRC, is more common in the proximal colon (Fig. 1.4.3) (Kloor and Knebel Doeberitz, 2016; Stoffel and Boland, 2015).

IBD patients have a 5-8 fold increase in risk for developing CRC, or colitis-associated cancer (CAC), which represents ~2-3% of all CRCs (Grivennikov and Cominelli, 2016). A key difference between CAC and other forms of CRC, is the mutation signature, with *APC* and *KRAS* mutations occurring at significantly lower rates in CAC patient tumors (Robles et al., 2016). CACs also have the capacity to develop within years or decades of active IBD, which is much quicker than sporadic CRCs (Grivennikov and Cominelli, 2016). Multiple aspects of the immune system implicated in IBD are also implicated in either CAC or CRC pathogenesis; these include PRRs, autophagy, and cytokines (IL-1β, IL-6, IL-17A, IL-23, and TNF) (Neurath, 2014; Pope et al., 2016).

Cancer type or mutation signature also appear to influence the type of cancer-associated inflammation (Lasry et al., 2016). For example, MSI CRCs and Lynch syndrome are characterized by the presence of frameshift peptide (FSP) antigens

that induce specific T cell responses within the tumor microenvironment and peripheral blood (Kloor and Knebel Doeberitz, 2016). However, this creates additional selective pressure on MSI tumors to develop immune evasion mutations, supported by the observation that ~30% of MSI CRCs have beta2-microglobulin mutations, which disrupts antigen presentation on tumor cells (Kloor and Knebel Doeberitz, 2016). Examples of mutation-specific inflammation include the loss of barrier function and increased inflammation associated with p53 loss and resistance to TGF-β's tumor-suppressive effects with activating mutations in *KRAS*. Additionally, nonsteroidal anti-inflammatory drugs (NSAIDS), appear to be an effective prevention strategy for sporadic and hereditary CRCs (Lasry et al., 2016). Although the exact mechanisms for NSAID prevention are still unclear, this further supports the theme that inflammation is an enabling characteristic of cancer, capable of driving tumor progression (Hanahan and Weinberg, 2011).

Mouse models of colonic adenoma-carcinoma progression

Numerous mouse models have been developed to study CRC either through genetic manipulation, chemical treatments, a combination of both, or xenografts where cells are transplanted either orthotopically or subcutaneously (Jackstadt and Sansom, 2016; Lee et al., 2016; West et al., 2015). The most commonly used model is the multiple intestinal neoplasia (Min or $Apc^{Min/+}$) model, which has a loss of function mutation in one of the *Apc* alleles (Jackstadt and Sansom, 2016). Although this is the most common mutation observed in human CRC, the distribution of

adenomas in mice differ, with the majority restricted to the small intestine (West et al., 2015). Differences in stem cell division rates, which are higher in the mouse small intestine, but lower in the colon (which is the opposite trend compared to humans), may partially explain the difference in tumor distribution (Tomasetti and Vogelstein, 2015a), although this hypothesis is controversial (Ashford et al., 2015; Podolskiy and Gladyshev, 2016; Song and Giovannucci, 2015; Tomasetti and Vogelstein, 2015b; Wu et al., 2016). Noteworthy, selective deletion of *Apc* in intestinal epithelial cells using Cdx2-cre recombinase transgenic mice showed tumorigenesis predominantly in the colon (Hinoi et al., 2007). A popular CAC model, where the chemical carcinogen, azoxymethane (AOM), in conjunction with DSS is used to induce cancer, has demonstrated multiple components of the immune system contribute to tumorigenesis (Pope et al., 2016; Subramaniam et al., 2016). Interestingly, some CRC mouse models are able to replicate the associated tumor distribution in human CRCs. For example, mice that express the epidermal growth factor receptor (EGFR) ligand HB-EGF (*HBUS* mice) develop serrated polyps in the cecum, similar to the proximal distribution observed in human serrated polyposis (Bongers et al., 2014). Nevertheless, the relationship between microbial niche and spatial distribution of carcinogenic lesions is poorly understood.

The role of the microbiota in CRC

Another feature shared between IBD and CRC, is an association with microbiota dysbiosis. Similar, to dysbiosis in IBD there appear to be differences

depending on whether the sampled microbiota is from the luminal or mucosa-associated compartment (Flemer et al., 2016). The majority of studies have used stool samples to profile the human CRC microbiota and found consistent increases in *Alistipes*, *Anaerococcus*, *Escherichia*, *Fusobacterium*, *Parabacteroides*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, and *Solobacterium* and decreases in *Roseburia* (Fig. 1.4.4) (Borges-Canha et al., 2015; Chen et al., 2012; Feng et al., 2015; Wang et al., 2012; Wu et al., 2013; Yu et al., 2015a; Zeller et al., 2014). In the mucosal compartment, *F. nucleatum*, Enterotoxigenic *B. fragilis* (ETBF) and *E. coli* (*pks*+) have increased prevalence in CRC mucosal tissue and *F. nucleatum* is enriched in adenoma and adenocarcinoma tissue (Fig. 1.4.4) (Arthur et al., 2012; Boleij et al., 2015; Borges-Canha et al., 2015; Buc et al., 2013; Chen et al., 2012; Kostic et al., 2012, 2013; Raisch et al., 2014; Shen et al., 2010; Yu et al., 2016). Few microbiota studies differentiate between the types of CRC when describing associated microbiota alterations, which could explain some of the variation seen across studies since the microbiota may be affected by CRC type or location (Flemer et al., 2016). For example, invasive *F. nucleatum* is more prevalent in proximal hyperplastic and sessile serrated adenomas (2 of the 3 histological categories for serrated polyps) and proximal CRCs (Fig. 1.4.3) compared to proximal/distal traditional adenomas (Yu et al., 2016).

The majority of mouse models that have investigated the role of the microbiota in CRC either with antibiotics or GF mice, suggest the microbiota promotes tumorigenesis (Pope et al., 2016; Schwabe and Jobin, 2013). Other microbiota members besides bacteria likely play a role in CRC pathogenesis, as a recent study

demonstrated a protozoan found within the mouse microbiota, increased tumorigenesis in *Apc*$^{Min/+}$ mice (Chudnovskiy et al., 2016). The protist, *Tritrichomonas musculis* (*T. mu*), induced IL-18 through epithelial inflammasome activation to promote Th1 and Th17 immunity, which conferred protection against acute enteric pathogens such as *Salmonella enterica* serovar Typhimurium but increased susceptibility to colitis and cancer (Chudnovskiy et al., 2016). Intriguingly, protists from the same class (parabasalids) are found in healthy human fecal samples with a prevalence ranging from 11.5-31.6%, however their role in human health and disease is unknown (Chudnovskiy et al., 2016).

The human CRC-associated bacteria: *pks*+ *E. coli*, ETBF and *F. nucleatum* have been investigated in CRC mouse models, providing insight into how these bacteria contribute to tumorigenesis. *E. coli* that possess a genotoxic island, polyketide synthase (*pks*), produce the genotoxin colibactin which causes DNA damage and induces colorectal cancer in an inflammation dependent manner (Arthur et al., 2012; Cuevas-Ramos et al., 2010). Interestingly, recent *in vivo* studies suggest *pks* expression may be regulated by host inflammation, while *in vitro* work suggests *pks* expression is dependent on *E. coli* iron sensors (Fur and RyhB) and production requires an *E. coli* heat shock protein (HtpG or Hsp90$_{Ec}$) (Arthur et al., 2014; Garcie et al., 2016; Tronnet et al., 2016). ETBF produce a toxin, *B. fragilis* toxin (Bft), which elicits host inflammation and modulates multiple aspects of the host immune response in an IL-17A dependent manner (DeStefano Shields et al., 2016; Garrett, 2015; Pope et al., 2016; Thiele Orberg et al., 2016). Although, *F. nucleatum* is not considered pro-inflammatory in mice, it is still capable of

modulating host immune responses through expansion of myeloid-derived immune cells and its adhesive Fap2 surface protein that binds to inhibitory receptor T cell immunoglobulin and ITIM domain (TIGIT), potentially promoting immune evasion (Gur et al., 2015; Kostic et al., 2013; Mima et al., 2015). Fap2 also appears to play a role in *F. nucleatum* localization and attachment to tumors, through binding of the host polysaccharide, Gal-GalNAc, which is highly expressed in human CRC adenocarcinomas (Abed et al., 2016). The functional role of dysbiosis in CRC development is still unclear as tumorigenesis in GF AOM/DSS mice associated with stools from CRC and healthy patients correlated with community composition rather than donor health status (Baxter et al., 2014).

Microbial biofilms have recently been associated with CRC and were more prevalent on proximal (right-sided) CRCs (89%) compared to distal (left-sided) CRCs (13%) (Fig. 1.4.3) (Dejea et al., 2014). The study defined biofilms as dense bacterial ($>10^9$ bacteria/mL) invasions of the mucus layer that spanned at least 200 μm of epithelial surface (Fig. 1.4.4) (Dejea et al., 2014). Bacteria associated with adenoma and CRC biofilms, identified by FISH staining, included Lachnospiraceae, Fusobacteria and Enterobacteriaceae (although the latter 2 were only found in a subset of tumors) (Dejea et al., 2014). Biofilms were also identified in 13% of healthy patients and strikingly, increased epithelial IL-6 and Stat3 activation was observed in the biofilm positive normal tissue from both CRC and healthy patients (Dejea et al., 2014). Biofilm positive CRC tissue also had increased levels of acetylated polyamines compared to biofilm negative CRC tissue, suggesting metabolites may contribute to the pathogenesis of biofilm associated CRC (Johnson et al., 2015a).

Multispecies bacterial biofilms have since been identified on additional types of CRC, with a prevalence that was relatively equal for proximal CRCs (52.1%), distal CRCs (55.6%), and sessile serrated adenomas (48.5%) compared to normal mucosa (20%) (Yu et al., 2016). It is unclear what other bacteria were present in these cases, as only universal bacteria and *F. nucleatum* FISH probes were used (Yu et al., 2016). The contrast in biofilm prevalence rates between these studies may be due to different definitions of biofilms and divisions of the colon (proximal to hepatic flexure vs splenic fixture, respectively) (Fig. 1.4.3) (Yu et al., 2016). Interestingly, neither biofilm presence nor *F. nucleatum* within biofilms correlated with *F. nucleatum* invasion into the epithelial cells, suggesting the bacteria's invasive capacity is independent from its biofilm forming capacity (Yu et al., 2016).

Given the theory that the appendix is a biofilm repository for replenishing the colonic microbiota, it is interesting to note that appendectomy was associated with a 14% higher incidence of CRC in a cohort of Taiwanese patients, with a higher risk for rectal cancer (left-sided) compared to cancer of the cecum-ascending colon (right-sided) (Fig. 1.4.3) (Wu et al., 2015). Similarly, there are also correlations between appendectomy and increased *C. difficile* infections and CD (but not UC), supporting the idea that the appendix may play a role in microbiota-associated disease (Andersson et al., 2003; Im et al., 2011).

Ultimately, multiple factors including microbiota composition and organization, host genetics, age, diet and environmental conditions shape the outcomes of host-microbiota interactions. Thus, the surrounding context must be accounted for when trying to understand the mechanisms of microbiota-associated immune effects and

diseases. In Chapter 2, we examined how the microbiota promotes systemic neutrophil development and function in the developing zebrafish. We found colonization of GF zebrafish with a conventional microbiota increased neutrophil numbers and altered neutrophil localization and functions. Additionally, we show that the acute phase protein SAA, is also induced upon microbiota colonization and mediates neutrophil migratory behaviors. Finally, *in vitro* experiments revealed NF-κB is activated upon SAA exposure and is required within neutrophils for SAA-dependent migration.

In Chapter 3, we utilized gnotobiotic *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice to examine the capacity of CRC-associated microbes to induce inflammation and colorectal cancer. We also investigated the functional role of human biofilm associated microbes in CRC development. We found colon tumorigenesis in SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice correlated with inflammation and was dependent on the microbiota, while small intestine tumors correlated with age and were independent from the microbiota. *F. nucleatum* and *pks*+ *E. coli* had differential capacities to induce inflammation and tumorigenesis, with only the latter promoting colon tumorigenesis in a colibactin dependent manner. Finally, we showed that biofilm forming microbes promote tumorigenesis, suggesting bacterial organization also contributes to CRC pathogenesis.

1.4. Figures

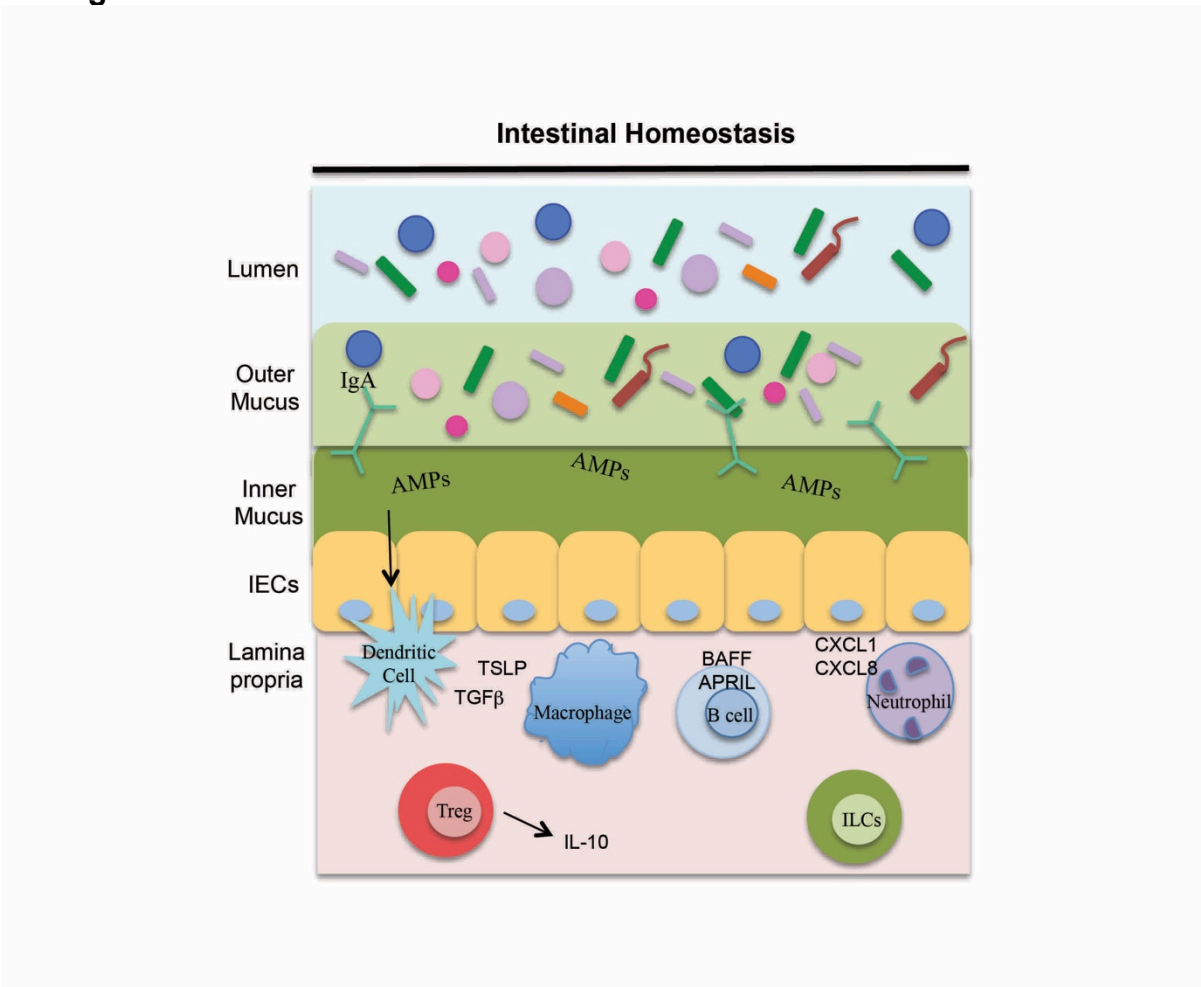


Figure 1.4.1. The colon microbiota and immune system under homeostatic conditions. The inner mucus layer of the colon is mostly bacteria free and serves as a barrier between the microbiota and IECs. IECs secrete mucus and AMPs to maintain separation and control microbiota composition. IECs also produce TSLP and TGFβ, which promotes tolerogenic macrophages and dendritic cells. Macrophages and dendritic cells actively sample antigens from the microbiota. Neutrophils prevent bacterial translocation across the epithelial barrier if recruited to the colon by chemokines. ILCS coordinate signals between the microbiota and the innate and adaptive immune system. Tregs mediate tolerance to the microbiota through IL-10 production. B cells are primarily devoted to IgA production and are stimulated by BAFF and APRIL from IECs.

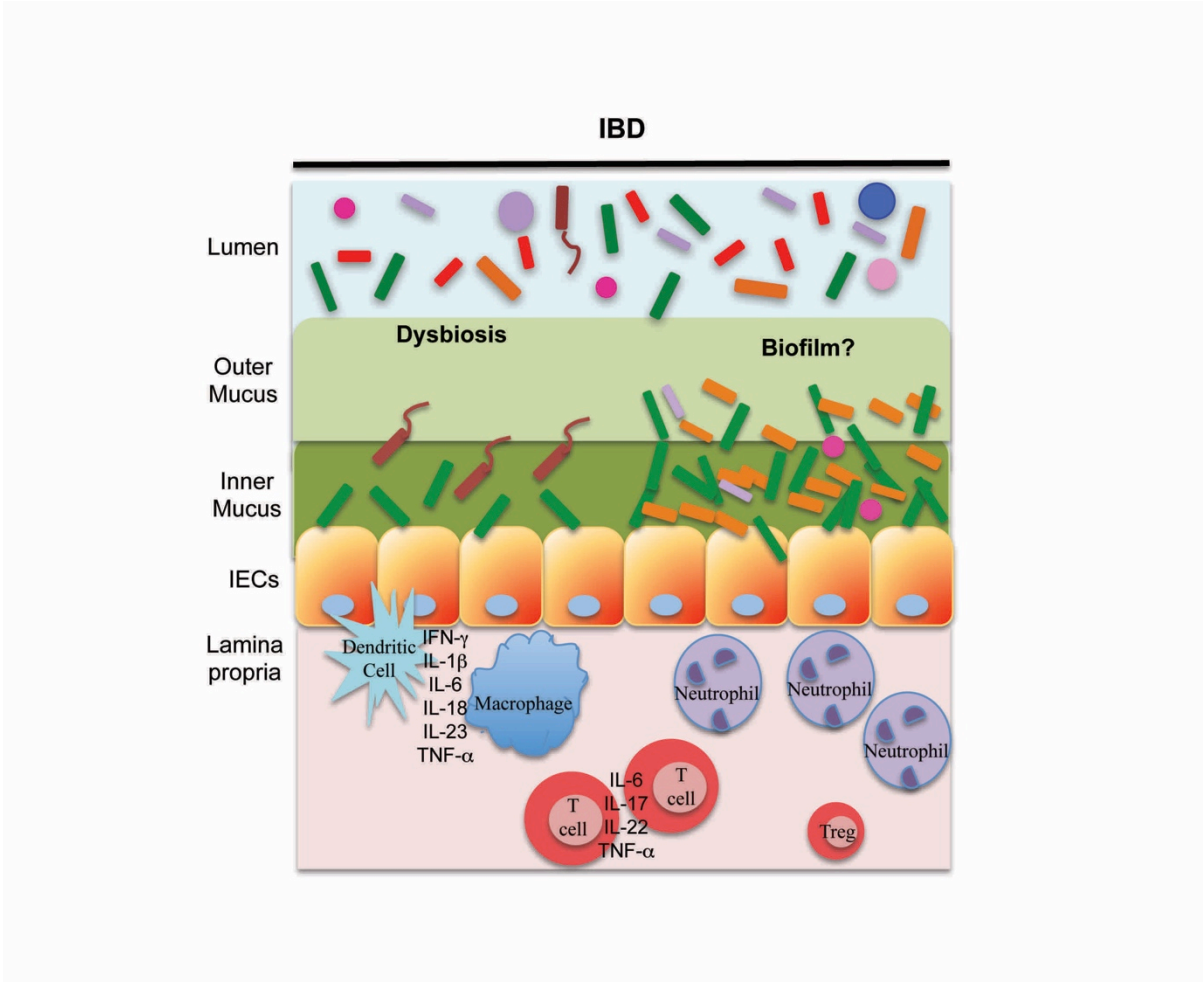


Figure 1.4.2. Immune and microbiota alterations associated with IBD. Immune alterations associated with IBD include an increase in neutrophils, increased pro-inflammatory cytokine production by macrophages, dendritic cells, and effector T cells, and Tregs with insufficient suppressive capacities. Bacterial dysbiosis associated with IBD includes increased Enterobacteriaceae (AIEC) and suphate-reducing bacteria, a decrease in *Faecalibacterium prausnitzii* and obligate anaerobes (*Bifidobacterium*). High density, bacterial biofilms (>10^9 bacteria/mL) that adhere to the epithelium have been observed at an increased frequency in IBD patients compared to controls and were associated with a predominance of *B. fragilis* and Enterobacteriaceae.

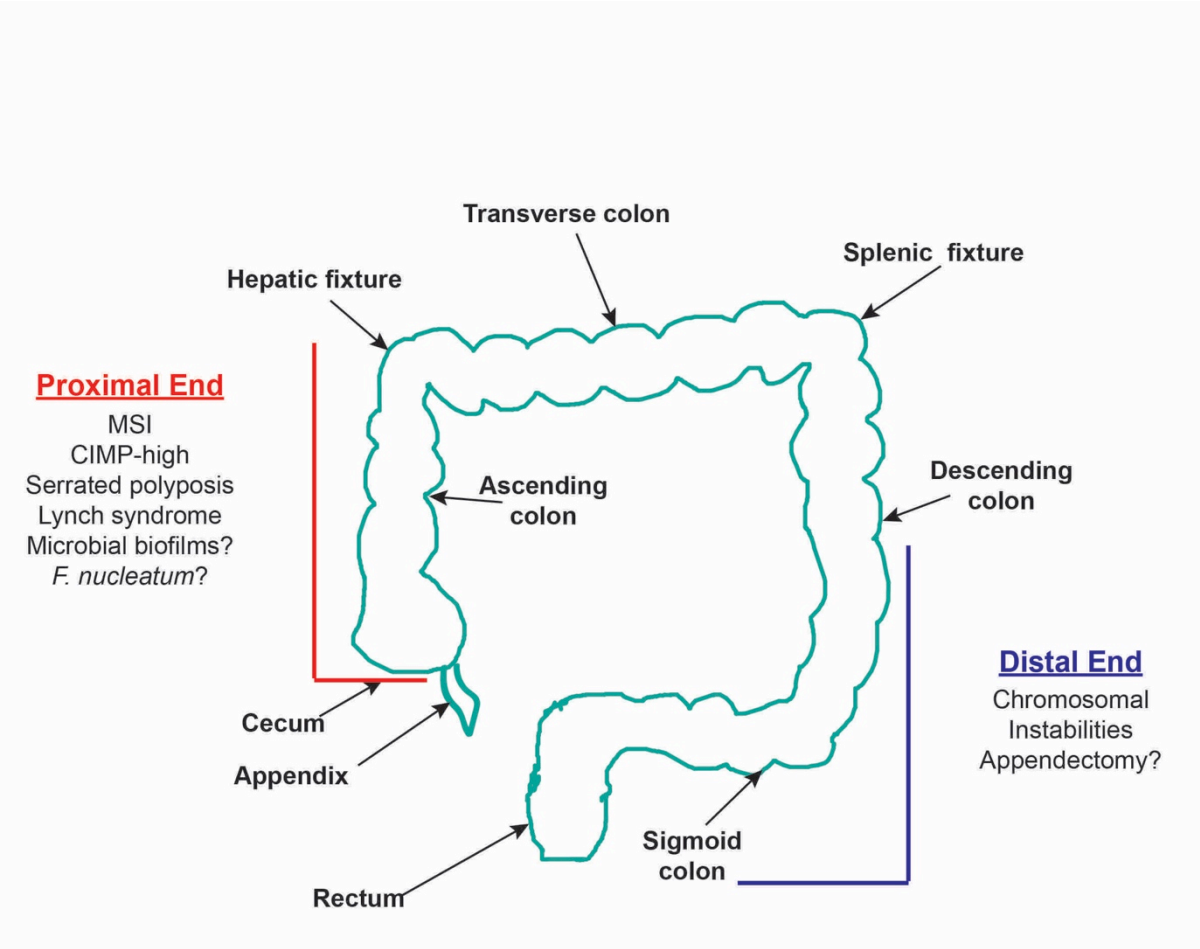


Figure 1.4.3. Spatial distribution of CRC types and associated microbiota changes. The anatomy of the human colon is depicted, along with a listing of the proximal versus distal associations observed in human CRCs. The proximal (right-sided) region is bracketed in red, while the distal (left-sided) region is bracketed in blue. The hypermutated forms of sporadic CRC: MSI and CIMP-high are more frequently associated with the proximal colon. Serrated polyposis and Lynch syndrome are hereditary CRCs and also more frequently associated with the proximal region. Microbial biofilms are associated with proximal CRCs according to Dejea et al. 2014 but not Yu et al. 2016, which could be due to different colon divisions and biofilm definitions. Invasive *F. nucleatum* has been observed in serrated polyposis. Nonhypermutated sporadic CRCs with chromosomal instabilities (chromosomal and subchromasomal changes) are more frequently associated with the distal colon. Additionally, one study suggests appendectomy is associated with a higher risk for rectal cancers.

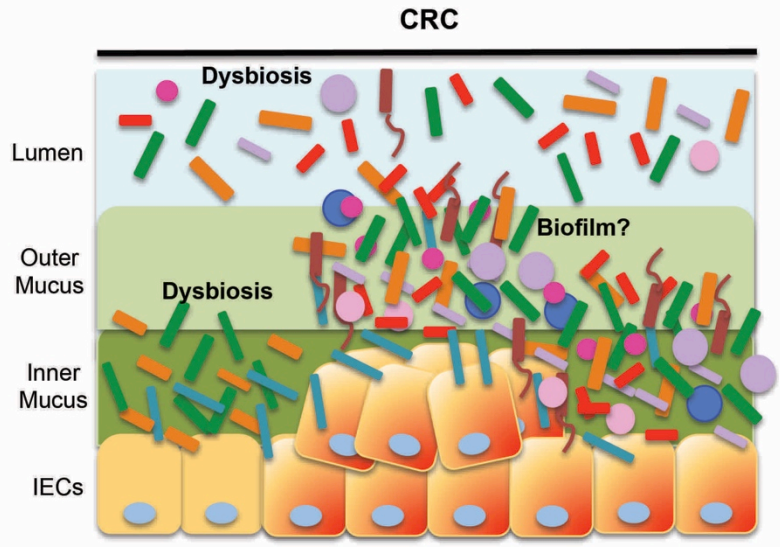


Figure 1.4.4. Microbiota dysbiosis and organizational changes associated with CRC. Luminal dysbiosis in CRC patients is associated with increased *Alistipes*, *Anaerococcus*, *Escherichia*, *Fusobacterium*, *Parabacteroides*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, and *Solobacterium* and decreases in *Roseburia*. Mucosal-associated bacteria in CRC include *F. nucleatum*, Enterotoxigenic *B. fragilis*, and *pks+ E. coli*, with *F. nucleatum* also enriched in adenoma and adenocarcionoma tissue. Dense bacterial biofilms of $>10^9$ bacteria/ml spanning at least 200 μm of epithelial surface have been associated with tumor and normal-flanking tissue from right-sided CRCs.

CHAPTER 2 : COMMENSAL MICROBIOTA STIMULATE SYSTEMIC NEUTROPHIL MIGRATION THROUGH INDUCTION OF SERUM AMYLOID A[1]

2.1. Overview

Neutrophils serve critical roles in inflammatory responses to infection and injury, and mechanisms governing their activity represent attractive targets for controlling inflammation. The commensal microbiota is known to regulate the activity of neutrophils and other leukocytes in the intestine, but the systemic impact of the microbiota on neutrophils remains unknown. Here we utilized *in vivo* imaging in gnotobiotic zebrafish to reveal diverse effects of microbiota colonization on systemic neutrophil development and function. The presence of a microbiota resulted in increased neutrophil number and myeloperoxidase expression, and altered neutrophil localization and migratory behaviors. These effects of the microbiota on neutrophil homeostasis were accompanied by an increased recruitment of neutrophils to injury. Genetic analysis identified the microbiota-induced acute phase

[1] Kanther, M*., **Tomkovich, S*.**, Xiaolun, S., Grosser, M.R., Koo, J., Flynn, E.J., Jobin, C[*]., and Rawls[*], J.F. (2014). Commensal microbiota stimulate systemic neutrophil migration through induction of serum amyloid A. Cell Microbiol *16*, 1053–1067.

I am co-first author on this previously published manuscript. I performed the following experiments: validation of morpholino knockdown, in vivo cell tracking anaysis of EGFP-expressing neutrophils, and mouse neutrophil isolation and migration assay. I generated some of the figures and contributed to the writing of the methods, results, discussion and figure legends.

protein serum amyloid A (Saa) as a host factor mediating microbial stimulation of tissue-specific neutrophil migratory behaviors. *In vitro* studies revealed that zebrafish cells respond to Saa exposure by activating NF-κB, and that Saa-dependent neutrophil migration requires NF-κB-dependent gene expression. These results implicate the commensal microbiota as an important environmental factor regulating diverse aspects of systemic neutrophil development and function, and reveal a critical role for a Saa-NF-κB signaling axis in mediating neutrophil migratory responses.

2.2. Introduction

Leukocytes such as neutrophils and macrophages are key mediators and effectors of inflammatory stimuli and represent attractive therapeutic targets for controlling acute and chronic inflammation. The complex community of microorganisms residing within the intestine (gut microbiota) has been identified as an important environmental factor regulating leukocyte function within the intestinal compartment (Abt and Artis, 2009). However, the presence of microbiota appears to also have profound systemic effects on leukocytes. Peripheral neutrophils collected from germ-free (GF) rodents display reduced phagocytosis, microbicidal activity, and production of superoxide anion and nitric oxide compared with ex-GF animals colonized with normal microbiota (conventionalized or CONVD) (Clarke et al., 2010; Ohkubo et al., 1990). Similarly, macrophages collected from peritoneal exudate in GF animals display reduced superoxide anion production and microbicidal activity, and impaired chemotaxis compared with CONVD controls (Czuprynski and Brown,

1985; Jungi and McGregor, 1978; Mitsuyama et al., 1986; Mørland and Midtvedt, 1984; Oliveira et al., 2005). The importance of the microbiota on systemic inflammation is further underscored by reports that multiple rodent models of spondyloarthritis do not develop disease when raised under GF conditions (Rehákova et al., 2000; Taurog et al., 1994), but disease can be initiated upon gut colonization with specific bacteria (Rath et al., 1996; Sinkorová et al., 2008).

Although recent research has yielded an abundance of new information about the impact of gut microbiota on intestinal leukocyte biology and immunity (Abt and Artis, 2009), gut microbiota effects on systemic leukocyte biology remain relatively unresolved. Our current information of the systemic effects of microbiota on neutrophils is largely derived from *ex vivo* experiments conducted on neutrophils collected from peripheral blood or bone marrow from GF and CONVD mammals (Clarke et al., 2010; Ohkubo et al., 1990). However, studies generated from *ex vivo* neutrophils may not be representative of the systemic population and do not fully recapitulate the native physiologic context of live tissues. Finally, mammals are not amenable to the high-resolution *in vivo* microscopy required to comprehensively define the systemic impact of microbiota on neutrophils. As a result, the specific aspects of systemic neutrophil activity affected by microbiota are not fully understood.

The zebrafish has several features that make it an attractive model to study the roles of commensal microbiota on systemic neutrophil biology. First, zebrafish are optically transparent from fertilization through early adulthood, permitting high-resolution imaging of host–microbe interactions in the intact physiologic context of a

living vertebrate (Kanther et al., 2011; Rawls et al., 2007). Second, the zebrafish has innate and adaptive immune systems that share extensive homology with those of humans and other mammals (Kanther and Rawls, 2010). Likewise, the digestive tracts of zebrafish and mammals are similar, including an intestine, pancreas, liver and gall bladder (Ng et al., 2005; Wallace et al., 2005). Third, we have developed methods for rearing zebrafish under GF conditions and colonizing GF zebrafish with members of the commensal microbiota (Pham et al., 2008).

Previous analyses of gnotobiotic zebrafish and mice have revealed that the presence of a microbiota causes extensive alterations in diverse aspects of host immunity and physiology. Reciprocally, host-mediated mucosal factors such as antimicrobial proteins, IgA, mucins, and inflammation alter microbial community composition and function (reviewed in (Abt and Artis, 2009; Hooper et al., 2012; Kanther and Rawls, 2010; Tremaroli and Bäckhed, 2012)). This complex interplay between host and microbial factors is central to the maintenance of homeostasis. However the host signaling pathways that mediate microbial cues to regulate systemic leukocyte responses remain unresolved.

Serum amyloid A (Saa) is a circulating HDL-associated apolipoprotein and acute phase protein. The Saa gene family (3 in humans, 4 in mice, 1 in zebrafish) is conserved across vertebrates (Fig. S2.1), suggesting important biological roles. *Saa* genes are expressed by multiple tissues including liver, intestinal epithelium (Eckhardt et al., 2010), and macrophages (Meek et al., 1992) and are markedly induced by diverse inflammatory stimuli (Uhlar and Whitehead, 1999) including gut microbiota (Hooper et al., 2001; Ivanov et al., 2009; Kanther et al., 2011; Rawls et

al., 2006). Serum Saa protein level is a salient biomarker for inflammatory disorders including IBD (Noble et al., 2008; Okahara et al., 2005), necrotizing enterocolitis, sepsis (Ng et al., 2010), and chronic obstructive pulmonary disease (Bozinovski et al., 2008). The precise roles of Saa in inflammation remain elusive because both pro- and anti-inflammatory actions have been reported. Reported pro-inflammatory roles for Saa include stimulation of extracellular matrix (ECM)-degrading enzymes such as MMP9 (Lee et al., 2005), recruitment of neutrophils and monocytes (Badolato et al., 1994; Connolly et al., 2010; Su et al., 1999), suppression of neutrophil apoptosis (Christenson et al., 2008), stimulation of granulocytosis (He et al., 2009), opsonization of Gram-negative bacteria (Shah et al., 2006), Nlrp3 inflammasome activation (Ather et al., 2011; Niemi et al., 2011), and stimulation of pro-inflammatory cytokines such as IL1β (Cheng et al., 2008; Lee et al., 2005; Niemi et al., 2011; Patel et al., 1998). In contrast, numerous reports cite anti-inflammatory effects of Saa on neutrophils, including inhibition of MPO production (Renckens et al., 2006), oxidative burst (Gatt et al., 1998; Linke et al., 1991), and migration (Gatt et al., 1998), and induction of IL10 expression (Cheng et al., 2008; De Santo et al., 2010; Shah et al., 2006). These diverse effects may be due to Saa's ability to stimulate signaling events through multiple transmembrane receptors, including formyl peptide receptor 2 (Fpr2/Fprl1) (Su et al., 1999), receptor for advanced glycosylation end-products (RAGE) (Cai et al., 2007), scavenger receptor class B type I (Scarb1/CLA-1) (Baranova et al., 2005), and Toll-like receptor 2 (Tlr2) (He et al., 2009). The signal transduction pathways that act downstream of Saa to regulate gene expression include extracellular signal-regulated kinases 1 and 2 (ERK1/2),

p38, c-Jun N-terminal kinase (JNK), Akt and NF-κB (Baranova et al., 2005; He et al., 2009; Jijon et al., 2005). However, the relationship between these pathways and the distinct immune cellular responses evoked by Saa remain unclear. Importantly, *in vivo* genetic analysis of Saa has been complicated by the fact that the human and mouse genomes encode 3 and 4 paralogous Saa genes respectively (Fig. S2.1). We previously showed that colonization with a normal microbiota in zebrafish results in NF-κB-dependent induction of *saa* expression in the distal intestine, liver and swim bladder (Kanther et al., 2011). However, the *in vivo* roles of Saa in systemic neutrophil biology, as well as neutrophil requirements for NF-κB in these responses, remain unclear. In this study, we took advantage of the fact that the zebrafish genome encodes only a single Saa gene to define the requirement for Saa in microbiota-induced neutrophil responses. Our results reveal novel roles for the microbiota on systemic neutrophil biology including increased number and migratory behavior and suggest that Saa-dependent neutrophil migration requires NF-κB signaling.

2.3. Materials and Methods

Animal husbandry. All experiments using zebrafish and mice were performed using protocols approved by the Animal Studies Committee of the University of North Carolina at Chapel Hill. Conventionally raised wild-type (TL strain) and *Tg(BACmpx:GFP)*^{i114} [hereafter referred to as *Tg(mpx:GFP)*] (Renshaw et al., 2006) zebrafish were maintained as described (Flynn et al., 2009; Kanther et al., 2011). Production using *in vitro* fertilization methods, colonization, maintenance and sterility

testing of GF zebrafish was performed as described (Pham et al., 2008). GF and CONVD animals were reared at an average density of 1.3 animals per ml in sterile vented tissue culture flasks (Cellstar) housed in an air incubator at 28.5°C on a 14 h light cycle. Wild-type 8- to 12-wk-old C57BL/6 mice were maintained under specific pathogen free conditions.

***In vivo* imaging.** For time-lapse imaging, zebrafish were anesthetized in 4x Tricaine (MS-222; Sigma-Aldrich), and then mounted in 1% low melting point agarose containing 1x Tricaine on glass bottom dishes (MatTek Corporation). Solidified agarose containing fish was then covered in sterile GZM containing 2x Tricaine. Timelapse movies were captured using a Zeiss 510 Meta Laser Scanning Confocal Microscope at a rate of 1 frame every 15 s for 5 or 15 min. For live whole animal imaging, zebrafish were anesthetized as described above, mounted in 3% methylcellulose, and imaged using a Leica M205 FA stereomicroscope.

Tail wounding assay. GF and CONVD 6dpf *Tg(mpx:GFP)* zebrafish were anesthetized as described above. Fish were mounted in 3% methylcellulose on a 35 mm Petri dish by placing only the anterior part of the fish into the methylcellulose. The posterior part of the fish was covered with sterile GZM. Fish were then observed under a LeicaS6E StereoZoom stereomicroscope with a Leica L2 cold light illuminator. Tail amputations were performed posterior to the end of the notochord using a scalpel. GF and CONVD animals were revived in sterile GZM containing antibiotics (AB-GZM) (Pham et al., 2008), and kept at 28.5°C. Fish were collected at

time points indicated and euthanized in 8 × Tricaine. Larvae were fixed in 4% paraformaldehyde overnight at 4°C, and washed 3 times for 10 min and 3 times for 1 h in PBS + 0.2% Tween. Fish were then mounted in 3% methylcellulose and imaged using a Leica M205 FA stereomicroscope. Numbers of GFP(+) neutrophils posterior to the notochord were quantified in 8–10 fish for each condition.

Morpholino injections and validation. Zebrafish embryos at the 1–2 cell stage were injected with morpholinos (GeneTools LLC) targeting saa (saa.i2e3, 0.9 pmol per embryo; GTCCTTTGCACTTCAAAAATAGAGT), or standard control MO (0.9 pmol per embryo; Gene Tools LLC) using a Drummond Nanoject II microinjector. Efficacy of splice-blocking MOs was measured by RT-PCR. cDNA was prepared from pools of whole larvae at 6dpf as described (5–15 larvae per pool) (Rawls et al., 2007), and 10 ng of cDNA was used as a template in PCR reactions using gene-specific primers (forward: CTTGCTGTGCTGGTGATGTT; reverse: AGTCTTCTGGGGGT CATCTTC). We resolved PCR amplicons on 2% agarose gels to detect morphant transcripts (Fig. S2.2).

Flow cytometry analysis. *Tg(mpx:GFP)* zebrafish were reared under GF and CONVD conditions. GF and CONVD 6dpf larvae were pooled and killed (50 animals per condition per experiment). Excess media was removed and animals were finely chopped using sterile razor blades in a 10 cm Petri dish, diluted in 1.5 ml 5% fetal bovine serum in Hanks' balanced salt solution (FBS/HBSS), and then transferred to sterile Eppendorf tubes. Cells were pelleted at 1000 *g* for 5 min at 4°C, and cell

pellets were washed with 1 ml FBS/HBSS and pelleted again at 1000 *g* for 5 min at 4°C. Cell pellets were then treated with 500 µl 10 mg ml^{-1} collagenase/ dispase liberase (Roche) solution in FBSS/HBSS for 35 min at room temperature with vigorous shaking. Digestion was stopped using 500 µl stopping solution [100 µl 0.5 M EDTA (pH 8.0) in 9.9 ml FBS/HBSS]. Cells were pelleted at 1000 *g* for 5 min at 4°C. The cell pellets were resuspended in 500 µl FBS/HBSS and passed through 40 µm mesh filter (BD Falcon), and the mesh was washed twice with 250 µl FBSS/HBSS. Cells were then brought to final volume of 1 ml of FBS/HBSS prior to sorting using a MoFlo sorting flow cytometer (Beckman Coulter). Conventionally raised non-transgenic and transgenic controls (pools of 50 fish per genotype) were prepared as above and used to define fluorescence and cell size gates. Single cell suspensions from GF and CONVD zebrafish were sorted for GFP fluorescence using Summit software. For RNA analysis GFP(+) neutrophils were collected in 1 ml cold FBSS/HBSS and stored at 4°C. These cells were then pelleted at 1000 g for 5 min at 4°C. The supernatant was then removed and replaced with 1 ml Trizol (Invitrogen). Cells were then prepared as described below for quantitative RT-PCR analysis.

Cell tracking analysis. Tracking analysis of EGFP-expressing neutrophils was performed in Volocity (Improvision, Perkin Elmer). The locations of individual cells were tracked in each frame of time-lapse images (5 or 15 min) captured as described above using Volocity's 'track objects manually' tool. Tracking was performed as described in the Volocity User Guide. 3–5 time-lapse movies per

experimental condition were analyzed. For tissue-specific analyses, tracked cells were categorized as either caudal hematopoietic tissue (CHT), intestine, or fin cells based on their location for the duration of the movie.

Zebrafish cell culture and stimulation. PAC-2 zebrafish embryonic fibroblast cells were grown at 28°C in Leibowitz L-15 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and penicillin G (50 U ml^{-1}; Life Technologies) in 0.5% CO_2. Cell lines were used between passages 15 and 35. Cells were grown to near confluency (90%) in 6-well plates (Costar), and were stimulated with LPS (10 μg ml^{-1}; from *Escherichia coli* 0111:B4; Sigma) or SAA (1, 4 μM; PeproTech) for the specified amount of time in media containing 1% FBS. Immunofluorescence assays for RelA/p65 were performed as described (Kanther et al., 2011).

Western immunoblot analysis. PAC-2 cells were stimulated with LPS (10 μg ml^{-1}) or SAA (4 μM) at specified time points. Cells were harvested and lysed in 1x Laemmli buffer, and the protein concentration was measured using a Bio-Rad quantification assay (Bio-Rad Laboratories). Western blot for IκB α (S32; Cell Signaling) and actin (MP Biomedicals) was performed as described previously (Kanther et al., 2011).

Transfection and luciferase activity assays. For transfections, PAC-2 cells were seeded into 12-well tissue culture plates ($\sim 5 \times 10^5$ cells per well) and grown in 1 ml

medium with 1% FBS at 28°C to ~70% confluency. Transfections with 0.2 μg ml^{-1} of the previously described pikbaa:Luc were performed using Lipofectamine 2000 (Invitrogen) as described by the manufacturer (Kanther et al., 2011). After 24 h transfection, fresh medium was supplied and cells were stimulated with LPS (10 μg ml^{-1}) or SAA (4 μM) for 24 h. Cells were then lysed and luciferase activity was determined using an LMax luminometer microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were normalized for extract protein concentrations measured with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR analysis. To isolate RNA from whole zebrafish larvae, 6dpf larvae (5–30 larvae per group) were anesthetized in 4x Tricaine (SigmaAldrich), placed in 1 ml Trizol (Invitrogen) taking care to remove any excess media, and then repeatedly passed through a 25-gauge needle (BD Syringe) until homogenized. Samples were vortexed for 30 s, incubated at room temperature for 5 min, supplemented with 0.2 ml chloroform per sample, vortexed for 30 sec, and incubated at room temperature for 2 min. The samples were then centrifuged at 12 000 *g* for 15 minutes at 4°C, and then the colorless upper phase containing the RNA was transferred to a new RNAse-free tube. An equal volume of 70% RNAse-free ethanol was added to each tube containing the upper phase, and then RNA was isolated using PureLinkTMRNA Mini Kit (Ambion) following the manufacturer's specifications. To isolate RNA from primary sorted zebrafish neutrophils or cultured zebrafish PAC2 fibroblasts, we used Trizol (Invitrogen) following manufacturer's specifications. Total RNA was used in reverse transcription and quantitative PCR assays using gene-

specific primers for *18S* (forward: CACTTGTCCCTCTAAGAAGTTGCA; reverse: GGTT GATTCCGATAACGAACGA), *mpx* (forward: TCCAAAGCTATG TGGGATGTGA; reverse: GTCGTCCGGCAAAACTGAA), *ncf1* (forward: TTCATCTCGCCGTCAGACTCGTTT; reverse: TGTAC ACATAGTGCTGGCTGGGAA), *il1b* (forward: TGGACTTCG CAGCACAAAATG; reverse: GTTCACTTCACGCTCTTGGATG), *ikbaa* (forward: GCCGTGCAGATCATCAAAC; reverse: CCGC TGTAGTTAGGGAAGGT), and *mmp9* (forward: CATCACTG AAATCCAGAAGGAGCTT; reverse: GTTCACCATTGCCTGA GATCTTC) as described (Kanther et al., 2011).

Neutrophil isolation and migration assay. Mice were injected intraperitoneally with 2.5 ml of 3% Fluid Thioglycollate Medium (Difco Laboratories) previously autoclaved for 15 min under 15 psi. Mice were euthanized with CO_2 intoxication and neutrophils in the peritoneal cavity were retrieved by lavage with 10 ml of ice-cold HBSS supplemented with 1.5 mM ethylene diamine-tetraacetic acid (EDTA). Neutrophils were resuspended in 0.5% FBS RPMI 1640 medium and pretreated for 1 h with the NF-κB inhibitor BAY-11–7082 (25 μM; Calbiochem), or cycloheximide (50 μg ml^{-1}; Sigma). Cells were plated at ~2 × 10^6 per insert in 6-well Transwells (Corning) with 3 μm pore in the presence of SAA (25 μg ml^{-1}) and incubated at 37°C and 5% CO_2 for 2.5 h. Neutrophils were imaged and counted as previously described (Sun et al., 2012).

Phylogenetic analysis. Protein sequences were exported from GenBank into the Cipres Science Gateway v3.2 (Miller et al., 2010) and aligned using Muscle. Phylogenetic trees were inferred using maximum likelihood in RAxML HPC2 7.3.1 on XSEDE using a GAMMA model and a BLOSUM62 protein substitution matrix. The best-scoring ML tree was identified and prepared using Dendroscope v1.4. Rapid bootstrap resampling (1000 replicates) was used to test the robustness of inferred topologies. Multiple sequence alignments were prepared using Boxshade.

Statistical methods. Statistical analysis was performed using unpaired two-tailed Student's t-test, or one-way analysis of variance (ANOVA) followed by Tukey's post-test. Values were calculated using GraphPad Prism software, and $P < 0.05$ was considered significant.

2.4. Results

Microbiota promotes increased neutrophil number and pro-inflammatory gene expression

To investigate the impact of the commensal microbiota on zebrafish myeloid lineages, we queried results from a microarray-based functional genomic comparison of gene expression in whole zebrafish at 6 days post fertilization (dpf) that had been raised GF or conventionalized since 3dpf (CONVD). Functional categorization of the resulting list of microbiota-regulated transcripts revealed enrichment for genes involved in leukocyte development and function (Kanther et al., 2011). CONVD zebrafish displayed relative increases in transcript levels for 17

genes known to be specifically expressed by myeloid leukocytes, including the zebrafish homologue of mammalian myeloperoxidase *mpx* (also called *mpo*; Table 1) (Kanther et al., 2011; Rawls et al., 2004, 2006). Our previous whole-mount *in situ* hybridization analysis of *mpx* mRNA in GF and CONVD zebrafish suggested that this increase in *mpx* transcript level could be due to increased neutrophil number or increased *mpx* mRNA levels in individual neutrophils (Kanther et al., 2011). To test if these transcript differences were associated with alterations in neutrophil number, we used transgenic *Tg(mpx:GFP)* zebrafish that robustly express GFP specifically in neutrophils (Renshaw et al ., 2006). Stereomicroscopic evaluation of GFP(+) neutrophil number and localization in whole 6dpf GF and CONVD zebrafish revealed a qualitative increase in neutrophil number throughout the animal (Fig. 2.6.1A). Flow cytometry of GFP(+) neutrophils from dissociated 6dpf *Tg(mpx:GFP)* fish confirmed a significant increase in total steady-state number of neutrophils per animal in CONVD compared with GF animals (Fig. 2.6.1B). Quantitative RT-PCR in flow-sorted neutrophils from GF and CONVD larvae revealed significant increases in *mpx* mRNA in sorted neutrophils from CONVD animals (Fig. 2.6.1C). Colonization with a commensal microbiota therefore results in significant increases in neutrophil *mpx* expression together with increases in steady-state neutrophil number.

Microbiota regulates tissue distribution and migration of neutrophils

We imaged whole 6dpf GF and CONVD *Tg(mpx:GFP)* zebrafish to evaluate the effect of microbiota on neutrophil localization and migration. Consistent with our

previous *mpx* RNA whole-mount *in situ* hybridization results (Kanther et al., 2011), CONVD zebrafish displayed increased GFP expression in the kidney, a site of definitive hematopoiesis (Fig. 2.6.1A). Since the intestine harbors microbial communities that are markedly denser than that of the surrounding water, we analyzed the frequency and distribution of GFP(+) neutrophils associated with intestines dissected from *Tg(mpx:GFP)* GF and CONVD larvae. We observed increased numbers of GFP(+) neutrophils in the intestines of CONVD *Tg(mpx:GFP)* zebrafish compared with GF controls, most significantly in the proximal region (segment 1) of the intestine (Fig. 2.6.1D). To determine if these changes in localization were associated with altered neutrophil migratory behaviors, we used confocal microscopy to quantify migration of individual neutrophils in live 6dpf GF and CONVD *Tg(mpx:GFP)* fish. Compared with GF controls, neutrophils in CONVD animals displayed significantly elevated migration velocity and decreased meandering (i.e. increased directional migration) compared with GF controls (Fig. 2.6.2, Movies S1 and S2). These results indicate that the microbiota regulates systemic neutrophil localization and migratory activity.

Microbiota promotes neutrophil recruitment to extra-intestinal injury

To determine if the observed effects of microbiota on neutrophil number, localization, and migration have functional consequences, we used a well-established injury model in which a portion of the tail fin in larval zebrafish is resected and the recruitment of leukocytes to the wound is quantified over time

(Renshaw et al., 2006; Yoo and Huttenlocher, 2011). Although early (1 h) GFP(+) neutrophil recruitment to the wound was slightly higher in GF animals compared with CONVD controls, later evaluation at 3, 6 and 15 h after injury revealed significantly more neutrophils recruited to the wound in CONVD animals (Fig. 2.6.3). These results confirm that colonization with a microbiota augments the host's capacity for recruiting neutrophils to extra-intestinal injury.

Saa is required for increases in neutrophil migration and suppression of inflammatory biomarkers following colonization with a microbiota

We next sought to define the role of zebrafish *saa* on neutrophil responses to the microbiota. Injection of zebrafish embryos with morpholino antisense oligonucleotides (MO) targeting *saa* resulted in partial knockdown of *saa* transcript through 6dpf (Fig. S2.2A). By comparing 6dpf GF and CONVD *Tg(mpx:GFP)* zebrafish injected with a MO targeting *saa* (*saa*-MO) or a standard negative control MO (ctrl-MO), we found that *saa* knockdown did not qualitatively alter the effects of the microbiota on neutrophil tissue distribution (Fig. 2.6.4A). Using a computational approach to quantify GFP(+) neutrophil number in whole live zebrafish (Ellett and Lieschke, 2012), we observed that the microbiota-induced increases in neutrophil number (Fig. 2.6.4B) and *mpx* mRNA levels (Fig. 2.6.4C) were also not affected by *saa* knockdown. In contrast, *in vivo* imaging of 6dpf GF and CONVD *Tg(mpx:GFP)* fish revealed striking tissue-specific *saa*-dependent alterations in microbiota-induced neutrophil migration behavior (Movies S3–5 and S6). In ctrl-MO animals, neutrophils associated with the CHT, intestine, and fin displayed significantly increased

migration velocity in the presence of a microbiota. In *saa*-MO animals, the microbiota caused a similar increase in fin neutrophils but failed to increase neutrophil migration velocity in the CHT and intestine (Fig. 2.6.4F). The presence of a microbiota in ctrl-MO animals caused a significant reduction in the meandering index of neutrophils associated with the CHT but not those in the intestine or fin. In *saa*-MO animals, the effect of the microbiota on CHT neutrophil meandering was significantly attenuated (Fig. 2.6.4G). Strikingly, neutrophils associated with the intestine in *saa*-MO animals displayed a microbiota-dependent decrease in meandering that was not observed in ctrl-MO controls (Fig. 2.6.4G). These results suggest multiple novel *in vivo* roles for Saa in regulating tissue-specific neutrophil migratory behaviors. These alterations in microbiota-induced neutrophil migration in *saa*-MO fish were accompanied with significant increases in microbiota-dependent induction of inflammatory biomarkers *ncf1* and *il1b* (Fig. 2.6.4D and E). These results reveal a potential anti-inflammatory role for *saa* in suppressing inflammatory gene expression and complex tissue-specific roles in neutrophil migration responses to commensal microbiota.

Saa-dependent induction of neutrophil migration requires NF-κB activity

Saa has been shown to promote neutrophil migration in mammals (Connolly et al., 2010; He et al., 2009) and we have shown that SAA activates NF-κB signaling in mammalian cells (Jijon et al., 2005). Because the NF-κB transcription factor has been linked to neutrophil migration (Penzo et al., 2010), we hypothesized that increased Saa levels in response to microbiota might activate NF-κB and NF-κB-

dependent immune cell migration. To test this, we turned to cell culture where the cell autonomous roles of SAA and NF-κB could be readily evaluated. Culture methods for purified zebrafish neutrophils have not been established, therefore we first tested the effects of SAA on the PAC2 zebrafish fibroblast cell line. Western blot analysis showed that SAA induced phosphorylation of the NF-κB protein inhibitor IκBα, a key process for canonical NF-κB activity (Fig. 2.6.5A). Immunofluorescence analysis showed that SAA promoted nuclear translocation of the NF-κB transcriptional subunit RelA/p65 (data not shown). To confirm that SAA functionally impacts NF-κB signaling, we investigated transcriptional activity using pikbaa:Luc gene reporter system (Kanther et al., 2011). Luciferase activity increased ~3-fold in pikbaa:Luc-transfected PAC2 cells following SAA stimulation, a level similar to LPS stimulation (Fig. 2.6.5B). Importantly, increased NF-κB activity was associated with SAA-induced accumulation of NF-κB target genes *mmp9* and *ikbaa* mRNA (Fig. 2.6.5C and D). These findings demonstrate that SAA induces NF-κB signaling and expression of NF-κB target genes in zebrafish cells. We next sought to directly test the functional impact of NF-κB signaling in Saa-induced neutrophil migration using mouse peritoneal neutrophils. Using a transwell migration assay, we observed that peritoneal neutrophil motility in response to SAA increased by ~2-fold (Fig. 2.6.6A and B). Neutrophil migration was reduced by 82% when the NF-κB inhibitor Bay 11–7082 (BAY) was co-incubated with SAA (Fig. 2.6.6A and B). Treatment with the protein synthesis inhibitor cycloheximide (CHX) strongly attenuated SAA-induced neutrophil transmigration (73%), suggesting that NF-κB-mediated gene expression is

necessary for neutrophil movement (Fig. 2.6.6A and B). These findings implicate microbiota-induced SAA expression as an important host response regulating neutrophil behavior.

2.5. Discussion

The majority of microbes on the human body reside in the intestine, where they are known to contribute significantly to intestinal physiology and mucosal immunity. There is, however, increasing evidence that the influence of the microbiota extends beyond the confines of the intestine to other tissues and their pathologies (McFall-Ngai et al., 2013). Therefore, the identification of the cellular and molecular mechanisms by which the microbiota shapes the systemic physiology of animal hosts is an important research objective. An improved understanding of the microbiota's impact on systemic leukocyte function is particularly needed due to the implication of the microbiota in the etiology of inflammatory diseases in intestinal and extra-intestinal compartments. Previous analysis of the microbiota's impact on neutrophil biology in mammals has been limited to *ex vivo* comparisons of neutrophils collected from peripheral blood or bone marrow (Clarke et al., 2010; Ferencík et al., 1985; Ohkubo et al., 1990, 1999). Here we have utilized the transparency of the zebrafish to provide the first comprehensive view of the microbiota's systemic impact on *in vivo* neutrophil function. Our results reveal diverse consequences of microbiota colonization on neutrophil homeostasis and behavior, as well as recruitment of neutrophils to injury. We also show a mechanistic role for a SAA-NF-κB signaling axis in microbiota-dependent neutrophil migration.

These findings underscore the potential of the microbiota to influence the systemic physiology of animal hosts and provide an important new conceptual framework for understanding the microbiota's roles in inflammatory diseases.

Our observations of increased systemic neutrophil number in CONVD compared with GF zebrafish larvae reveal a novel role for the microbiota in defining the steady state neutrophil population. Systemic neutrophil number might be influenced by differences in microbiota composition or husbandry practices, because a recent comparison of starved GF and conventionally raised zebrafish larvae in a different zebrafish facility did not reveal differences in neutrophil number (Galindo-Villegas et al., 2012). Inflammatory stimuli can regulate the hematopoietic compartment in zebrafish, as injection with LPS induces myelopoiesis (Liongue et al., 2009) and bacterial infection or tail wounding induces mobilization of neutrophils from the CHT (Deng et al., 2013; Yoo and Huttenlocher, 2011). Our study using commensal microbial colonization adds a novel aspect to bacteria–host interaction by showing that microbial cues regulate myelopoietic programs.

In vivo imaging of GFP(+) neutrophils in gnotobiotic zebrafish revealed significant influences of microbiota on neutrophil localization and migratory behavior. We detected a quantitative increase in neutrophil localization in the intestines of CONVD versus GF zebrafish, with the most marked increases in the proximal intestine. This is consistent with a previous study by Bates and colleagues that reported an increase in MPO(+) cell number in the distal intestine of CONVD versus GF zebrafish (Bates et al., 2007). These microbiota-associated increases in intestinal neutrophil number may be due to tissue-specific alterations in neutrophil

recruitment or retention, or may simply reflect the observed overall increase in systemic neutrophil number. Sites of hematopoiesis are dynamic during zebrafish development, and occur in the CHT and kidney at the larval stages under study here (for review see (Kanther and Rawls, 2010). We observed salient qualitative increases in GFP(+) neutrophil localization to the kidney region in CONVD zebrafish. The significance of this microbiota-induced neutrophil localization remains unknown, and could be indicative of altered granulopoiesis in the kidney hematopoietic tissue or linked to microbiota-induced NF-κB activation in the adjacent swim bladder (Kanther et al., 2011). Time-lapse *in vivo* microscopy revealed that microbiota-induced alterations in neutrophil localization were accompanied by significantly increased neutrophil migration velocity in all evaluated tissues and decreased neutrophil meandering in the CHT region. These observed tissue-specific influences of the microbiota on neutrophil behavior underscore the utility of *in vivo* imaging in the zebrafish for defining the regional impact of microbial colonization status.

These systemic impacts of the microbiota on neutrophil development are predicted to have diverse functional consequences on host immunity and inflammation. In support, we found that the presence of a microbiota is associated with significant alterations in inflammatory gene expression in neutrophils. Our previous genomic comparison of whole GF and CONVD zebrafish larvae revealed that transcripts encoding multiple myeloid markers including neutrophil-specific *mpx* were increased in the presence of the microbiota (Kanther et al., 2011). Moreover, we previously identified individual bacterial species sufficient to induce *mpx* in gnotobiotic zebrafish (Kanther et al., 2011; Rawls et al., 2004, 2006) and showed

that this response requires functional bacterial flagella (Rawls et al., 2007). Here we found that *mpx* mRNA levels were increased in sorted neutrophils from CONVD compared with GF zebrafish larvae. This is consistent with previous analysis of MPO levels in neutrophils from gnotobiotic mammals (Ferencík et al., 1985), suggesting an evolutionarily conserved role for microbiota in controlling neutrophil gene expression programs. The breadth and impact of microbiota-induced alterations in neutrophil transcription remain to be defined. However, a recent genetic analysis in zebrafish revealed that *mpx* functions to downregulate H_2O_2 gradients established at sites of injury and thereby contributes to the resolution of inflammation (Pase et al., 2012). This data suggests that increased *mpx* expression in neutrophils in the presence of the microbiota might serve as an anti-inflammatory response to commensal microbial colonization. Consistent with a previous study (Galindo-Villegas et al., 2012), we also found that the presence of a microbiota significantly increased the number of neutrophils recruited to a fin injury. This could be due to increased recruitment or retention of neutrophils in the wound in the presence of a microbiota, or could reflect the observed overall increase in systemic neutrophil number in those animals. Although GF neutrophils express less *mpx* transcript, other microbiota-induced responses must cause this wound recruitment phenotype because *mpx*-deficient neutrophils develop normally and migrate normally to a fin wound (Pase et al., 2012). Notably, GF fish recruited more neutrophils exclusively at an early time point (1 h) after injury, suggesting neutrophils might be slower to arrive but accumulate in greater numbers in the wound of colonized animals. Together, these results establish that the microbiota regulates neutrophil function as well as

development, and suggest that identification of the underlying molecular mechanisms could provide potential therapeutic targets for controlling inflammation.

Our results identify a novel role for Saa in regulating neutrophil migratory behavior in response to microbiota colonization. SAA proteins are known to be produced by multiple vertebrate tissues and cell types in response to various stimuli including gut microbiota. Indeed, we showed that zebrafish *saa* transcript levels in distal intestine, liver, and swim bladder are elevated upon colonization with a microbiota via *myd88*-dependent and NF-κB-dependent mechanisms (Kanther et al., 2011). However the functional consequence of commensal microbiota-induced SAA on neutrophil activity remained unknown. The existence of a single Saa orthologue in zebrafish allowed us to test the requirement of *saa* function on neutrophil responses to the microbiota using MO knockdown. Microbiota-induced alterations in neutrophil number, localization and *mpx* levels were unaffected by *saa* knockdown, suggesting that these host responses do not require *saa*. In contrast, neutrophil migratory responses to the microbiota were strongly affected by *saa* knockdown. Microbiota-induced increases in neutrophil velocity were attenuated in the CHT and intestines of *saa*-MO fish but not in the fin. This reveals novel tissue-specific roles for *saa* in neutrophil migration, and suggests that neutrophils located in these tissue compartments might have different sensitivities or accessibility to Saa protein. The ability of the microbiota to induce a lower meandering index in the CHT was also attenuated following *saa* knockdown, suggesting that this host response also requires Saa. Knockdown of *saa* additionally caused an unexpected reduction in meandering index in the intestine, consistent with an increased inflammatory tone in

that tissue. In support of this notion, *saa* knockdown was associated with increased expression of inflammatory biomarkers *il1b* and *ncf1*, reminiscent of increased susceptibility to DSS colitis in mice deficient for *Saa1* and *Saa2* (Eckhardt et al., 2010). Importantly, MO injection resulted in only a partial knockdown of wild-type *saa* transcript. Therefore *saa* may have additional functions that could be revealed by stronger loss of function approaches.

Saa induces neutrophil migration in mammals as well as zebrafish. Saa is known to activate NF-κB signaling in mammalian cells, and NF-κB has also been linked to mammalian neutrophil migration. To test whether Saa induces neutrophil migration by activating NF-κB, we turned to cell culture platforms where the cell autonomous effects of Saa can be readily evaluated. We find that zebrafish fibroblasts, like mammalian cells, respond to Saa exposure by inducing the NF-κB signaling pathway and downstream transcriptional targets. Transwell migration assays using murine peritoneal neutrophils revealed that Saa-dependent induction of neutrophil migration requires NF-κB-dependent gene expression. We previously found that the ability of the microbiota to induce zebrafish *saa* required NF-κB (Kanther et al., 2011), indicating that the NF-κB pathway is involved at multiple steps in this process. Together, our data support a model in which the presence of a microbiota results in NF-κB-dependent induction of *saa* in multiple tissues, which leads to systemic NF-κB dependent increases in neutrophil migration. In parallel, the microbiota causes altered neutrophil number, localization, and *mpx* expression using *saa*-independent mechanisms. Since the NF-κB pathway regulates gene transcription in multiple tissues and cell types (Kanther et al., 2011), *in vivo* analysis

of the cell-autonomous roles of NF-κB in neutrophil behavior will require new approaches for controlling the NF-κB pathway specifically in the neutrophil lineage. Although this study focused exclusively on neutrophils, we anticipate that the microbiota might have similar effects on other leukocyte lineages. Additional studies are needed to determine the similarities and differences between leukocyte responses to colonization by commensal microbiota and infection with pathogenic microbes. Of particular interest are the mechanisms by which gut microbes might regulate aspects of hematopoiesis and mobilization of immune cells to distinct target tissues such as the gut. An improved understanding of how commensal and pathogenic microbes control systemic neutrophil function could lead to the development of new probiotic, antibiotic and pharmacologic approaches for controlling neutrophil activity and inflammation to reduce incidence and severity of human IBD and other inflammatory diseases.

2.6. Figures and Tables

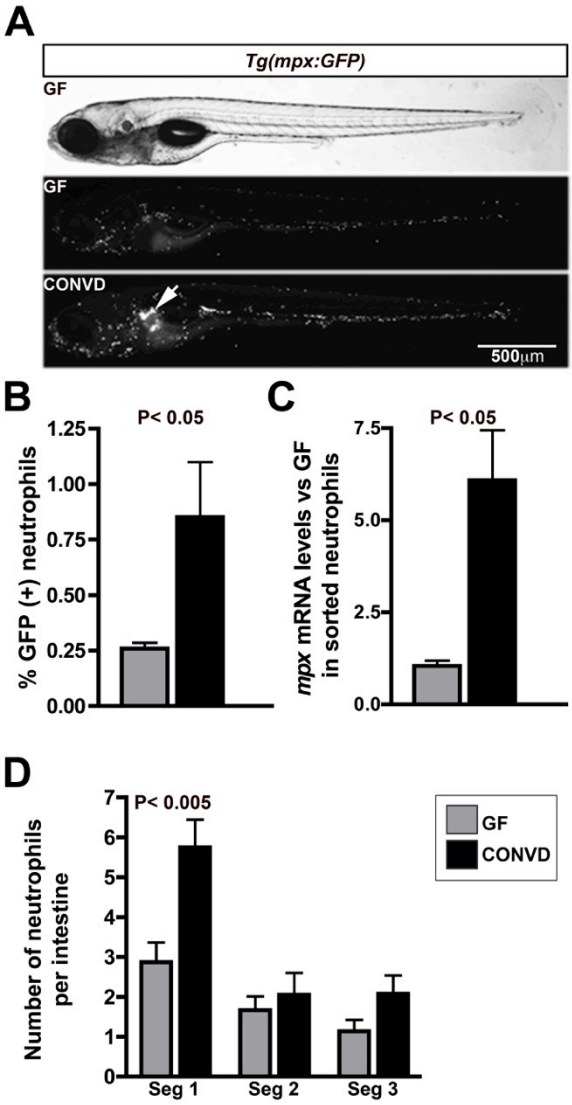


Figure 2.6.1. Microbiota regulates neutrophil localization, number and stimulates inflammatory biomarkers. A. Live 6dpf *Tg(mpx:GFP)* zebrafish show GFP(+) neutrophil localization as a function of microbial status. Note the increased neutrophil localization in the kidney (white arrow) in CONVD animals. B. Flow cytometry analysis reveals that the percent frequency of GFP(+) neutrophils in dissociated 6dpf *Tg(mpx:GFP)* zebrafish is higher in CONVD compared with GF animals. C. qRT-PCR for *mpx* mRNA levels in sorted neutrophils. D. Quantification of mean total number of GFP(+) neutrophils associated with dissected intestines by segment. Data are representative of 8–10 guts per microbial condition from two biological replicates. Significant Student's *t*-test *P*-values are shown.

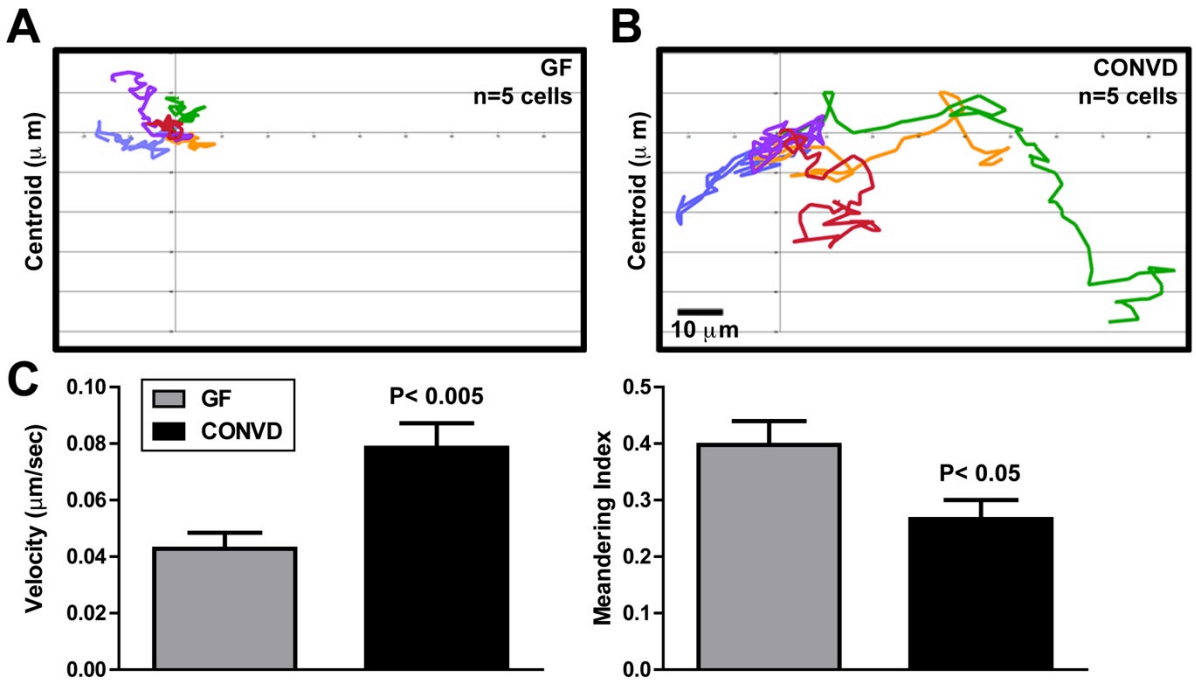


Figure 2.6.2. Microbiota induces systemic neutrophil migration. Neutrophil tracking analysis of the CHT from 15-min movies of live 6dpf GF (A) and CONVD (B) *Tg(mpx:GFP)* zebrafish reveals increased migratory activity in CONVD. C. Quantification of neutrophil migration velocity and meandering index in 6dpf GF and CONVD zebrafish (calculated from 29 neutrophils per condition). Significant Student's *t*-test *P*-values are shown. See also Movies S1 and S2.

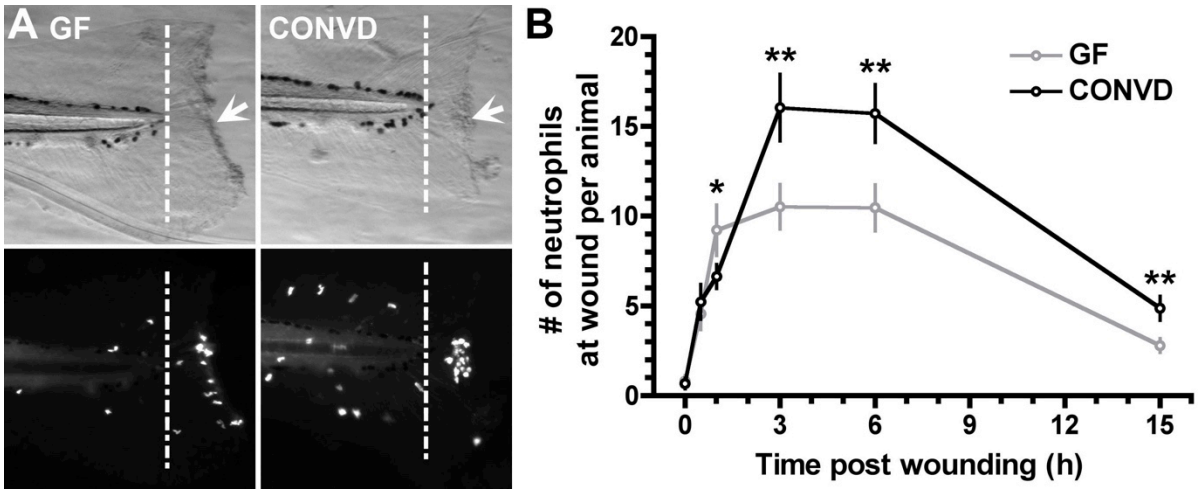


Figure 2.6.3. Microbiota promotes neutrophil recruitment to tail wounds in GF and CONVD *Tg(mpx:GFP)* zebrafish. A. Brightfield and GFP fluorescence images of 6dpf GF and CONVD zebrafish tails 3 h post injury. B. Mean numbers of neutrophils recruited to wound site (posterior to the end of the notochord marked by white dashed line) at time points post injury as indicated. Data represents 8–10 fish per condition per time point. Significant Student's *t*-test *P*-values are shown: **P* < 0.05 and ***P* < 0.005 vs GF.

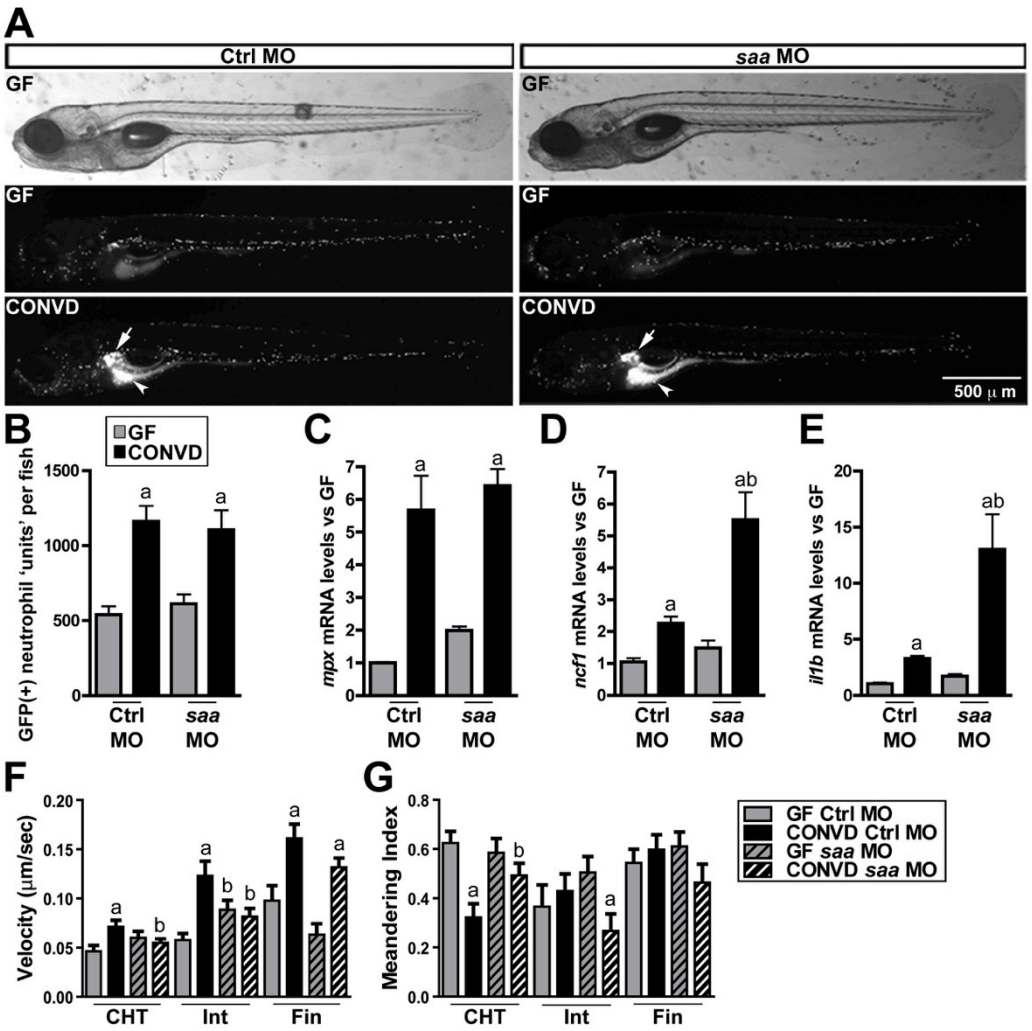


Figure 2.6.4. *saa* mediates systemic neutrophil migration in response to the microbiota. Comparisons of 6dpf GF and CONVD *Tg(mpx:GFP)* zebrafish injected with either standard control (Ctrl MO) or a morpholino targeting *saa* (*saa* MO). A. Images of whole live 6dpf *Tg(mpx:GFP)* zebrafish show GFP(+) neutrophil localization including increased concentration of neutrophils in the kidney (white arrow) and intestine (white arrow head) in CONVD Ctrl MO and *saa* MO zebrafish. B. Neutrophil units quantified by GFP densitometry from whole animal images similar to those shown in A. C. qRT-PCR for *mpx* mRNA in sorted neutrophils. qRT-PCR for *ncf1* (D) and *il1b* (E) mRNA in whole 6dpf zebrafish. Quantification of neutrophil velocity (F) and meandering index (G) in the CHT, intestine (Int), and fin (calculated from 7–29 neutrophils per tissue per condition). Student's *t*-test *P*-values are indicated: a, $P < 0.05$ compared with GF condition in same genotype; b, $P < 0.05$ compared with Ctrl MO in same microbial condition. See also Movies S3–S6.

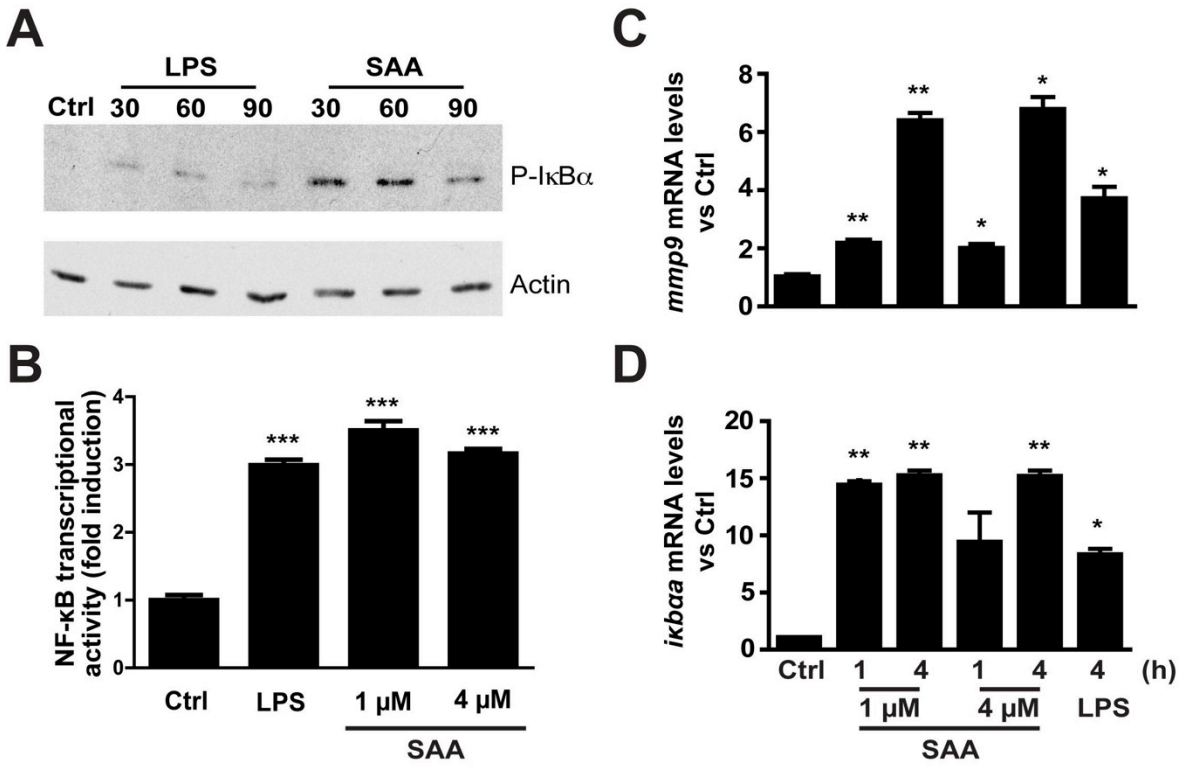


Figure 2.6.5. SAA stimulation of a zebrafish cell line results in activation of the canonical NF-κB pathway and induces expression of NF-κB target genes. A. Western blot of zebrafish PAC-2 cells shows rapid phosphorylation of IκBα proteins after LPS (10 μg ml^{-1}) or SAA (4 μM) stimulation. B. Zebrafish PAC-2 cells transfected with *ikbaa*-luciferase gene reporter (pikbaa:Luc) show increased luciferase activity upon stimulation with LPS (10 μg ml^{-1}) or SAA (1, 4 μM). C and D. qRT-PCR using primers for *mmp9* and *ikbaa*, predicted NF-κB target genes, demonstrate induction upon stimulation of PAC-2 cells with LPS (10 μg ml^{-1}) or SAA (1, 4 μM) normalized to 18S ribosomal RNA [rRNA]). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

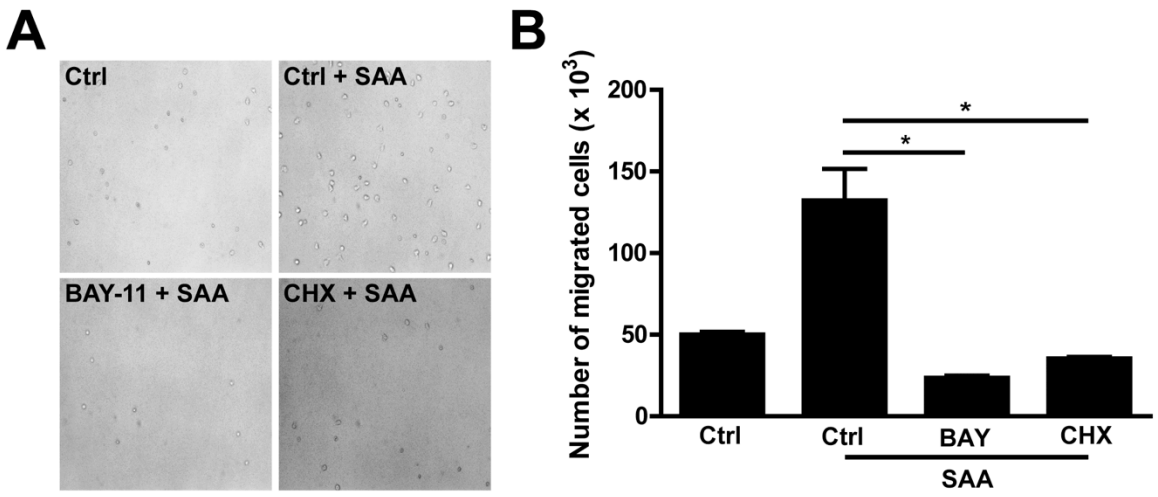


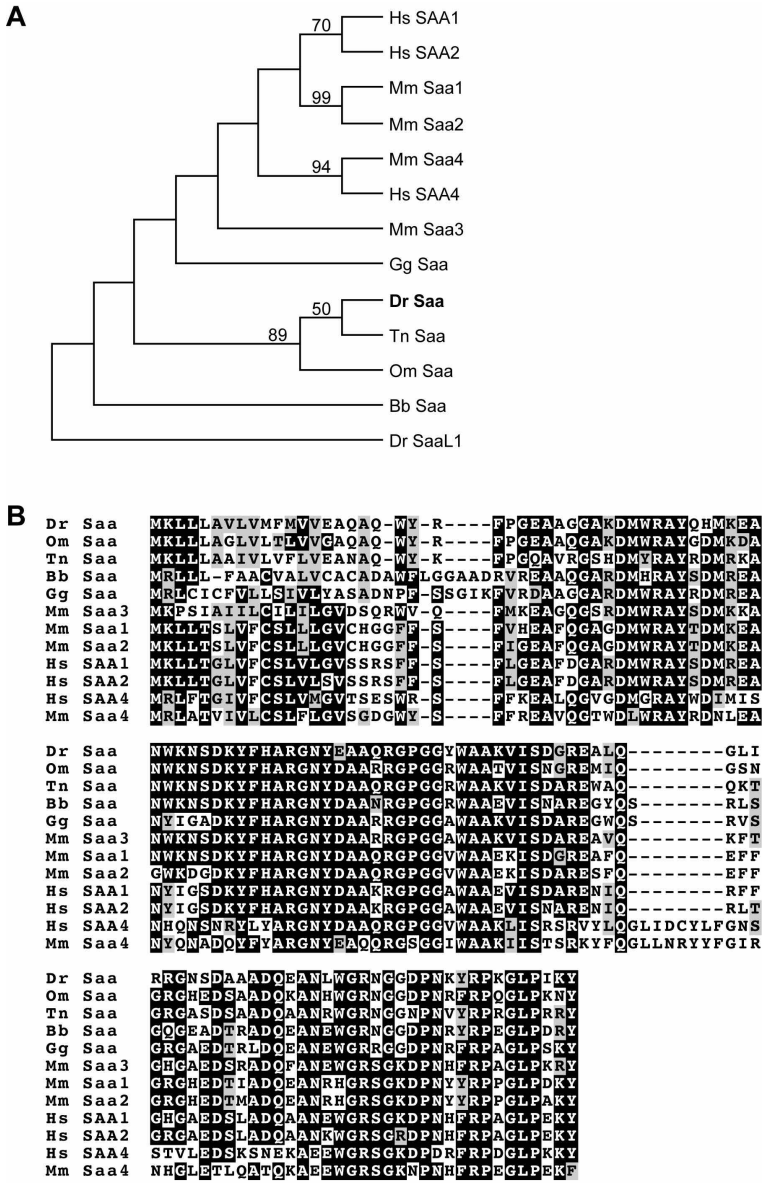
Figure 2.6.6. SAA promotes neutrophil migration and requires NF-κB and protein synthesis. A. Peritoneal isolated murine neutrophils were pretreated for 1 h with BAY 11–7082 (BAY, 25 μM) or cycloheximide (CHX, 50 μg ml^{-1}) and then plated into the top well of a Transwell system. The cells' migration in response to SAA (2.08 μM, 25 μg ml^{-1}) in the bottom well was enumerated after 2.5 h. Representative light images of neutrophils migrated into bottom wells. Magnification 200x. B. Quantitative measurements of migrated neutrophils. Data are expressed as mean ± SEM. *P < 0.05. Results are representative of two independent experiments.

Gene name	FC[a]	Reference[b]
myeloid-specific peroxidase (*mpx*)	8.0	(Bennett et al., 2001; Lieschke et al., 2001)
matrix metalloproteinase 9 (*mmp9*)	7.2	(Yoong et al., 2007)
microfibrillar-associated protein 4 (*mfap4*)	6.4	(Zakrzewska et al., 2010)
matrix metalloproteinase 13a (*mmp13a*)	5.1	(Qian et al., 2005; Yoong et al., 2007)
neutrophil cytosolic factor 1 (*ncf1*)	3.0	(Qian et al., 2005)
lymphocyte cytosolic plastin 1 (*lcp1*)	2.2	(Bennett et al., 2001)
CCAAT/enhancer binding protein, beta (*cebpb*)	2.1	(Thisse et al., 2001)
coronin, actin binding protein, 1A (*coro1a*)	2.0	(Song et al., 2004)

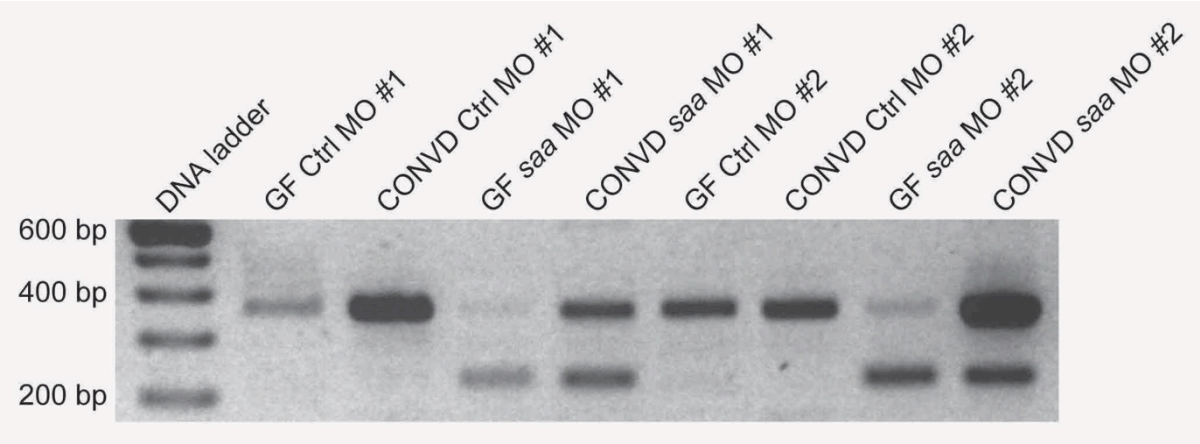
Table 2.1. Elevated transcript levels for myeloid lineage genes in CONVD compared with GF zebrafish larvae.

a. Transcript fold change (FC) in 6dpf CONVD compared to GF zebrafish larvae from (Kanther et al., 2011).

b. Reference establishing myeloid lineage expression for the respective zebrafish gene.



Supplemental Figure 2.1. Phylogenetic analysis of Saa protein sequences. A. Maximum likelihood phylogenetic tree of human (*Homo sapiens*, Hs) SAA1 (NP_000322), SAA2 (NP_110381) and SAA4 (NP_006503); mouse (*Mus musculus*, Mm) Saa1 (NP_033143), Saa2 (NP_035444), Saa3 (NP_035445) and Saa4 (NP_035446); chicken (*Gallus gallus*, Gg) Saa (ADF56353); rainbow trout (*Oncorhynchus mykiss*, Om) Saa (NP_001117908); Tetraodon (*Tetraodon nigroviridis*, Tn) Saa (CAF99678); amphioxus (*Branchiostoma belcheri*, Bb) Saa (BAB97379); and zebrafish (*Danio rerio*, Dr) Saa (NP_001005599) and SaaL1 (NP_956429). Zebrafish SaaL1 is included as an outgroup. Bootstrap support (≥ 50%) is shown as results from 1000 bootstrap replicates. B. Multiple sequence alignment of Saa proteins. Identical residues are highlighted in black, and similar residues are highlighted in grey.



Supplemental Figure 2.2. PCR validation of *saa* MO knockdown. Injection of 1-cell stage embryos with 9 pmol saa.i2e3 morpholino (*saa* MO) results in ~ 150 bp reduction in a subset of *saa* transcripts in both GF and CONVD larvae at 6dpf. This is consistent with saa.i2e3 MO causing exclusion of exon 3 (154 bp) from *saa* transcripts. These *saa* MO-induced splicing defects were not observed following injection of a standard control morpholino (Ctrl MO). Data from two biological replicate pools (#1 and #2; 5–15 larvae per pool) are shown. Note that the total *saa* transcript levels are increased in CONVD compared with GF controls, but that the magnitude of *saa* transcript increase varies between replicates.

Supplemental movies available online:
http://onlinelibrary.wiley.com/doi/10.1111/cmi.12257/full

Movie S1. Neutrophil migration in GF zebrafish larvae. Live 6dpf *Tg(mpx:GFP)* GF zebrafish imaged at a rate of one frame every 15 s for 15 min reveals decreased neutrophil migration compared with CONVD controls (compared with Movie S2).

Movie S2. Neutrophil migration in CONVD zebrafish larvae. Live 6dpf *Tg(mpx:GFP)* CONVD zebrafish imaged at a rate of one frame every 15 s for 15 min reveals increased neutrophil migration compared with GF controls (compared with Movie S1).

Movie S3. Neutrophil migration in GF Ctrl-MO zebrafish larvae. Live 6dpf *Tg(mpx:GFP)* GF zebrafish injected with standard control MO (Ctrl MO) were imaged at a rate of one frame every 15 s for 5 min (compared with Movies S4–S6).

Movie S4. Neutrophil migration in CONVD Ctrl-MO zebrafish larvae. Live 6dpf *Tg(mpx:GFP)* CONVD zebrafish injected with standard control MO (Ctrl MO) were imaged at a rate of one frame every 15 s for 5 min (compared with Movies S3, S5 and S6).

Movie S5. Neutrophil migration in GF *saa*-MO zebrafish larvae. Live 6dpf *Tg(mpx:GFP)* GF zebrafish injected with *saa* MO were imaged at a rate of one frame every 15 s for 5 min (compared with Movies S3, S4 and S6).

Movie S6. Neutrophil migration in CONVD *saa*-MO zebrafish larvae. Live 6dpf *Tg(mpx:GFP)* CONVD zebrafish injected with *saa* MO were imaged at a rate of one frame every 15 s for 5 min (compared with Movies S3–S5).

CHAPTER 3 : GNOTOBIOTIC *Apc*$^{Min/+}$;*Il10*$^{-/-}$ MICE SHOW THE LOCATION SPECIFIC ROLE OF BACTERIA IN CARCINOGENESIS[2]

3.1. Overview

Inflammation and microbiota are critical components of intestinal tumorigenesis. In this study, we investigated the impact of inflammation and microbes on the development of carcinogenesis using a spontaneous model of colon cancer. We generated germ free *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice and exposed them to various microbial conditions. Spearman analysis showed colon tumorigenesis significantly correlated with inflammation in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice, while small intestine tumors significantly correlated with age in specific-pathogen-free (SPF) housed *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice. Germ-free (GF) *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice that are conventionalized by either natural SPF microbiota acquisition or via SPF microbiota gavage have significantly more colon tumors compared to GF mice, suggesting bacteria promote tumorigenesis primarily in the colon. While *Fusobacterium nucleatum* failed to

[2]**Tomkovich, S.**, Yang, Y., Winglee, K., Dejea, C.M., Gauthier, J., Pope, J.L., Ferraguti, D., Mühlbauer, M., Sun, X., Perez-Chanona, E., Liu, X., Martin, P., Oswald, E., Sears, C., Fodor, A. and Jobin, C. Gnotobiotic *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice show the location specific role of bacteria in carcinogenesis. (Manuscript in final phase of submission).

I performed or helped with all experiments except for the *F. nucleatum* associations, generated the figures and contributed to the writing of the methods, results and discussion sections.

induce inflammation and tumorigenesis in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice, *pks*+ *Escherichia coli* promote tumorigenesis in the *Apc*$^{Min/+}$;*Il10*$^{-/-}$ model in a colibactin dependent manner. Finally, human intestinal biofilm forming microbes promote intestinal tumorigenesis in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. In conclusion, colon tumorigenesis is influenced by both inflammation and presence of a specific set of bacteria in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice.

3.2. Introduction

Colorectal cancer (CRC), the third most common type of malignancy and the third leading cause of cancer-related deaths in the United States (Siegel et al., 2016), involves both genetic and environmental factors. Among the genomic changes associated with CRCs, loss-of-function mutations in the *Apc* (*adenomatous polyposis coli*) gene, a regulator of the WNT signaling pathway, are the most prevalent (~80% of CRCs) (Cancer Genome Atlas Network, 2012; Fearon, 2011) and are considered the initiating event in ~80% of CRCs (Fearon, 2011). Of the environmental factors, the gut microbiota is increasingly appreciated as a key player in CRC pathogenesis. CRC patients often carry a microbiota distinctive from the healthy population (Vogtmann and Goedert, 2016). Microbes can modulate CRC development by generating genotoxins, or indirectly and more commonly by mediating inflammatory and immune responses (Brennan and Garrett, 2016; Lasry et al., 2016). Inflammation is not only a hallmark of CRC (Lasry et al., 2016), but also an established risk factor for CRC as supported by epidemiological data from individuals with inflammatory bowel diseases (IBD) (Beaugerie and Itzkowitz, 2015).

While human studies provide valuable correlation data on CRC, much of the mechanistic insight into the disease etiology is obtained from mouse models. Mouse CRC models can be categorized into two classes: spontaneous and chemical-induced (Jackstadt and Sansom, 2016). Spontaneous CRC mice carry mutations in genes frequently mutated in human CRCs. The multiple intestinal neoplasia (Min) mouse (referred to as $Apc^{Min/+}$ hereafter), a commonly used animal model of intestinal carcinogenesis, carries a point mutation in one allele of the *Apc* (adenomatous polyposis coli) gene and is susceptible to spontaneous intestinal adenoma formation, although predominantly in the small bowel without exhibiting chronic intestinal inflammation (Moser et al., 1990; Su et al., 1992). Noticeably, inflammation enhances development of colon cancer in this model as seen with the use of dextran sulfate sodium (DSS) (Cooper et al., 2001; Tanaka et al., 2006), by specifically deleting *Apc* gene in epithelial cells (Grivennikov et al., 2012), and by genetically introducing defective IL-10 signaling (Dennis et al., 2013, 2015; Huang et al., 2006).

Mouse models have been an unparalleled tool for understanding the roles of microbes and inflammation in CRC pathogenesis. A general pro-tumorigenic role for the microbiota was demonstrated in the $Apc^{Min/+}$ model, as the mice display reduced tumor load in the small and/or large intestine when derived into germ-free (GF) conditions (Dove et al., 1997; Li et al., 2012). The mechanisms by which microbes promote development of CRC are diverse and somewhat specific to each microorganism. For example, enterotoxigenic *Bacteroides fragilis* promotes CRC through induction of Th17 response in $Apc^{Min/+}$ mice (Wu et al., 2009), polyketide

synthase (*pks*)+ *E. coli* via production of colibactin in azoxymethane (AOM)/*Il10*$^{-/-}$ mice (Arthur et al., 2012), and *Fusobacterium nucleatum* by fostering a carcinogenic immune microenvironment (expansion of tumor-permissive myeloid-derived suppressor cells, upregulation of *Ptgs2* (*Cox-2*), *Il1β*, *Il6*, *Il8*, *Tnf*α, etc) in *Apc*$^{Min/+}$ mice (Kostic et al., 2013). Importantly, inflammation and colonic polyposis in mice with *Apc* deficiency and T cell-specific deletion of *Il10*, *Apc*$^{\Delta 468}$;CD4^{Cre}*Il10*$^{f/f}$ mice, can be attenuated by antibiotic treatment (Dennis et al., 2013), suggesting that microbiota-driven inflammation underlies colitis-associated CRC. However, the relationship between genetic susceptibility, microbial status and development of CRC is unclear.

Here we report the impact of microbial manipulation using mouse and human biota on the development of intestinal neoplasia in GF *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. We found that inflammation status correlates with tumorigenesis and the microbiota is essential for colorectal but not small intestinal neoplasia. Finally, we showed differential ability of human tissue-associated bacteria to promote CRC in genetically susceptible mice.

3.3. Materials and Methods

Animals. The University of Florida Institutional Animal Care and Use Committee approved all animal experiments. 129/SvEv *Apc*$^{Min/+}$ mice were derived GF and crossed to GF 129/SVEv *Il10*$^{-/-}$ mice to generate GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice were transferred to the SPF breeding suite and bred

for 2-3 generations. SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice were either transferred to an SPF housing suite after weaning or remained in the breeding suite, mice transferred to the SPF housing suite were sacrificed at 12, 16 and 20 weeks of age. SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice older than 20 weeks were retired breeders from the SPF breeding suite.

Bacterial strains and culture conditions. *F. nucleatum* strains were provided by Dr. Emma Allen-Vercoe (University of Guelph), including the IBD clinical isolate EAVG_016, and CRC isolates CC53, CC7/3JVN3C1, CC7/5JVN1A4, CC2/3Fmu1, CC2/3FmuA and CC7/4Fmu3 (used for the 20-week colonization experiment in *Apc*$^{Min/+}$ mice). *E. coli* NC101 or NC101 Δ*clbP* were cultured from glycerol stocks in LB broth, then diluted 1:10 in fresh LB medium and cultured at 37°C before harvesting for gavage. *F. nucleatum* strains were cultured in Brain Heart Infusion Broth (BHI) (AS-872, Anaerobe Systems) statically at 37°C in an anaerobic chamber (type B Vinyl, Coy Laboratory). Enumeration of *F. nucleatum* was done by anaerobically plating serial dilutions of culture or fecal materials on fastidious anaerobic agar supplemented with 5% sheep blood.

***E.coli* NC01 *clbP* mutation.** Inactivation of gene *clbP* was performed by using the lambda Red recombinase method (Datsenko and Wanner, 2000) using primers clbP-P1 (TTCCGCTATGTGCGCTTTGGCGCAAGAACATGAGCCTATCGGGGCGCAAgtgtaggctggagctgcttc) and clbP-P2

(GTATACCCGGTGCGACATAGAGCATGGCGGCCACGAGCCCAGGAACCGCCcat atgaatatcctccttag). The allelic exchange was confirmed by PCR using primers IHAPJPN29 and IHAPJPN30 (Nougayrède et al., 2006).

SPF microbiota preparation. Cecal and fecal contents were collected from wild type 129/SvEv mice that were housed under SPF conditions in the animal facility at the University of Florida. 1 gram of the contents was suspended in 10 ml sterile PBS, broken down using pipette tips, and vortexed. After settling for 2 min, the supernatant was transferred to a new tube, mixed with equal volume of sterile 20% glycerol, and frozen at –80°C.

Mouse colonization. 7-12 week GF *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$;*Il10*$^{-/-}$ were transferred to SPF conditions or gnotobiotic isolators as described above. SPF stock microbiota was diluted 1:10^6 and 200 ul of this mixture was gavaged to each mouse. *E. coli* NC101 or NC101 Δ*clbP* was gavaged at 10^8 colony-forming units (CFU)/mouse. *F. nucleatum* was gavaged at 10^8 CFU/mouse when a single strain was used, or 10^8 CFU per strain per mouse when a mixture of strains were used. BHI medium weekly gavaged mice were used as control for *F. nucleatum* experiments.
Mice were euthanized at indicated time points. The small intestine, cecum and colon were cut open longitudinally and macroscopic tumors were counted. About 1 X 0.5 cm snips were taken from the proximal and distal colon, flash frozen in liquid nitrogen and stored at -80°C until analysis. The rest of the colon was Swiss rolled and fixed in 10% neutral buffered formalin solution. Swiss rolls were processed,

paraffin-embedded, sectioned and H&E stained by the Molecular Pathology Core at the University of Florida. Histological scoring of inflammation was performed blindly as described previously (Arthur et al., 2012) and calculated as the average between the proximal and distal colon region scores.

Extraction of human tissue-associated bacteria. Patient tissues were collected and screened for biofilms as described previously (Dejea et al., 2014). In brief, CRC patient tumor (T) and normal flanking (NF) tissues were collected via surgical resection and healthy control patient tissues were collected from the right and left colon by colonoscopy biopsy (bx). Part of the patient tissues were fixed in Carnoy's solution and biofilm status was assessed by fluorescence in situ hybridization (FISH) with a universal bacterial probe and bacterial density was quantified with ImageJ. Bacterial biofilms were defined as biofilm positive if there were $>10^9$ bacteria/mL for CRC patients or 10^8 bacteria/mL for colonoscopy biopsies that were within the mucus layer and spanned at least 200 μm of the epithelial surface. Biofilm negative (BF-) and positive (BF+) inoculums were prepared from 3mm diameter tissue pieces that were collected from healthy or CRC patients and stored at -80°C. Each inoculum was composed of tissue from 5 different patients. The BF-bx and BF+ bx were pooled from separate groups of healthy patients while the BF+NF and BF+T were from the same set of CRC patients. All inoculums were prepared anaerobically by mincing and homogenizing tissue pieces in PBS. GF mice were transferred to a gnotobiotic isolator and gavaged with 100-200 microliters of inoculum.

Immunohistochemistry (IHC). IHC was performed as described previously (Arthur et al., 2012). Briefly, Swiss roll sections were deparaffinized, rehydrated, and boiled in 10mM citrate buffer for antigen retrieval. For CTNBB1, the mouse anti-CTNNB1 antibody (1:300 overnight) (6101503, BD Transduction Laboratories) and mouse on mouse (M.O.M.) peroxidase kit (PK-2200, Vector Labs) were used. For PCNA, sections were blocked with 1% BSA, incubated with anti-PCNA clone PC10 (M087901-2, Dako) mouse monoclonal antibody (1:300, 30 minutes), followed by 1:1000 goat anti-mouse biotin secondary antibody (31800 Fisher), and then incubated with streptavidin-horseradish peroxidase (18-152, Millipore). Liquid DAB+ (K3467, Dako) was used according to manufacturer instructions for development.

Fecal DNA extraction and 16S qPCR. DNA was extracted using phenol:chloroform separation followed by DNeasy Blood & Tissue Kit (69506, Qiagen). qPCR was performed on CFX384 Touch Real-Time PCR Detection System (1855485, Bio-rad) using the SsoAdvanced™ Universal SYBR Green Supermix (1725274, Bio-rad). The following primers were used: Fuso_F GGATTTATTGGGCGTAAAGC, Fuso_R GGCATTCCTACAAATATCTACGAA; and Eubacteria_F GGTGAATACGTTCCCGG, Eubacteria_R TACGGCTACCTTGTTACGACTT.

16S rRNA sequencing. The V1-V3 region hypervariable region of the 16S rRNA gene was amplified using primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3'). Both the forward and the reverse primers contained universal Illumina paired-end adapter sequences, as well as unique

individual 4-6 nucleotide barcodes between PCR primer sequence and the Illumina adapter sequence to allow multiplex sequencing (Supplemental Table 3.1). PCR products were visualized on an agarose gel, before samples were purified using the Agencourt AMPure XP kit (A63881, Beckman Coulter) and quantified by qPCR with the KAPA Library Quantification Kit (KK4824, KAPA Biosystems). Equimolar amount of samples were then pooled and sequenced with an Illumina MiSeq.

16s rRNA sequencing analysis. Taxonomic ranks were assigned for the forward reads using the RDP (ribosomal database project) classifier (Wang et al., 2007) version 2.2 with confidence set to 80%. Reads were grouped by genera and the counts were normalized and $\log_{10}$ transformed (McCafferty et al., 2013) using the following formula:

$$log_{10}\left(\frac{RC}{n} \text{ x } \frac{\sum x}{N} + 1\right)$$

where RC is the read count for a particular OTU in a particular sample, *n* is the total number of reads in that sample, the sum of *x* is the total number of reads in all samples and N is the total number of samples. The Principle Coordinate Analysis (PCoA) was generated from the Bray-Curtis distance of the normalized and $\log_{10}$ transformed counts using the capscale function in the vegan R package (Oksanen et al., 2015; R Core Team, 2015).

Genera significant for biofilm group (BF-bx, BF+bx, BF+NF or BF+T) were detected using the lme function in the R nlme package, with the REML method(Pinheiro et al., 2016) to fit a generalized mixed linear model of the form:

$$genera \sim group + 1|cage + \varepsilon$$

where genera indicates the log_{10} normalized abundance of a particular genera, group indicates the biofilm group and 1|cage indicates that we used the cage as a random effect. We then ran an ANOVA analysis on the above model to generate p-values for biofilm group. We filtered genera absent in more than a quarter of the samples. The p-values for cage were calculated using an ANOVA of this model and a model with the cage removed (genera ~ group + ε). The p-values were then adjusted for multiple hypothesis testing using the method of Benjamini & Hochberg (Benjamini and Hochberg, 1995). The heatmap was generated using the R function ggplot2 (Wickham, 2009). The code and tables used to generate the 16S rRNA sequencing figures can be found at: https://github.com/afodor/biofilm.

qPCR examination of inflammatory cytokines. RNA was extracted from frozen tissue snips using Trizol reagent followed by phenol:chloroform separation. After DNA removal using the Turbo DNA-free Kit (AM1907, Ambion), 1 ug of RNA was used for cDNA synthesis using iScript cDNA Synthesis Kit (1708891, Bio-rad). qPCR was performed on CFX384 Touch Real-Time PCR Detection System (1855485, Bio-rad) using the SsoAdvanced™ Universal SYBR Green Supermix (1725274, Bio-rad). The following primers were used: IL6_F CGGAGGCTTGGTTACACATGTT, IL6_R CTGGCTTTGTCTTTCTTGTTATC; TNFα_F ATGAGCACAGAAAGCATGATC, TNFα_R TACAGGCTTGTCACTCGAATT; IFNγ_F ACGCTTATGTTGTTGCTGATGG, IFNγ_R

CTTCCTCATGGCTGTTTCTGG; IL-1β_F GCCCATCCTCTGTGACTCAT, IL-1β_R AGGCCACGGTATTTTGTCG; IL17A_F GCCCTCAGACTACCTCAACC, IL17A_R ACACCCACCAGCATCTTCTC; IL-22_F CATGCAGGAGGTGGTGCCTT, IL-22_R CAGACGCAAGCATTTCTCAG; 36B4_F TCCAGGCTTTGGGCATCA, 36B4_R CTTTATTCAGCTGCACATCACTCAGA; and GUSB_F CCGATTATCCAGAGCGAGTATG, GUSB_R CTCAGCGGTGACTGGTTCG. 36B4 and Gusb were used as references. Relative fold gene expression was calculated using the delta delta Ct method.

Statistical analysis. Statistics were calculated with Graphpad Prism using the Mann-Whitney nonparametric test or with the Spearman nonparametric correlation analysis.

3.4. Results

Inflammation promotes development of CRC in $Apc^{Min/+}$;$Il10^{-/-}$ mice

To investigate the interaction between microbial status, inflammation and CRC development, we interbred *Il10*$^{-/-}$ mice to *Apc*$^{Min/+}$ mice (129SvEV background) to generate *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. Colon and cecal tumors increased dramatically in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ compared to *Apc*$^{Min/+}$ mice (colon tumor mean= 5.03 vs 0.73, respectively $p< 0.0001$; cecal tumor mean= 0.51 vs 0, respectively $p< 0.0005$) (Fig. 3.6.1A and C) whereas small bowel lesions remained similar between the two genotypes (mean= 0.84 vs 2.23 $p= 0.2914$) (Fig. 3.6.1D). Histological assessment

showed presence of colonic neoplastic lesions in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice (Fig. 3.6.1E) and as expected, increased inflammation in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice compared to *Apc*$^{Min/+}$ mice (combined score mean = 1.32 vs 0.23, respectively $p < 0.0001$) (Fig. 3.6.1B). Moreover, Spearman analysis showed a significant correlation between development of CRC and extent of inflammation in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice ($r = 0.7441$ $p < 0.0001$) whereas no such correlation is observed in *Apc*$^{Min/+}$ mice ($r = -0.0968$ $p = 0.6135$) (Fig. 3.6.1F). Furthermore, development of small intestinal neoplasia did not correlate with the state of colitis (*Apc*$^{Min/+}$;*Il10*$^{-/-}$ $r = 0.2364$; *Apc*$^{Min/+}$ $r = 0.2526$; $p > 0.05$) (Fig. 3.6.1F). Interestingly, endpoint age was a significant contributor to tumorigenesis in the small bowel of both *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice ($r = 0.6548$, 0.8208, respectively $p < 0.0001$) but only weakly contributed to neoplasia in the large bowel of *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice ($r = 0.2561$ $p = 0.0256$) (Fig. 3.6.1G).

Due to its role in promoting cellular proliferation, we evaluated the distribution of nuclear Catenin Beta 1 (CTNNB1) and proliferating cell nuclear antigen (PCNA) in actively inflamed and neoplastic regions of *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ colons. Nuclear CTNNB1 and PCNA staining was mostly restricted to the crypt bases in *Apc*$^{Min/+}$ mice (Fig. 3.6.2B and D). In contrast, the colonic mucosa from *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice showed areas of CTNNB1 and PCNA staining extending the full crypt length in some cases (Fig. 3.6.2A and C). In addition, expression of proliferative IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA increased in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ compared to *Apc*$^{Min/+}$ mice (Fig. 3.6.2E). Furthermore, *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice with a high number of tumors (>2) had significantly increased TNFα, IFNγ and IL-1β mRNA compared to low tumor

number (≤2) *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice (Fig. 3.6.2F). Taken together, these data suggest that the heightened inflammatory and proliferative state observed in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ compared to *Apc*$^{Min/+}$ mice increased propensity for colorectal tumor formation and progression.

Bacteria are essential for development of colon tumorigenesis in Apc$^{Min/+}$*;Il10*$^{-/-}$ *mice.*

To stringently evaluate the impact of bacteria on CRC development, we derived *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice in GF conditions and then performed microbial manipulation by either gavaging the mice with specific-pathogen-free (SPF) biota or transferring them into SPF conditions. Importantly, colon tumorigenesis was practically abolished in GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice (mean= 0) compared to SPF conditions (Fig. 3.6.3A,D). Interestingly, SPF gavage enhanced colon tumor loads compared to passive SPF colonization of *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice (mean= 3.86 vs 1 respectively p= 0.0126) (Fig. 3.6.3A,D), although colitis scores and most inflammatory cytokine expression (IL-6, TNFα, IFNγ, IL-22 and IL-17a) were not significantly different (colitis score means = 1.86 vs 2.36 respectively p=0.46) (Fig. 3.6.3B, 3.6.4D). Colon inflammation and tumors were practically absent in GF and SPF gavaged *Apc*$^{Min/+}$ mice (data not shown), suggesting inflammation is a key component of bacteria-mediated tumorigenesis. Development of small bowel neoplasia in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice (Fig. 3.6.3C, data not shown) was not significantly impacted by microbial colonization, suggesting a stronger genetic

contribution to cancer in the small intestine compared to the colon in this model. GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ colons had reduced nuclear CTNNB1 and PCNA (Fig. 3.6.4A-C) and decreased inflammatory cytokine expression (Fig. 3.6.4D) compared to SPF mice, indicating bacteria play a significant role in the increased inflammatory and proliferative state in SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice.

Gnotobiotic experiments reveal specific microbial requirements for CRC development in Apc$^{Min/+}$*;Il10*$^{-/-}$ *mice.*

Fusobacterium spp. have been linked to the development of CRC (Brennan and Garrett, 2016) and recent studies showed increased carcinogenesis in *F. nucleatum*-colonized *Apc*$^{Min/+}$ mice (Kostic et al., 2013; Yu et al., 2015b). To investigate the interplay between microbiota and *F. nucleatum* in CRC, we transferred GF *Apc*$^{Min/+}$ to SPF conditions and gavaged them with SPF microbiota followed by weekly gavage with a *F. nucleatum* CRC clinical isolate for 20 weeks. Interestingly, presence of *F. nucleatum* failed to enhance carcinogenesis in these mice (Fig. 3.6.5A,C). Daily gavage of an *F. nucleatum* isolate from patients with inflammatory bowel diseases did not show tumorigenic or inflammatory activity in these mice either (data not shown). We next transferred GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice to SPF conditions, gavaged them with SPF microbiota and then introduced a mixture of 6 *F. nucleatum* strains obtained from CRC patients by weekly gavage for 16 weeks. Although *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice developed more inflammation and tumors than *Apc*$^{Min/+}$ mice, presence of *F. nucleatum* species did not influence carcinogenesis nor inflammation (Fig. 3.6.5B,C). To rule out the possibility that the SPF biota down-

modulate *F. nucleatum* carcinogenic properties, we transferred GF *Apc*$^{Min/+}$ mice to a gnotobiotic isolator and associated these mice with a mixture of 6 *F. nucleatum* CRC clinical isolates (single gavage). Surprisingly, presence of *F. nucleatum* isolates failed to enhance carcinogenesis in *Apc*$^{Min/+}$ mice (Fig. 3.6.5D) despite the presence of high CFU counts (mean= 10^7 CFU/g of stool). A similar lack of colitis and carcinogenesis development was observed in *F. nucleatum* colonized *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice (data not shown).

To further study the relationship between microbial status and carcinogenesis in gnotobiotic *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice, we colonized these mice with *E. coli* NC101, a strain carrying the genotoxic island *pks*. We previously showed that removing *pks* from *E. coli* NC101 decreased development of CRC in the AOM/*Il10*$^{-/-}$ mouse model (Arthur et al., 2012). We next mono-associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice by oral gavage (10^8 CFU/mouse) with an *E. coli* NC101 mutant deficient for *ClbP*, the *pks* gene necessary for colibactin activation (Δ*clbP*). We found that wild type NC101-colonized mice developed significantly more colon tumors than *E. coli* NC101 Δ*clbP* associated mice (mean= 1.71 vs 0.17 respectively p= 0.0023) (Fig. 3.6.6A). The finding that NC101 Δ*clbP* has diminished carcinogenic capacity compared to NC101 was confirmed in another model of colitis-associated colorectal, the AOM/*Il10*$^{-/-}$ model (Arthur et al., 2014) (mean= 2 vs 5 tumors respectively p= 0.039, data not shown). Importantly, deletion of *clbP* did not compromise the ability of *E. coli* NC101 to induce inflammation (colitis score mean= 2.5, 2.675 respectively p= 0.76) (Fig. 3.6.6B, D). Presence of a functional *pks* did not influence development of small intestinal tumors in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice (mean= 0.29, 0.8 respectively p= 0.22) (Fig.

3.6.6C). Overall, these findings show that *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice are sensitive to microbial status and develop site specific tumors.

Multispecies bacterial biofilms (BF) were recently associated with right-sided CRCs and were also found in a subset (13%) of healthy patients (Dejea et al., 2014). To evaluate the carcinogenic potential of human biofilm-forming bacteria, we gavaged GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice with 4 different inoculums pooled from healthy patient colonoscopy biopsies (BF- or BF+ bx) or CRC patient surgical resections (BF+ normal flanking tissue: NF and BF+ tumor tissue: T). Each group of mice were maintained in separate gnotobiotic isolators for the duration of the experiment (12 weeks). Surprisingly, all 3 groups of mice that were associated with BF+ inoculums developed significantly more colon and small intestine tumors (Fig. 3.6.7A, B) compared to BF-bx associated mice. Moreover, the pro-tumorigenic effect of biofilm forming bacteria is independent of inflammation, as colitis (Fig. 3.6.7C) and the majority of inflammatory cytokines (Fig. 3.6.7D) were not significantly different between BF- and BF+ associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice.

To gain insight into the microbial communities associated with biofilms and carcinogenesis, we used 16S rRNA sequencing to determine the bacterial composition in the human inoculums, as well as the stool (1 and 12 week) and distal colon (DC) tissues collected from transplanted mice after 12 weeks. ANOSIM analysis reported striking differences between BF- and BF+ groups (Fig. 3.6.7E) in both the stool and DC tissue compartments. At the genus level, there were 24 OTUs in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice that were significantly different between the BF- and BF+ associated groups after transplantation into mice (Fig. 3.6.7F). Six of the 24 genera

were significantly increased in both the stool and distal colon tissue compartments of BF+ associated mice (*Clostridium XVIII*, *Erysipelotrichaceae incertae sedis*, *Escherichia Shigella*, *Eubacterium*, *Parabacteroides*, and *Robinsoniella*). These findings suggest biofilm forming bacteria are pro-carcinogenic and are associated with a specific microbiota composition.

3.5. Discussion

Genetics and environmental factors play an important role in CRC development, with increasing attention directed toward the intestinal microbiota as a key environmental component (Pope et al., 2016). In general, the microbiota is thought to play a pro-carcinogenic role in CRC with numerous CRC mouse models demonstrating tumor reduction in antibiotic treated or germ-free mice (Schwabe and Jobin, 2013). Here we utilized gnotobiotic $Apc^{Min/+}$ and $Apc^{Min/+}$;$Il10^{-/-}$ mice to define the relationship between inflammation, microbial status and tumorigenesis. We observed that despite genetic susceptibility in both $Apc^{Min/+}$ and $Apc^{Min/+}$;$Il10^{-/-}$ mice, colonic inflammation in the latter mice foster development of tumors. Gnotobiotic experiments revealed that *E. coli* colibactin but not *F. nucleatum* promote tumorigenesis, suggesting an intricate interaction between host genetics and bacteria. Importantly, microbial organization is important for development of CRC as bacteria obtained from biofilm positive human but not biofilm negative tissues promote carcinogenesis in gnotobiotic $Apc^{Min/+}$;$Il10^{-/-}$ mice.

We observed an inflammation dependent increase in colon tumorigenesis in 129 SvEv $Apc^{Min/+}$;$Il10^{-/-}$ mice, which is in line with previous reports on *Il10* deficient

C57BL/6 *Apc*$^{Min/+}$ and *Apc*$^{\Delta 468}$ mice (Dennis et al., 2013; Huang et al., 2006). However, in the small intestine compartment, tumors developed at a comparable rate regardless of *Il10* status in *Apc*$^{Min/+}$ mice, which is in contrast to previous findings showing a delay in small intestinal polyp formation in *Il10* deficient *Apc*$^{\Delta 468}$ mice (Dennis et al., 2015). Possible explanations for the differences in small intestine tumor formation may be due to genetic background differences which have been shown to strongly modulate tumor multiplicity, particularly in the small intestine of *Apc*$^{Min/+}$ mice (Dietrich et al., 1993; Kwong and Dove, 2009).

The interaction between bacteria and the host in the context of intestinal carcinogenesis is complex. One study, using the chemical AOM/DSS regimen, reported that GF mice developed more colonic tumors than mice colonized with a complex biota, suggesting certain bacteria can have a beneficial role in CRC (Zhan et al., 2013). Since microbial composition is a key determinant of colon tumor burden in AOM/DSS mice (Zackular et al., 2013, 2016), this chemical model may better capture the protective functions of bacteria than the genetic *Apc*$^{Min/+}$ mouse model.

Nevertheless, the role of bacteria in *Apc*$^{Min/+}$ intestinal tumorigenesis is also complicated with a report showing fewer tumors in the middle region of the small intestine in GF *Apc*$^{Min/+}$ mice (Dove et al., 1997) while another report showed reduced tumors throughout the intestine in GF *Apc*$^{Min/+}$ mice (Li et al., 2012). The difference in tumor distribution is not clear. Our finding that bacteria promote colon tumors in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice is in line with a study showing reduced colon polyp numbers in *Apc*$^{\Delta 468}$;CD4^{Cre}*Il10*$^{f/f}$ mice following broad-spectrum antibiotic treatment (Dennis et al., 2013).

Numerous studies have implicated *Fusobacterium* spp., in particular *F. nucleatum*, as carcinogenic based on associative studies showing the presence of the bacterium in the luminal and mucosal compartment of human CRC patients using genomic analyses (Ahn et al., 2013; Castellarin et al., 2012; Feng et al., 2015; Flanagan et al., 2014; Kostic et al., 2012; McCoy et al., 2013; Wu et al., 2013; Yu et al., 2015a, 2015b; Zackular et al., 2014; Zeller et al., 2014). In addition, daily gavage of *F. nucleatum* (strain EAVG_002; 7/1 or ATCC 25586) for 8 weeks was shown to promote intestinal tumorigenesis in C57BL/6 $Apc^{Min/+}$ mice (Kostic et al., 2013; Yu et al., 2015b). Subsequent studies using tumor cell transplantation models showed FadA binds E-cadherin to promote tumor growth and Fap2 mediates *F. nucleatum* colonization via an affinity for the polysachharide Gal-GalNAc, which is overexpressed in tumors (Abed et al., 2016; Rubinstein et al., 2013). Surprisingly, $Apc^{Min/+}$ and $Apc^{Min/+}$;$Il10^{-/-}$ mice colonized with various *F. nucleatum* isolates from CRC patients failed to promote intestinal tumorigenesis, in the presence (SPF) or absence of complex biota (gnotobiotic). The absence of tumorigenesis in mono-associated $Apc^{Min/+}$ mice was not due to poor colonization since a high load of *F. nucleatum* (10^7 CFU/g) was recovered from these mice. The discrepancy between our study and the one from Kostic et al. is unclear but could be the result of strain specific properties (EAVG_002 vs other strains tested here), mouse genetic background differences and different microbial environments, as microbial communities are notoriously different between institutions. Nevertheless, our gnotobiotic approach clearly showed that *F. nucleatum* failed to induced either inflammation or cancer, as opposed to *E. coli* pks+ mono-associated $Apc^{Min/+}$;$Il10^{-/-}$

mice. Thus, it is possible that only a select group of *F. nucleatum* strains possesses carcinogenic abilities, which require interaction with other specific members of the microbial community. It would be important to define these interactions and test a larger set of *F. nucleatum* strains to determine the role of these bactera in CRC pathogenesis.

Several studies have found an association between *pks*+ *E. coli* and human CRC patients (Arthur et al., 2012; Bonnet et al., 2014; Buc et al., 2013). Furthermore, *pks*+ *E. coli* isolates from mice or human CRC patients have a pro-tumorigenic effect in GF AOM/*Il10*$^{-/-}$, SPF *Apc*$^{Min/+}$, and SPF AOM/DSS mice (Arthur et al., 2012; Bonnet et al., 2014; Cougnoux et al., 2014). However, because the *pks*-associated *clbA* gene is implicated in the production of siderophores located in the enterobactin (*ent*) and yersiniabactin (HPI) loci (Martin et al., 2013), and our previous observation was based on removal of the entire *pks* island, it is unclear whether the decreased tumorigenesis observed in AOM/*Il10*$^{-/-}$ mice was the consequence of dual siderophore/colibactin impairment, or solely due to abolished *pks* activity. Using a mutant with defective ClbP, the key enzyme implicated in pre-colibactin cleavage and generation of the active form (Dubois et al., 2011), we demonstrate the colibactin-producing *E. coli* murine isolate NC101 is responsible for the pro-tumorigenic effect of the bacterium in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. Whether *clbA* contributes to colibactin-mediated tumorigenesis is still unclear and would need to be investigated, especially since a recent *in vitro* study showed that iron levels and *E. coli* iron sensors regulate *clbA* transcription and colibactin production (Tronnet et al., 2016). Since our studies were performed using a mono-association approach,

and therefore without competitive pressure from other microorganisms, the full extent of iron acquisition on *E.coli pks*+ induced carcinogenesis remains unclear.

Multiple studies have demonstrated an association between bacterial dysbiosis and human CRC (Borges-Canha et al., 2015), but the role of dysbiosis in cancer is still unclear. Furthermore, recent work suggests that bacteria organization may also contribute to CRC (Dejea et al., 2014; Johnson et al., 2015a). Interestingly, tumorigenesis was enhanced in all BF+ associated $Apc^{Min/+}$;$Il10^{-/-}$ mice including those associated with BF+ bacteria from healthy patients, suggesting a functional effect of biofilm derived bacteria in carcinogenesis. *Erysipelotrichaceae*, *Escherichia*, *Eubacterium*, *Holdemania*, *Oscillibacter*, and *Parabacteroides* were increased in BF+ associated mice, and have all been previously associated with CRC (Chen et al., 2012; Feng et al., 2015; Wu et al., 2013). Similarly*, Bifidobacterium* and *Streptococcus* were decreased in our BF+ associated mice and are also decreased in human CRC patients (Feng et al., 2015; Wu et al., 2013). Our ability to recapitulate some of the same dysbiotic signatures seen in human CRC, suggests a gnotobiotic approach with CRC mouse models will be a powerful tool for further elucidating the functional role of dysbiosis in CRC development.

It is interesting to note that inflammation developed in both BF- and BF+ bacteria-colonized $Apc^{Min/+}$;$Il10^{-/-}$ mice, while only the latter group displayed carcinogenesis. Therefore, although inflammation is a key component of tumorigenesis, the microbial environment (e.g. BF- and BF+ community) exposed to this inflammatory milieu is equally important for the pathogenesis.

The mechanisms by which the BF+ consortium promotes carcinogenesis in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice are unclear. Interestingly, polyamine levels increased in BF+ cancer patient tissue, although the exact polyamine source (human, microbial or a combination) is still unclear (Johnson et al., 2015a). Whether BF+ consortium generate specific metabolites with pro-carcinogenic properties remains to be investigated. Similarly important is the identification of the mechanisms contributing to biofilm formation and promotion of CRC. Some of these mechanisms may relate to fostering a niche that helps the bacteria cope with nutritional limitations in the host, promoting survival of the biofilm associated bacteria. For example, *in vitro* experiments along with a mouse model of chronic wound infection showed *Enterococcus faecalis* produces an amino acid metabolite that modulates siderophore production in a uropathogenic *E. coli* clinical isolate, fostering growth and colonization in low iron conditions (Keogh et al., 2016). Similar regulation may occur between different species of bacteria in intestinal biofilms.

Recent studies have attempted to dissect the contributions of intrinsic (organ specific stem cell division rates, aging) and extrinsic factors (hereditary mutations, lifestyle, environmental exposure, etc.), to overall cancer risk in humans (Podolskiy and Gladyshev, 2016; Tomasetti and Vogelstein, 2015a; Wu et al., 2016). However, the interplay between all these factors makes it difficult to tease out the various contributions using epidemiological data. Nevertheless, these studies suggest that small intestine cancers with a relatively low lifetime risk are driven by intrinsic risk factors, while 82.9% of the mutation signatures in CRCs are from extrinsic factors, correlating with a much higher lifetime risk (Wu et al., 2016). We postulate that one

of the extrinsic factors contributing to CRC risk is the microbiota, which not coincidentally is also affected by lifestyle and environmental factors (Conlon and Bird, 2015; O'Sullivan et al., 2015). Interestingly, the concentration of bacteria increases along the gastrointestinal tract with 10^3-10^4 bacteria/mL in the small intestine to 10^{11} bacteria/mL in the colon, mirroring the distribution of cancer risk along the human intestinal tract (Sender et al., 2016). Similarly, in the *Apc*$^{Min/+}$;*Il10*$^{-/-}$ model, age strongly correlates with small intestine tumor numbers while inflammation and bacteria composition play a strong role in colon tumorigenesis. Elucidating the mechanisms by which specific bacteria and bacterial organization promote carcinogenesis will generate important insight into the pathophysiology of CRC.

3.6. Figures and Tables

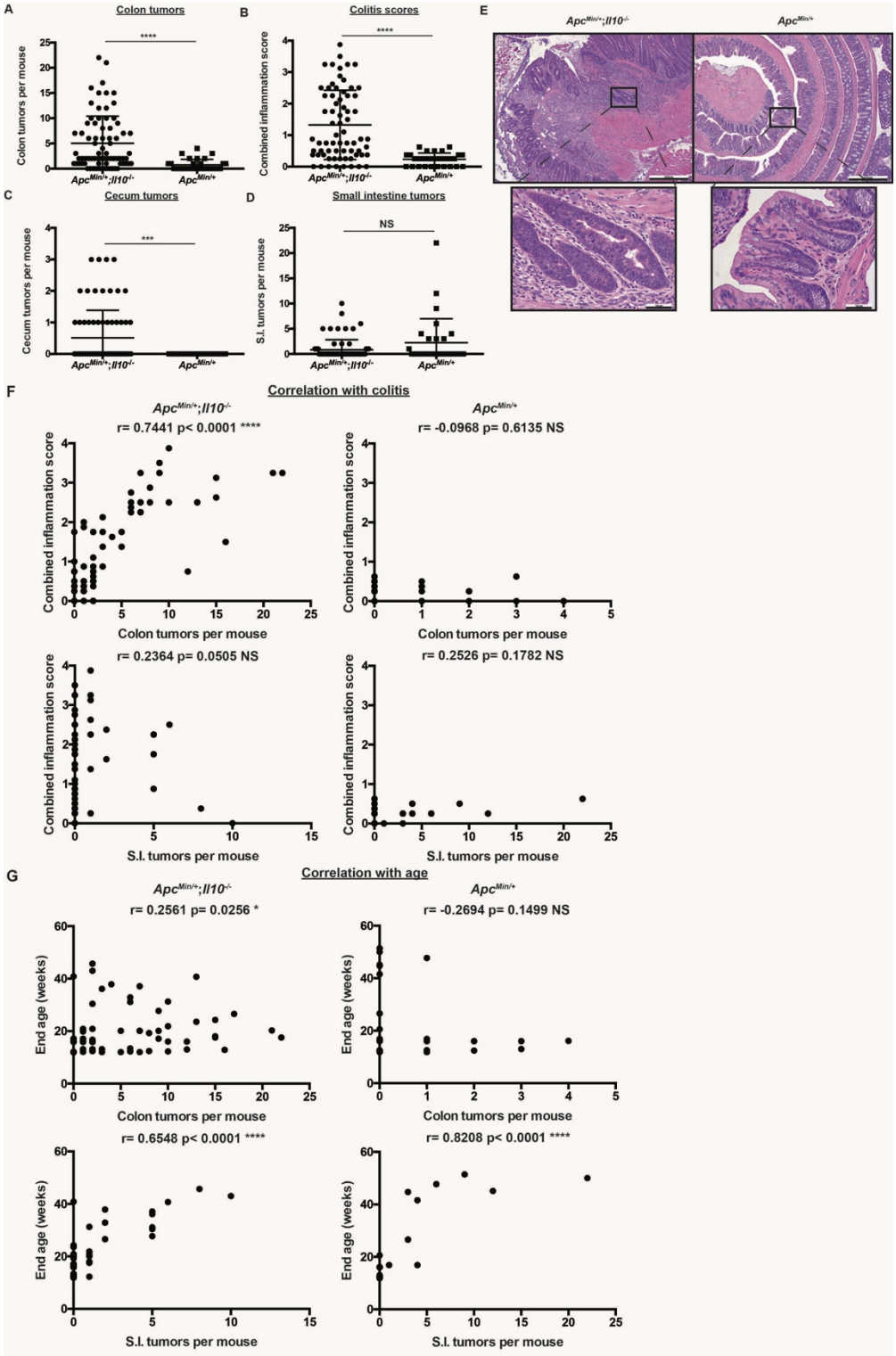


Figure 3.6.1. Inflammation fosters CRC development in genetically engineered mice. A) Macroscopic colon tumor counts from 12-51 week old SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice. **B**) Colon combined histological inflammation scores (the average of the proximal and distal inflammation scores) from SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice. **C-D**) Cecum and small intestine macroscopic tumor counts from SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice. **E**) Colon H&Es from 30-40 week old SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice (5X, 40X magnification). **F**) Relationship between colon inflammation score and macroscopic colon or small intestine tumors in SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice. **G**) Relationship between mouse endpoint age and macroscopic tumors in SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice. Spearman correlation r values and corresponding p values are noted in each panel. Statistics: ****p< 0.0001, ***p< 0.001, **p< 0.01, *p< 0.05, NS: not significant.

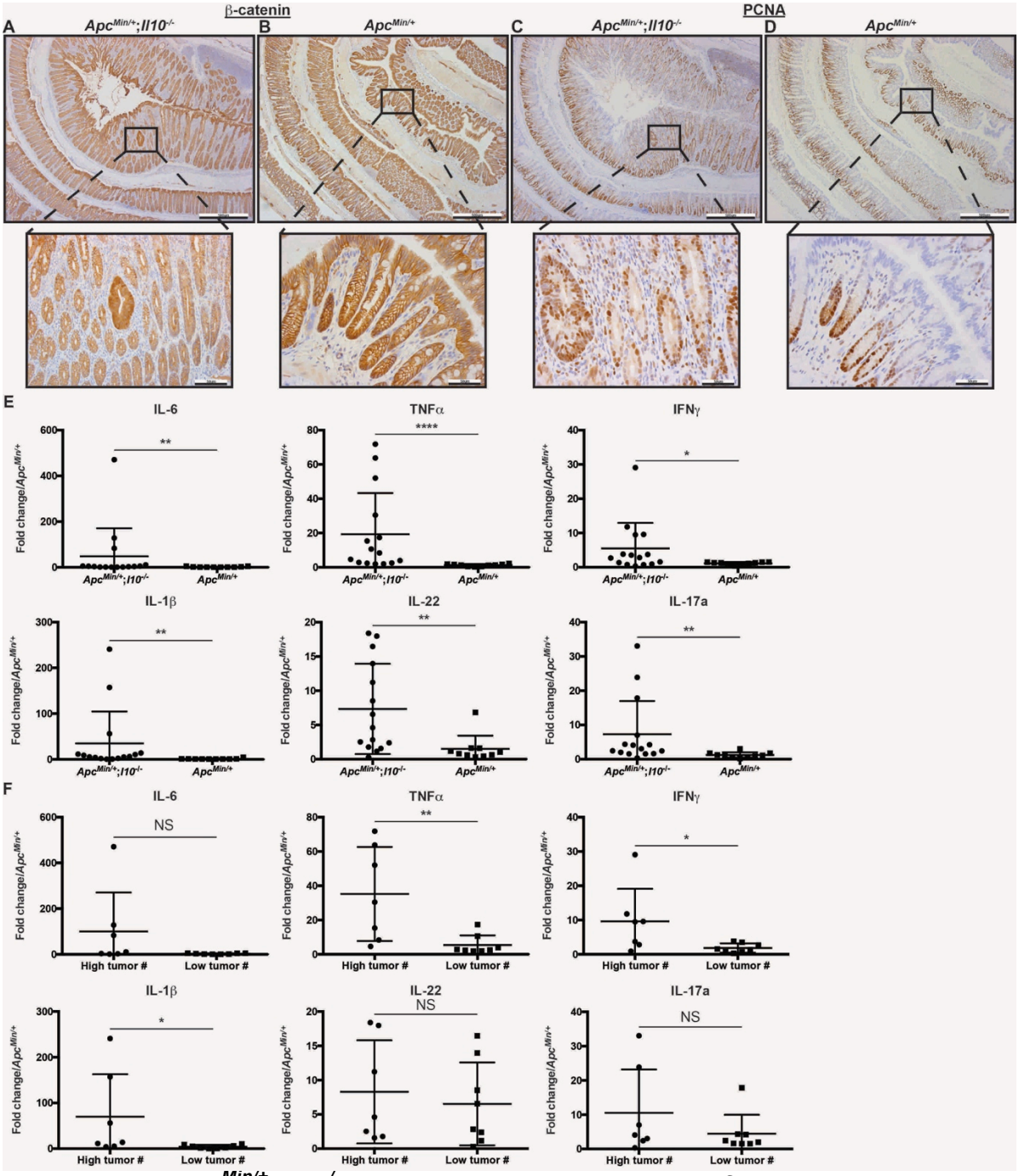


Figure 3.6.2. *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice have increased colon inflammation and proliferation. A-B) CTNNB1 immunohistochemistry (IHC) from ~16 week old SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ (**A**) and *Apc*$^{Min/+}$ (**B**) colons. **C-D**) PCNA IHC from SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ (**C**) and *Apc*$^{Min/+}$ (**D**) colons. **E**) IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA expression in 16-48 week old SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ proximal colon tissue snips with relative fold expression compared to *Apc*$^{Min/+}$ mice. **F**) IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA expression in 16-48 week old SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$

stratified by tumor number (high: > 2 tumors or low: ≤ 2 tumors) with relative fold expression compared to *Apc*$^{Min/+}$ mice.

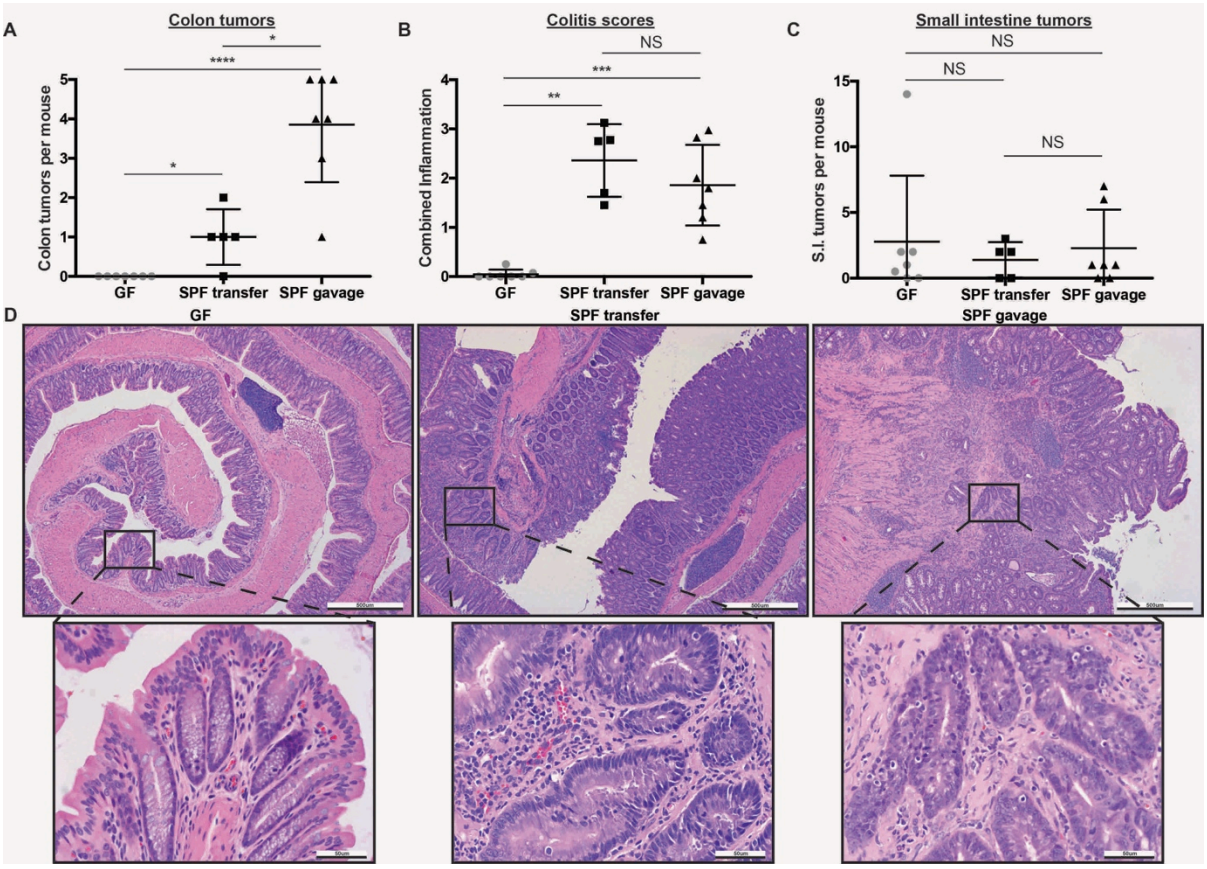


Figure 3.6.3. Bacteria promote colon inflammation and tumorigenesis in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. A-C) Colon macroscopic tumor counts (**A**), colitis scores (**B**), and small intestine tumor counts (**C**) from GF (n=7), SPF transferred (n=5) and SPF gavaged (n=7) *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. SPF transfer (transferred to SPF and allowed to naturally acquire their microbiota) and gavage (transferred to SPF and gavaged with the cecal and fecal contents from a wild type 129SvEv mouse) mice were sacrificed 16 weeks after transfer from GF. **D**) Colon H&Es from GF, SPF transferred, and SPF gavaged *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice.

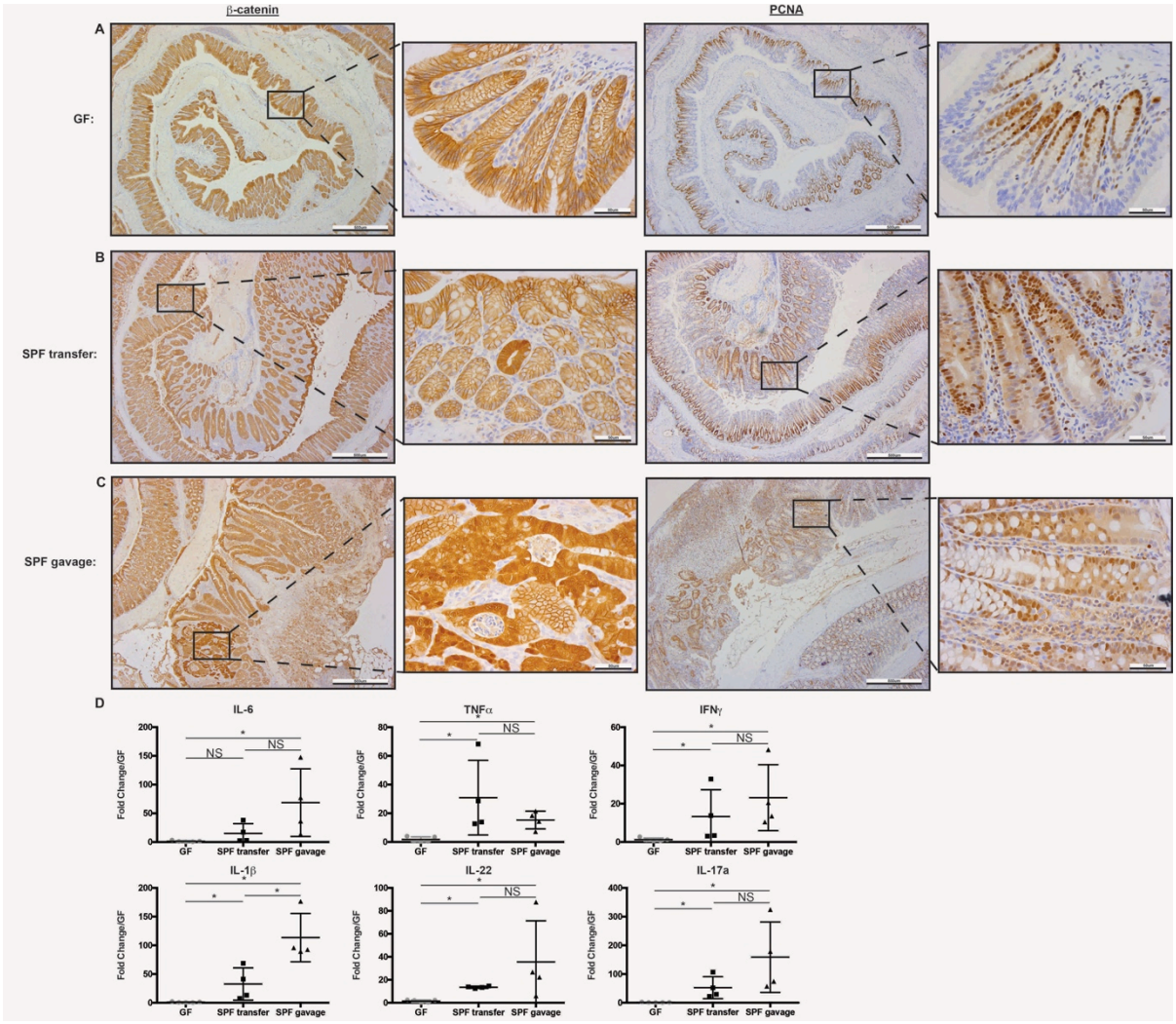


Figure 3.6.4. Microbiota promote colon inflammation and proliferation in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. A-C) β-catenin and PCNA IHC from GF (**A**), SPF transferred (**B**), and SPF gavaged (**C**) *Apc*$^{Min/+}$;*Il10*$^{-/-}$ colons. **D**) IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA expression in GF (n=5), SPF transfer (n=4), and SPF gavaged (n=4) *Apc*$^{Min/+}$;*Il10*$^{-/-}$ proximal colon tissue snips with relative fold expression compared to GF.

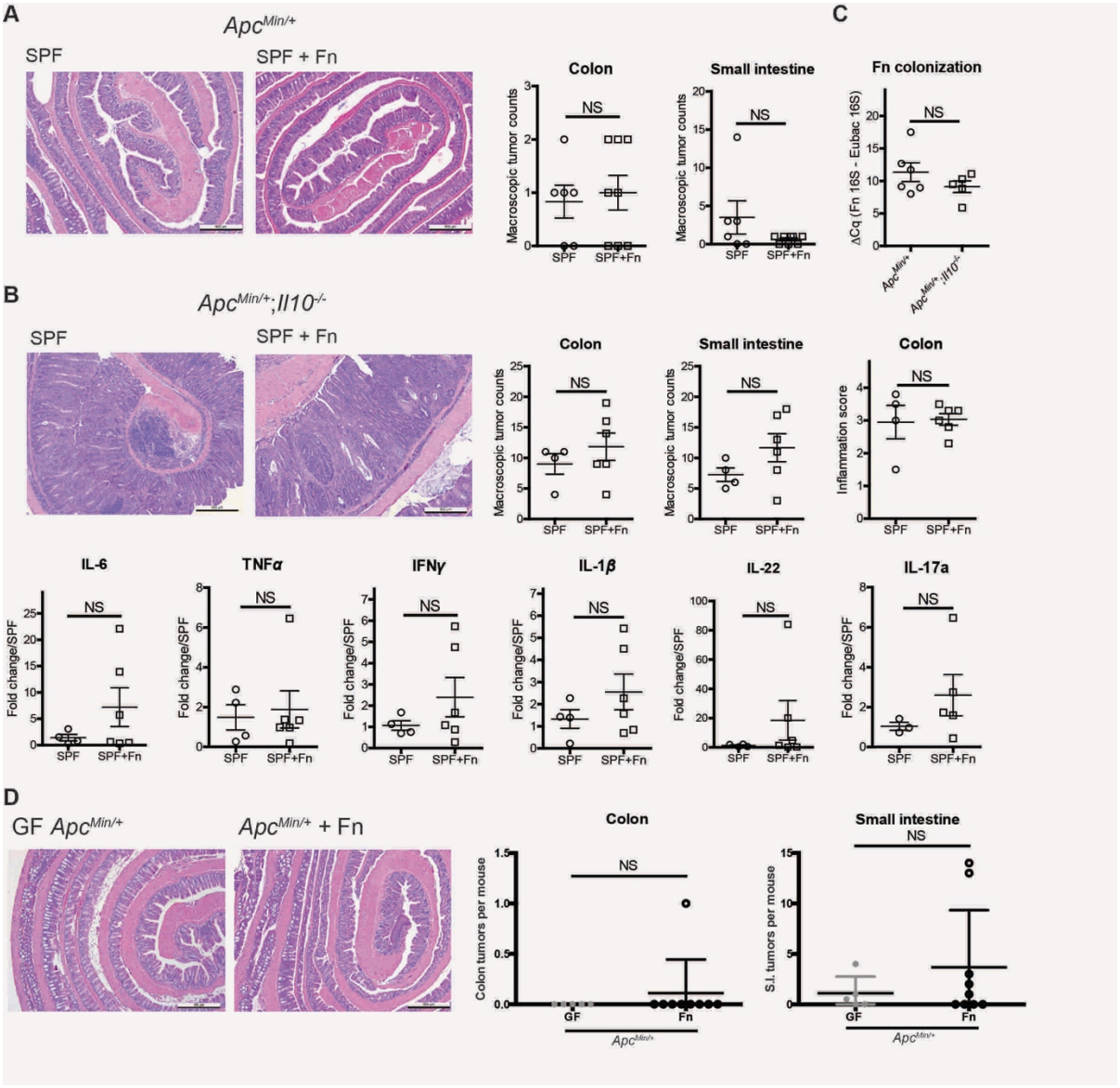


Figure 3.6.5. ***F. nucleatum*** **(Fn) does not exhibit pro-inflammatory and pro-tumorigenic activities. A**) GF *Apc*$^{Min/+}$ mice were transferred to SPF conditions and immediately gavaged with SPF microbiota. SPF+Fn mice received Fn (a single strain human CRC isolate) via weekly gavage. SPF (control) mice received weekly gavage of BHI medium. Tumorigenesis and inflammation were examined 20 weeks later. **B**) GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice were transferred to SPF conditions and immediately gavaged with SPF microbiota. SPF+Fn mice received Fn (a mixture of 6 human CRC isolates) via weekly gavage. SPF (control) mice received weekly gavage of BHI medium. Tumorigenesis and inflammation were examined 16 weeks later (top panel). IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA expression in SPF and SPF+Fn *Apc*$^{Min/+}$;*Il10*$^{-/-}$ distal colon snips (bottom panel). **C**) qPCR examination of fecal Fn levels in SPF+Fn *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$;*Il10*$^{-/--}$ mice. **D**) GF *Apc*$^{Min/+}$ mice were transferred to a gnotobiotic isolator and gavaged with Fn (a mixture of 6 human CRC

isolates). Tumorigenesis was examined 16 weeks later (GF n= 5 and Fn colonized n=9). In panels **A-B**, **D**, representative histology images of the colon are shown on the left. Macroscopic tumor counts are shown on the right.

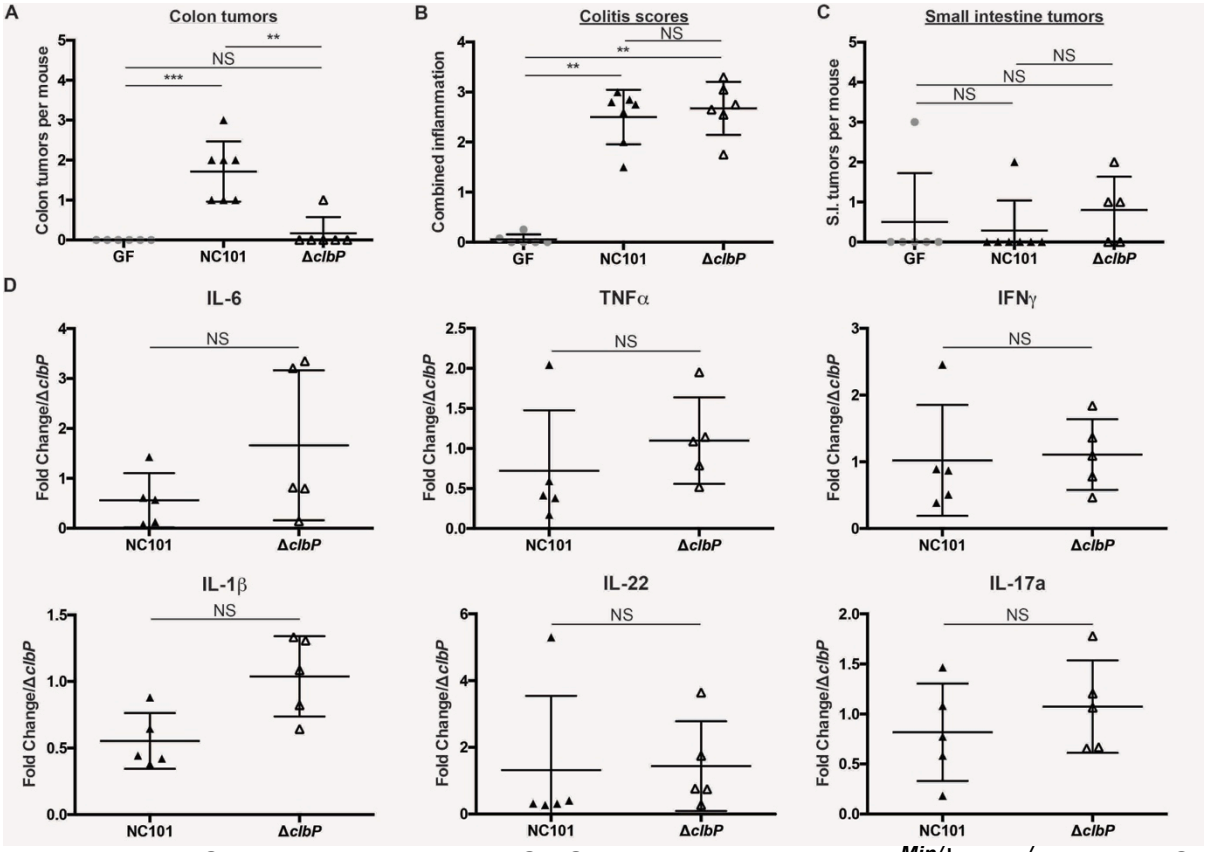


Figure 3.6.6. Colibactin promotes CRC development in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. A-C) Colon tumor counts (**A**), colitis scores (**B**), and small intestine tumor counts (**C**) from GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ (n=6) and 16 week *E. coli* NC101 (n=7) or Δ*clbP* (n=6) mono-associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. **D**) IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA expression in NC101 (n=5) or Δ*clbP* (n=5) mono-associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ distal colon snips with relative fold expression compared to Δ*clbP* mono-associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice.

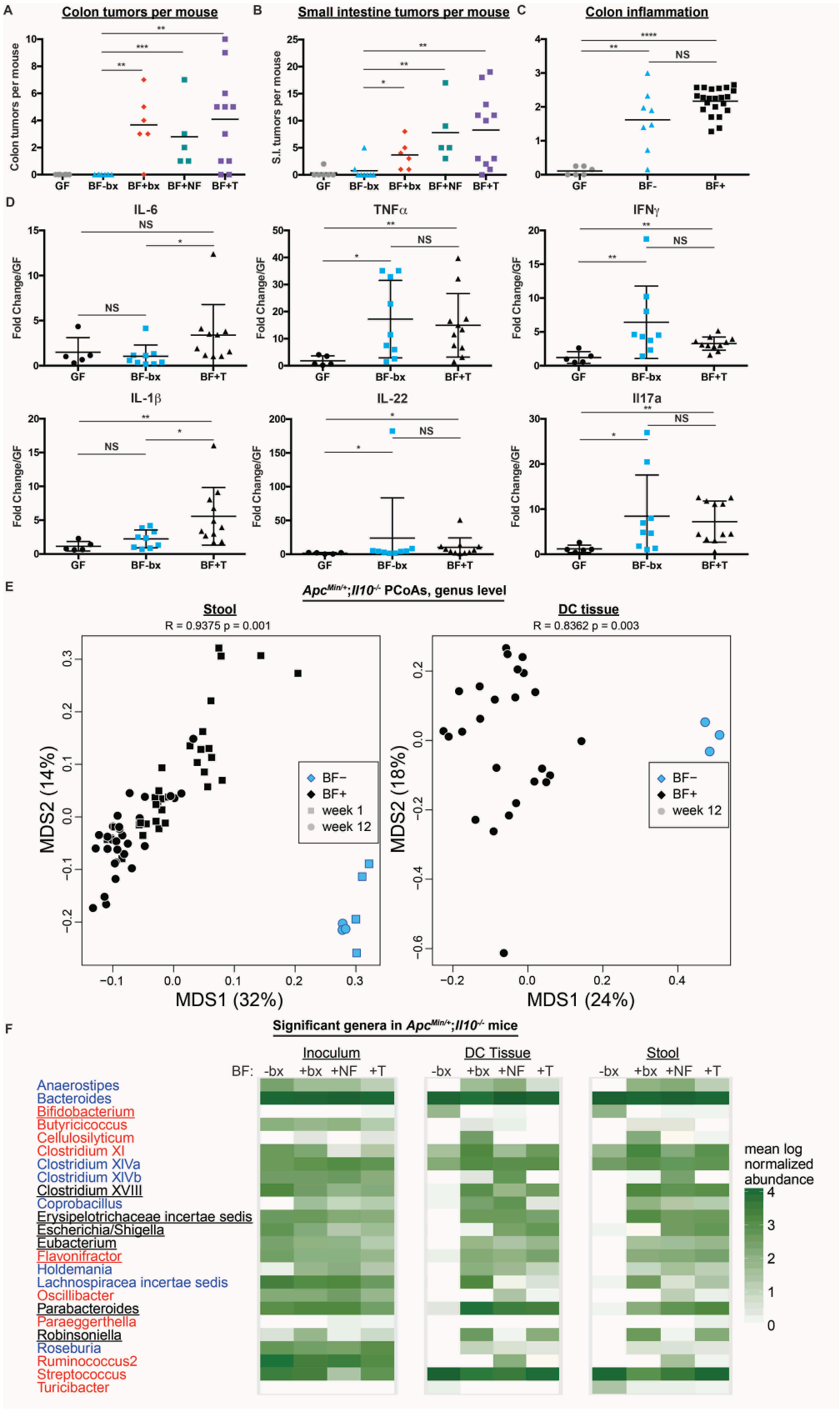

A
Colon tumors per mouse
Colon tumors per mouse
GF
BF-bx
BF+bx
BF+NF
BF+T
B
Small intestine tumors per mouse
S.I. tumors per mouse
C
Colon inflammation
BF-
BF+
NS
D
IL-6
TNFα
IFNγ
IL-1β
IL-22
Il17a
Fold Change/GF
E
Apc^Min/+;Il10^-/- PCoAs, genus level
Stool
R = 0.9375 p = 0.001
DC tissue
R = 0.8362 p = 0.003
MDS1 (32%)
MDS2 (14%)
MDS1 (24%)
MDS2 (18%)
BF−
BF+
week 1
week 12
F
Significant genera in Apc^Min/+;Il10^-/- mice
Inoculum
DC Tissue
Stool
BF: -bx +bx +NF +T
Anaerostipes
Bacteroides
Bifidobacterium
Butyricicoccus
Cellulosilyticum
Clostridium XI
Clostridium XIVa
Clostridium XIVb
Clostridium XVIII
Coprobacillus
Erysipelotrichaceae incertae sedis
Escherichia/Shigella
Eubacterium
Flavonifractor
Holdemania
Lachnospiracea incertae sedis
Oscillibacter
Parabacteroides
Paraeggerthella
Robinsoniella
Roseburia
Ruminococcus2
Streptococcus
Turicibacter
mean log normalized abundance

Figure 3.6.7. Human biofilm-associated bacteria promote tumorigenesis in gnotobiotic *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. A-B) Colon (**A**) and small intestine (**B**) tumor counts in GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice associated with BF- (n=8) or BF+ (n=6) inoculums pooled from colonoscopy biopsies (bx) from healthy patients and BF+ inoculums pooled from the normal flanking (NF) (n=5) and tumor tissue (T) (n=11) from CRC patient resections. **C**) Colon inflammation scores in GF, BF-, and BF+ (all 3 BF+ groups combined) associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. **D**) IL-6, TNFα, IFNγ, IL-1β, IL-22 and IL-17a mRNA expression in proximal colon tissue snips from GF (n=5), BF- (n=10), and BF+T (n=11) associated mice with relative fold expression compared to GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. **E**) Genus level PCoAs of BF- and BF+ associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ stool (1 and 12 week time points) and distal colon (DC) tissue microbiota (12 week time point). ANOSIM R and corresponding p value are noted for each compartment. **F**) Heatmap depicting mean $\log_{10}$ normalized relative abundances of genera that were significantly different between biofilm groups in the stool (red font), DC tissue (blue font), or both compartments (black font).

Name	Sequence
PE1_27F-1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTAAGAGTTTGATCCTGGCTCAG
PE1_27F-2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCATAGAGTTTGATCCTGGCTCAG
PE1_27F-3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCTAGAGTTTGATCCTGGCTCAG
PE1_27F-4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCAAGAGAGTTTGATCCTGGCTCAG
PE1_27F-5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGATAGAGTTTGATCCTGGCTCAG
PE1_27F-6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTACGAGAGTTTGATCCTGGCTCAG
PE1_27F-7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATTGGCAGAGTTTGATCCTGGCTCAG
PE1_27F-8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCTGAGAGTTTGATCCTGGCTCAG
PE1_27F-9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGACAAAGAGTTTGATCCTGGCTCAG
PE1_27F-10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATAAGAGTTTGATCCTGGCTCAG
PE1_27F-11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGCTAAGAGTTTGATCCTGGCTCAG
PE2_534R-1	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAGCATTACCGCGGCTGCTGG
PE2_534R-2	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTTACCATTACCGCGGCTGCTGG
PE2_534R-3	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTACATTACCGCGGCTGCTGG
PE2_534R-4	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCAGTATTACCGCGGCTGCTGG
PE2_534R-5	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAGCTATTACCGCGGCTGCTGG
PE2_534R-6	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGTGAATTACCGCGGCTGCTGG
PE2_534R-7	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTAGTGATTACCGCGGCTGCTGG
PE2_534R-8	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATGCGATTACCGCGGCTGCTGG
PE2_534R-9	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTGACTGTATTACCGCGGCTGCTGG
PE2_534R-10	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTCACTGTATTACCGCGGCTGCTGG
PE2_534R-11	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTCAGAGCTATTACCGCGGCTGCTGG
PE2_534R-12	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGCATGTATTACCGCGGCTGCTGG
PE2_534R-13	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATCGTGATTACCGCGGCTGCTGG
PE2_534R-14	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTACATCATTACCGCGGCTGCTGG

PE2_534R-15	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTCAGCATATTACCGCGGCTGCTGG
PE2_534R-16	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAGTCACGATTACCGCGGCTGCTGG
PE2_534R-17	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTACGAGTGCATTACCGCGGCTGCTGG
PE2_534R-18	CAAGCAGAAGACGGCATACGAGATCGGCATTCCTGCTGAACCGCTCTTCCGATCTGACCACTTATTACCGCGGCTGCTGG

Supplemental Table 3.1. V1-V3 16S rRNA MiSeq primer sequences. Unique barcode in red.

CHAPTER 4 : CONCLUSIONS AND FUTURE DIRECTIONS

4.1. The microbiota promotes systemic neutrophil function through SAA.

We demonstrated that microbes promote systemic immune function using gnotobiotic zebrafish. Specifically, we showed that colonizing germ-free (GF) zebrafish with a conventional microbiota increased neutrophil numbers and myeloperoxidase expression, altered neutrophil localization and migratory behaviors and facilitated neutrophil recruitment to sites of injury. We showed that the acute phase protein serum amyloid A (SAA) was also induced by the microbiota (Fig. 4.6.1). *In vitro* experiments revealed zebrafish cells respond to SAA exposure by activating nuclear factor (NF)-κB and neutrophils depend on NF-κB for SAA-dependent migration (Fig. 4.6.1). These findings contribute to the growing body of evidence that the microbiota affects both innate and systemic immunity. However, the mechanism(s) by which the microbiota mediates such a wide range of effects are still unclear. Our work suggests that the acute phase protein, SAA has the potential to mediate at least some of these effects.

4.2. Future directions regarding the roles of the microbiota and SAA in modulating systemic and intestinal immunity

What are the specific bacteria, bacterial components and metabolites that modulate systemic immunity and SAA induction?

Additional studies with gnotobiotic zebrafish, demonstrated members of the zebrafish microbiota have differential capacities to induce neutrophils in the intestine (Rolig et al., 2015). For example, *Vibrio* and *Aeromonas* induced neutrophil influx while *Shewanella* suppressed it, and *Shewanella*'s suppressive effect was dominant when associated with the other 2 strains (Rolig et al., 2015). Although, the authors only examined neutrophil response in the intestine, it is possible these bacterial specific effects extend to the periphery as well. Additionally, the authors show *Shewanella*'s suppressive effect is mediated through a secreted factor, as supernatant was also capable of suppressing neutrophil influx (Rolig et al., 2015). Although, it is unclear whether SAA had a role in modulating these bacterial specific effects our study showing SAA modulates migratory behaviors suggests it may. Other bacteria that may modulate SAA expression include the polyketide synthase (*pks*+) *Escherichia coli* Nissle, which decreased colitis and serum SAA concentrations in *Il10*$^{-/-}$ mice (Kamada et al., 2005; Olier et al., 2012; Sonnenborn and Schulze, 2009) and segmented filamentous bacteria (SFB), which induced SAA through adherence to epithelial cells (Atarashi et al., 2015; Sano et al., 2015).

Microbiota colonization was previously shown to induce *saa* in the zebrafish intestine in a myeloid differentiation primary response protein 88 (MyD88) dependent manner, so one possible mechanism modulating systemic neutrophil function may

be related to Toll-like receptor (TLR) recognition of microbial secreted factors (Kanther et al., 2011). Further supporting this notion, administering microbial-associated molecular patterns (MAMPs) (heat-killed *E. coli* Nissle or autoclaved cecal contents) to GF mice is able to restore most aspects of myelopoiesis (neutrophils and monocytes) in a TLR dependent manner (Balmer et al., 2014; Khosravi et al., 2014). Neutrophil development, homeostasis, and function have been shown to be microbiota-dependent in neonatal mice, but it is not known whether SAA also plays a role in mediating neutrophil function in that context (Deshmukh et al., 2014).

What cell types interact with the microbiota and produce SAA?

While our study did not examine the cellular source of SAA upon microbiota colonization, *in vitro* work by other groups demonstrated LPS stimulation of mouse CMT93 cells induced SAA3 (Eckhardt et al., 2010), while SFB adherence and growth on a mouse intestinal epithelial cell (IEC) cell line (mICcl2) induced SAA (SAA1-3) (Schnupf et al., 2015). While zebrafish have only 1 SAA, humans and mice have 3 and 4 forms, respectively (Kanther et al., 2014) and these could be induced by different microbes or microbial components. SFB mono-colonization of GF mice, revealed adherence of SFB primarily within the terminal ileum, which induced SAA1/2 expression from small intestinal epithelial cells and lead to T helper lymphocyte type 17 (Th17) cell induction (Atarashi et al., 2015; Sano et al., 2015). Additionally, SAA expression by small intestine IECs was further enhanced by IL-22

from group 3 innate lymphoid cells (ILC3s, cells that also respond to the microbiota) (Honda and Littman, 2016; Sano et al., 2015). It is unclear whether an SFB-like bacteria equivalent induces SAA in humans, although adherence to colon IECs does seem to play a role in Th17 cell induction in mice associated with human clinical isolates from Crohn's disease (CD) patients (Atarashi et al., 2015). Additionally, SFB has been implicated in regulation of pulmonary defense against *Staphylococcus aureus* and *Aspergillus fumigatus* lung infection by modulating the Th17 response in mice (Gauguet et al., 2015; McAleer et al., 2016), but it is unknown whether SAA plays a role in this extra-intestinal effect.

What other aspects of immunity or disease are impacted by the microbiota's effects on neutrophils or SAA?

Our studies focused on the developing zebrafish, but subsequent studies suggest the microbiota and SAA continues to impact host health. For example, neutrophil turnover is an important aspect of immune homeostasis, however a recent study suggests the microbiota can inhibit this process by promoting neutrophil ageing, which results in neutrophils that are more pro-inflammatory, exhibit impaired migration and contribute to diseases such as sickle-cell disease or septic shock (Zhang et al., 2015). Interestingly, neutrophil aging in mice was promoted by MAMPs in the blood that signal through neutrophil TLRs that are MyD88 dependent (Zhang et al., 2015). Thus, the systemic impact of microbiota on neutrophil function may extend to extra-intestinal diseases as well. Furthermore, the mechanisms by which the microbiota modulates local and systemic immunity has important implications for

acute infections and vaccine and cancer immunotherapy efficacy (Gorjifard and Goldszmid, 2016).

The microbiota and SAA may also impact the pathogenesis of intestinal diseases. For example, studies have shown an association between inflammatory bowel diseases (IBD) and SAA levels, but the role of the microbiota in this observation is unclear. Recent work suggests the microbiota may be mediating this response, as colonizing gnotobiotic *Il10*$^{-/-}$ mice with stool from healthy, CD or ulcerative colitis (UC) patients, revealed induction of *Saa3* in all 3 association types but *Saa3* was further enhanced in mice associated with microbes from CD and UC patients compared to controls (Nagao-Kitamoto et al., 2016). Interestingly, SAA may also play a role in colitis-associated cancer (CAC) in specific-pathogen-free (SPF) *Il10*$^{-/-}$ mice, as serum neutrophils and SAA concentrations increased progressively over time, mirroring the induction of colitis and colorectal adenocarcinoma (Berg et al., 1996). Additionally, the role of SFB as an inducer of Th17 cells via IEC production of SAA1/2 is intriguing given the potential tumorigenic role of Th17 cells and their associated cytokines in cancer, particularly as IL-17 has been implicated in the pathogenesis of ETBF induced colorectal cancer (CRC) in adenomatous polyposis coli deficient (*Apc*$^{\Delta 468}$) mice (Gagliani et al., 2014; Wu et al., 2009). Together, these studies suggest that members of the intestinal microbiota modulate SAA to affect both innate and adaptive immune functions, which impact local as well as systemic immunity.

4.3. CRC-associated bacteria have differential abilities to promote tumorigenesis in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice.

We evaluated the interplay between inflammation, microbes and CRC using *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice, a spontaneous model of colon cancer. SPF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice developed more colon tumors than *Apc*$^{Min/+}$ mice and colon tumor numbers significantly correlated with colitis scores, suggesting inflammation promotes colon tumorigenesis. Utilizing gnotobiotic *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice we showed that GF *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice developed almost no colon tumors, suggesting bacteria are essential for colon tumorigenesis.

We next evaluated whether specific human CRC-associated microbes promote CRC development in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. We found that *F. nucleatum* mono-associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ and *Apc*$^{Min/+}$ mice did not develop colon tumors or colitis despite high colonization. In contrast, *E. coli* NC101 mono-associated mice developed colon tumors in a colibactin dependent manner. Finally, we evaluated whether bacterial organization contributes to CRC development by associating gnotobiotic *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice with either biofilm negative or biofilm positive microbes from healthy or CRC patient tissue. We found colon tumors developed in biofilm positive associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice, regardless of donor health status (Fig. 4.6.2). Additionally, inflammation developed in both biofilm negative and positive associated mice, suggesting microbial organization and composition has a greater impact on tumorigenesis than inflammation.

4.4. Future directions for evaluating how human CRC-associated bacteria and microbial biofilms promote carcinogenesis

Does inflammation alter microbial activities during CRC progression?

One important next step will be to examine how host inflammation modulates the microbiota and what role this plays in cancer progression. Studies profiling the fecal microbiota in C57BL/6 *Apc*$^{Min/+}$; mice demonstrated dysbiosis (increased *Bacteroides* spp.) preceded neoplasia, suggesting dysbiosis may contribute to initiation of tumorigenesis (Son et al., 2015). Furthermore, two different *E. coli* NC101 heat shock proteins (*ibpB* and *ibpA*) have previously been shown to be upregulated in inflamed mono-associated *Il10*$^{-/-}$ mice (Patwa et al., 2011). Interestingly, *in vitro* experiments demonstrated *E. coli pks* requires the heat shock protein HtpG (Hsp90$_{Ec}$), a molecular chaperone for colibactin production (Garcie et al., 2016) suggesting there could be interplay between the heat shock response during inflammation and colibactin production. Additionally, work from our lab demonstrated 5 *pks* island genes were increased at 12 weeks in azoxymethane (AOM)/*Il10*$^{-/-}$ compared to *Il10*$^{-/-}$ mice suggesting inflammation and cancer alters *E. coli* gene expression (Arthur et al., 2014). Together, these 2 studies suggest inflammation and/or cancer has the capacity to alter *E. coli* microbial activities. Both of these 2 previous studies examined expression of *E. coli* in the luminal (cecal contents vs stool, respectively) compartment, so an important next step will be to examine gene expression in mucosal tissue-associated *E. coli*.

We showed that inflammation was not sufficient to promote CRC in *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice associated with either *E.coli* Δ*clbP* or biofilm negative microbes from human tissue. It would be interesting to see whether NC101 and biofilm positive microbes could promote CRC in a model without genetic predisposition to inflammation, such as the *Apc*$^{Min/+}$ mouse. Alternatively, another approach would be to treat *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice with an nonsteroidal anti-inflammatory drug (NSAID) such as mesalamine (5-aminosalicylic acid), which was previously implicated in reducing IBD patient biofilms (Swidsinski et al., 2005b, 2007) and study microbial gene expression, especially *pks* genes.

Are there colonization advantages for Enterobacteriaceae to maintain pks?

Another area of interest is whether acquiring *pks* confers colonization advantages to *E. coli*. Before the microbiota stabilizes around 2 years*, E. coli* is a relatively dominant member of the infant microbiota, but eventually settles to 10^7-10^8 CFU/gram in the colon (Secher et al., 2016). Approximately 30% of phylogenetic group B2 *E. coli* are *pks*+*,* and their prevalence is increasing among infants (Secher et al., 2016). Experiments with neonatal mice suggests early colonization with *pks*+ *E. coli* altered barrier function and increased immune activation (Secher et al., 2015). Of note, *pks* has been found within other Enterobacteriaceae, including *Klebsiella pneumonia* and may be highly prevalent in lab mice, with one university finding an 88% prevalence rate out of 51 tested *E. coli* isolates (García et al., 2016). Interestingly, group B2 *E. coli* appear to be a feature of Westernization as 0/24 *E.*

coli strains from an isolated group of Amerindians belonged to this phylogenetic group (Clemente et al., 2015). Could there be a colonization advantage to *pks* that is promoting its prevalence in Western countries? Intriguingly, a recent *in vitro* study showed *pks*+ *E. coli* inhibited the growth of multiple strains of *S. aureus* but no other tested bacteria including *Streptococcus*, *Enterococcus*, *Acinetobacter*, *Bacillus*, *Clostridium difficile*, *Pseudomonas aeruginosa*, and other Enterobacteriaceae (Faïs et al., 2016). It is unclear whether *pks* has a direct role in modulating colonization competition with other microbiota members *in vivo*.

Of note, *E. coli* Nissle contains *pks* but was originally isolated because of its ability to protect against the acute enteric pathogen, *Shigella*, and has been used clinically as a probiotic to treat IBD (Sassone-Corsi and Raffatellu, 2015; Schultz, 2008). *E. coli* Nissle has the ability to modulate multiple aspects of host immunity, including expansion of peripheral blood CD4+T cells, promotion of tight junctions, induction of immunoglobulin A (IgA) and IgM in infants, induction of antimicrobial peptides (AMPs such as β-defensin 2), and modulation of anti- and pro-inflammatory cytokines (Behnsen et al., 2013). Previous work suggests siderophores, which are involved in iron acquisition, contribute to Nissle's ability to reduce *Salmonella enterica* serovar Typhimurium colonization (Sassone-Corsi and Raffatellu, 2015). Strikingly, *E. coli* Nissle's probiotic ability to reduce inflammation in dextran sulfate sodium (DSS) and T cell transfer models of colitis was dependent on the *pks* gene *clbA*, a phosphopantetheinyl transferase capable of contributing to siderophore production (Martin et al., 2013; Olier et al., 2012). It is unclear whether *pks* plays a role in other aspects of Nissle's probiotic immunomodulatory effects besides

contributing to colonization resistance through siderophore modulation. It will also be important to directly assess the contribution of colibactin to *E. coli* Nissle's beneficial effects by deleting the peptidase ClbP, which would not affect siderophore production. Also, worthy of further investigation is what accounts for the striking contrast between *E. coli* Nissle's beneficial effects and the carcinogenic abilities of other *pks*+ *E. coli* strains.

Which biofilm associated bacteria promote tumorigenesis in $Apc^{Min/+};Il10^{-/-}$ mice?

Our 16S sequencing results identified a set of core bacteria (*Clostridium* XVIII, *Erysipelotrichaceae incertae sedis*, *Escherichia Shigella*, *Eubacterium*, *Parabacteroides*, and *Robinsoniella*) that are increased in the stool and distal colon tissue of biofilm positive associated mice, however their functional role is still unknown. An important question to address is whether microbes organized as biofilms in $Apc^{Min/+};Il10^{-/-}$ mice colonized with human-derived biofilm positive bacteria. A new set of biofilm association experiments will be needed address this question since biofilm assessment requires Carnoy's fixation, bacterial counts and fluorescence *in situ* hybridization (FISH) assay. We are currently performing metatranscriptomics on the tissue-associated bacteria to examine bacterial expression patterns that are associated with biofilm forming bacteria and tumorigenesis. Based off of our 16S sequencing results and observations of Enterobacteriaceae within IBD and CRC patients' biofilms, *E. coli* is a strong candidate bacteria (Dejea et al., 2014; Swidsinski et al., 2005b). Additionally, AIEC

are capable of producing biofilms *in vitro* and the *E. coli* type 1 pili, FimH has previously been associated with intestinal epithelial cell adherence (Dreux et al., 2013; Martinez-Medina et al., 2009).

A new strategy for isolating bacterial strains from the human microbiota was recently described that selects for spore-forming bacteria by treating fecal samples with ethanol to kill non-spore-forming vegetative cells (Browne et al., 2016). Spores are a feature of *C. difficile* biofilms *in vitro* and may also promote antibiotic resistance (Semenyuk et al., 2014). Screening fecal metagenomic datasets from healthy individuals for the spore-forming genomic signature revealed spore-forming bacteria are found in ~60% of bacterial genera and represent ~30% of the total intestinal microbiota (Browne et al., 2016). Exposing spore-forming isolates to environmental stressors such as oxygen exposure and disinfectants showed the microbiota spores' resilience was similar to *C. difficile* spores (Browne et al., 2016). Although, it is unknown whether spore-forming bacterial prevalence changes during IBD or CRC, strikingly 5 out of 10 genera that were significantly increased in biofilm positive associated mice are associated with spore-forming genera isolated in this study (Browne et al., 2016). Using the same approach as Browne et al. may facilitate isolation of these strains and would subsequently allow us to test their biofilm-forming capacities *in vitro*.

Both diet and obesity are risk factors for CRC and a recent study showed administering a high-fat diet (HFD) to mice altered the spatial distribution of the bacteria within the small intestine (Tomas et al., 2016). The HFD was 40% fat by weight and increased the amount of bacteria in the ileum intervillous zones as well

as altered bacterial composition through down-regulation of peroxisome proliferator-activated receptor-γ, which altered mucosal defenses (AMPs mucus secretion, barrier permeability) (Tomas et al., 2016). Similarly, administering dietary emulsifiers, common food additives, to mice altered microbial localization in the colon, increased the amount of adherent bacteria, altered composition, induced colitis in *Il10*$^{-/-}$ mice and was associated with obesity/metabolic syndrome (Chassaing et al., 2015). Thus, in addition to altering composition and being a risk factor for CRC, diet may also have the capacity to alter bacterial organization. Further investigations are needed to determine whether diet plays a role in the pathogenesis of biofilm associated colorectal cancer.

What are the mechanisms that promote formation of adherent biofilms associated with CRC?

One possible theory of how pathogenic intestinal biofilms form, arises from the observation that some of the anaerobic bacteria increased in CRC patient stool samples (*Fusobacterium*, *Parvimonas*, *Gemella* and *Peptostreptococcus*) are also found within the mouth (Feng et al., 2015; Flemer et al., 2016). Some of these bacteria form dental plaques or biofilms in the mouth, which can contribute to periodontal infections (Bao et al., 2015; Flynn et al., 2016). This is unlikely to be a driving mechanism based on our results though, as these genera were not significantly increased in our biofilm positive associated *Apc*$^{Min/+}$;*Il10*$^{-/-}$ mice. Another mechanism contributing to biofilm formation could be related to quorum sensing and

bacterial second messengers which have both been shown to regulate biofilm formation in enteric pathogens (Flemming et al., 2016; Tamayo et al., 2007).

An *in vitro* approach will facilitate the identification of mechanisms that contribute to biofilm formation, provided we are able to isolate candidate biofilm associated cancer-promoting bacteria from either human tissue or biofilm positive associated mice. *In vitro* approaches would include using the Calgary biofilm device system under anaerobic conditions (Sproule-Willoughby et al., 2010). Additionally, since host mucus may be an important contributing factor, growing the bacteria in dissolved mucus or coating the pegs of the Calgary biofilm device with intestinal mucus may facilitate growth under *in vitro* conditions (Bollinger et al., 2003, 2006; Reisner et al., 2006).

Future studies should also address the possibility that other microbiota members besides bacteria may contribute to biofilm associated pathogenesis. Human stool is comprised of 10^9 virus-like particles per gram and a metagenomic survey of DNA viruses from a single individual's stool over time indicates the majority of these are bacteriophages, which infect bacteria (Minot et al., 2013). Initial studies suggest bacteriophage composition shifts depending on age and health status (Lim et al., 2015; Manrique et al., 2016; Norman et al., 2015). Although the role of bacteriophages in microbiota associated intestinal diseases is unclear, transfer of bacteriophages has been demonstrated in fecal microbiota transplantation (FMT), a treatment used for refractory *Clostridium difficile* infections (Chehoud et al., 2016). Thus it is possible that gnotobiotic experiments, which transplant microbes from human stools or tissues into mice, may also be

transplanting bacteriophages. This could be a potential confounding factor to our biofilm findings if intestinal bacteriophages are able to facilitate biofilm formation. There is precedence for this in the context of cystic fibrosis airway secretions, where filamentous bacteriophage within *P. aeruginosa* boost biofilm viscosity through interaction with host (hyaluronan) and microbial polymers (Secor et al., 2015). Interestingly, a filamentous phage that infects *E. coli* was also able to interact with hyaluronan, a polysaccharide that's been shown to accumulate in the connective tissue of colon adenocarcinomas (Secor et al., 2015; Wang et al., 1996). Further investigations are needed to determine whether bacteriophages are a part of human mucosal-associated intestinal biofilms, and what role they may play. Metagenomic or metatranscriptomic sequencing could also be used to characterize potential viral or fungal members, and FISH probes could then be designed to evaluate presence within intestinal biofilms, although there may be resolution difficulties with visualizing viruses (Scupham et al., 2006; Vilas Boas et al., 2016).

What aspects of microbiota composition and organization contribute to left-sided vs right-sided CRCs?

The noted differences by Dejea et al. between tumor location and biofilm (Dejea et al., 2014) deserve further investigation because of the mutational and environmental differences between proximal (right-sided) and distal (left-sided) CRC (Shen et al 2015). However, biofilm positive associated $Apc^{Min/+}$;$Il10^{-/-}$ mice primarily developed CRC in the distal colon, indicating this model may not recapitulate all the underlying nuances of proximal CRCs associated with biofilms. Examining the role

of biofilm forming microbes in mice that express the epidermal growth factor ligand, HB-EGF (*HBUS*) mice (Bongers et al., 2014), a model of proximal CRC, may reveal additional insights into how biofilm-forming microbes promote proximal CRC.

Intriguingly, specific mouse microbes were already shown to mediate serrated polyp formation in *HBUS* mice, as CRC could be ameliorated via antibiotic treatment or rederivation (Bongers et al., 2014). The specific microbes associated with cecal serrated polyps included *Bilophila* and Desulfovibrio (Hydrogen sulphate/sulphite reducing bacteria) and *Eubacterium rectale* (*Clostridium* XIVA and XIVb), which was shown to invade the lamina propria within serrated polyps via FISH staining (Bongers et al., 2014). Both the host and sulphate-reducing bacteria produce hydrogen sulfide (H_2S) and some studies have characterized host H_2S as beneficial, promoting inflammation resolution and healing (Motta et al., 2015; Wallace and Wang, 2015), In contrast, there's also evidence that H_2S from *Bilophila wadsworthia* may promote colitis in *Il10*$^{-/-}$ mice in conjunction with a high saturated fat diet (Devkota et al., 2012). We recently observed that abundance of H_2S producing bacteria including *Atopobium parvulum* on intestinal mucosal interface correlates with severity of pediatric CD (Mottawea et al. in press). In addition, *A. parvulum* enhanced development of colitis in *Il10*$^{-/-}$ mice, a phenomenon associated with H_2S production (Mottawea et al. in press). Nevertheless, the role of hydrogen sulphate/sulphite reducing or H_2S producing bacteria in promotion of CRC is unclear. *Bilophila*, *Atopobium* and *Fusobacterium* were observed in all 5 of our initial biofilm positive inoculums, but were not consistently transplanted into the biofilm positive associated mice (found in 2, 0, or 1 out of 5 biofilm positive associations,

respectively). Given that *Fusobacterium* may also associate with proximally located CRCs (Yu et al., 2016), perhaps a mouse model prone to developing proximal tumors would facilitate the study of proximal-colonizing genera, which did not consistently colonize our $Apc^{Min/+}$;$Il10^{-/-}$ mice.

Another area of interest is what drives tumorigenesis in human distal (left-sided) CRCs that are mostly biofilm negative (Dejea et al., 2014). One possibility, suggested by the association between appendectomies and distal CRC risk could be a deficiency in proximal beneficial biofilms (Wu et al., 2015). For example, a recent study used an *in vitro* approach that measured the expressional response of preformed commensal *E. coli* (K12 MG1655) biofilms upon introduction of pathogenic enteric bacteria (Enteroaggregative *E. coli* and *Klebsiella pneumonia*) to identify genes involved in biofilm resistance to pathogen colonization (Da Re et al., 2013). One of the genes identified *yceP* (*bssS*) had previously been associated with biofilm formation, and mice precolonized with a *yceP* deficient mutant were more susceptible to enteric pathogen colonization (Da Re et al., 2013). Although only one type of commensal bacteria was examined and it is unclear whether this related to biofilm formation *in vivo* (Da Re et al., 2013), these findings do suggest some microbiota biofilms could have beneficial properties. Similarly, oral gavage of purified curli fibers, components of *E. coli* Nissle biofilms, ameliorated 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis in mice and *in vitro* induced anti-inflammatory IL-10 expression in macrophages though TLR2 (Oppong et al., 2015). Whether a biofilm protects or promotes disease, likely depends on a combination of factors, the vast majority of which are still unknown but likely relate to the overall microbial

composition, location, density, proximity to the epithelium, and host health and genetics.

4.5. Conclusion

The microbiota affects multiple aspects of innate and adaptive immunity. Understanding the mechanisms governing these interactions has the possibility to improve the efficacies of vaccines and cancer immunotherapies. Our studies with biofilm associated microbes from human patients suggest organization of the microbiota within the intestine may serve as another measure of host health in addition to composition. Microbial richness has been proposed as a measure of host health, with microbiota-associated diseases such as obesity, IBD, and CRC being associated with reduced diversity. However, given the impact that sampling location has on microbiota profiling, it may be better to evaluate both the organization and composition of the microbiota in both the luminal and mucosal-associated compartments along multiple sites of the gastrointestinal tract. Of even greater importance are functional studies aimed at identifying how microbial gene expression or metabolites change depending on microbial location and structure, during different health or disease states. We've demonstrated and discussed the wide range of factors that can impact host-microbiota interactions. Elucidating the exact mechanisms of a microbe's immunomodulatory or carcinogenic effects may allow a more targeted therapeutic approach that will also minimize the chances of having unintended consequences on other microbiota members.

4.6. Figures

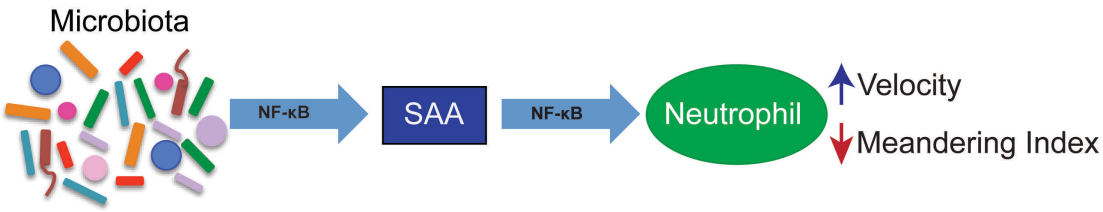


Figure 4.6.1. The microbiota promotes systemic neutrophil development and mediates neutrophil migration in an Saa-NF-κB dependent manner.

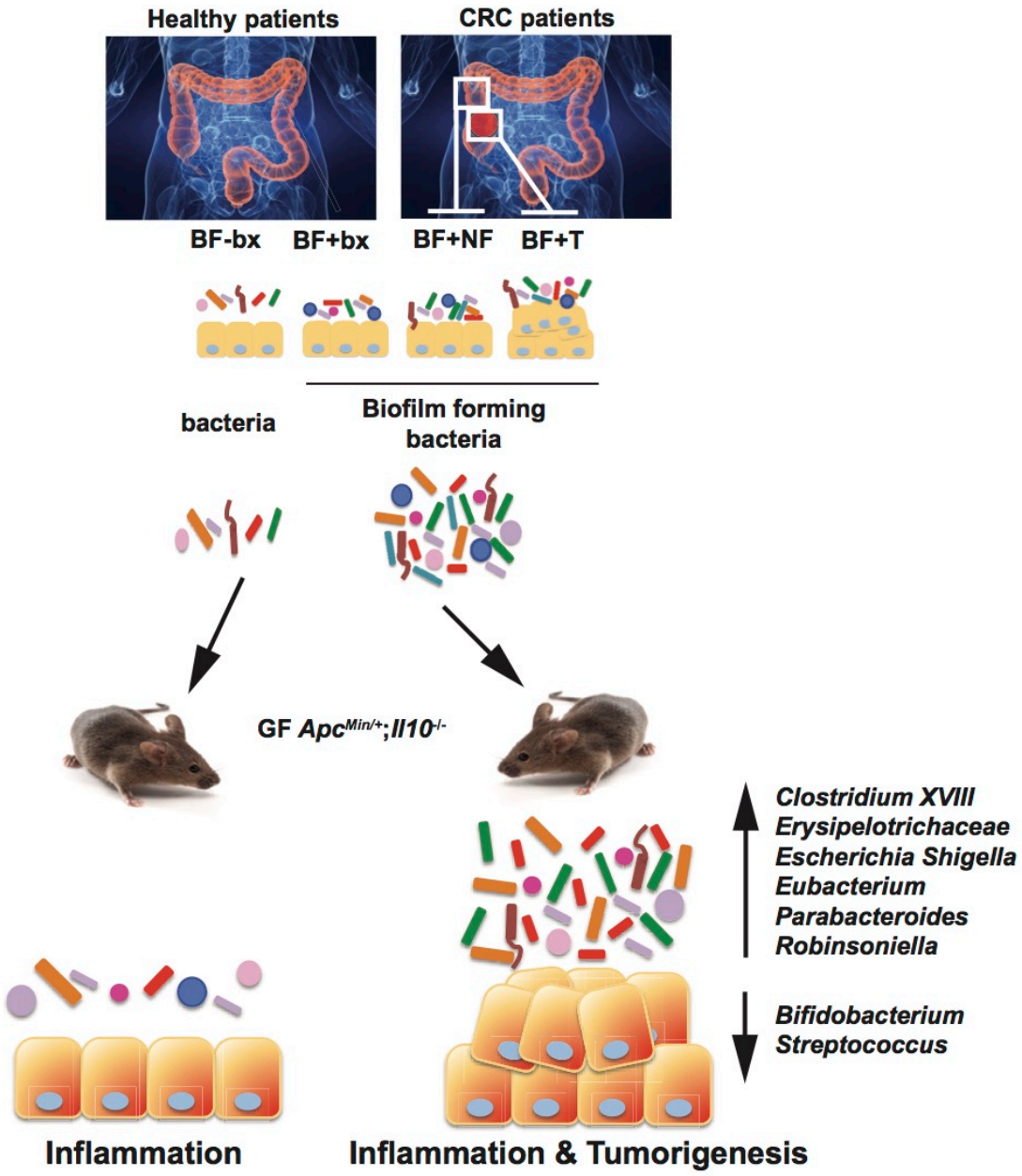


Figure 4.6.2. Microbial biofilms foster bacterial activities that promote colorectal cancer.

APPENDIX 1: MICROBIOTA AND HOST IMMUNE RESPONSES: A LOVE-HATE RELATIONSHIP[3]

Overview

A complex relationship between the microbiota and the host emerges early at birth and continues throughout life. The microbiota includes the prokaryotes, viruses and eukaryotes living among us, all of which interact to different extents with various organs and tissues in the body, including the immune system. Although the microbiota is most dense in the lower intestine, its influence on host immunity extends beyond the gastrointestinal tract. These interactions with the immune system operate through the actions of various microbial structures and metabolites, with outcomes ranging from beneficial to deleterious for the host. These differential outcomes are dictated by host factors, environment, and the type of microbes or products present in a specific ecosystem. It is also becoming clear that the microbes are in turn affected and respond to the host immune system. Disruption of this complex dialogue between host and microbiota can lead to immune pathologies such as inflammatory bowel diseases, diabetes and obesity. This review will discuss recent advances regarding the ways in which the host immune system and microbiota interact and communicate with one another.

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[3]**Tomkovich, S.** and Jobin, C. 2016. Microbiota and host immune responses: a love-hate relationship. *Immunology* 147(1), pp. 1–10.

I chose the majority of the papers we covered, did the majority of the writing and created the figures and tables.

Introduction

The microbiota refers to the population of microbes (prokaryotes, viruses and eukaryotes) living among us, outnumbering host cells by a factor of 10 (Belkaid and Hand, 2014; Mukherjee et al., 2015). The host immune system encompasses both the innate and adaptive immune systems, which work together to determine the class of microbial threat and direct the type and degree of immune response to the exposure (Iwasaki and Medzhitov, 2015). The immune system and microbiota develop and mature together, beginning at birth, or even potentially in the womb (Aagaard et al., 2014). This early coexistence is likely essential in shaping the immune system response to avoid unwanted immune reactions to intestinal microbial components. An inappropriate response to indigenous bacteria could have deleterious consequences for the host as seen with inflammatory bowel diseases (IBD).

The importance of the microbiota in shaping host immunity is best appreciated in germ-free (GF) models. GF housing conditions maintain a microorganism free environment and are a powerful system to dissect various aspects of host-microbe interactions. GF mice display an “underdeveloped” innate and adaptive immune system: reduced expression of antimicrobial peptides, reduced immunoglobulin A (IgA) production, fewer numbers of T cells types and increased susceptibility to microbial infections (Jain and Walker, 2015). The deficits of GF mice highlight the key role of microbes in bringing the immune system into a “combat ready” mode. Studies comparing monozygotic and dizygotic twins suggest that non-heritable

influences from the environment, including the microbiota, determine much of the immune variation seen in humans (Brodin et al., 2015). Alterations in the microbiota, referred to as dysbiosis, have been implicated as risk factors for IBD, cancers, multiple sclerosis, asthma, and type I diabetes; reinforcing the impact of the microbiota on host health. Diet also has profound effects on microbiota composition and metabolite production, both of which influence host immunity but this element will not be discussed here (for reviews, see (Jain and Walker, 2015; Tilg and Moschen, 2015)). This review will focus on recent advances in understanding how microbes and microbial components interact with host immunity and how these interactions influence host health.

Broad influence of microbiota on host immunity

Microbiota: intestinal effects

The establishment of a mature microbiota is a dynamic process during the first 2 years of life (Jain and Walker, 2015) and coincides with the development of the immune system. Throughout the early developmental period innate immune components play key roles in protecting the infant from pathogens and shaping microbiota assembly. IgA is found in breast milk and can prevent immune activation in infants by binding microbial antigens. Similarly, secretory IgA produced along the intestinal tract continues to be important for maintaining mucosal homeostasis through adulthood (Belkaid and Naik, 2013). The development of the mature microbiota is regulated by host immune system components, which can also be

influenced by microbiota members. Recent work with gnotobiotic mice suggests that Proteobacteria, the dominant phylum in newborns, triggers a Proteobacteria-specific IgA response in mice which plays a key role in controlling Proteobacteria levels in the adult microbiota (Mirpuri et al., 2014). Fecal IgA levels (low versus high) are partly controlled by members of the microbiota; a phenotype that is vertically transmissible and independent of host genetic factors (Moon et al., 2015). 16S rRNA sequencing revealed that *Sutterella* species are partly responsible for variable IgA levels, most likely by degrading IgA secretory component (Moon et al., 2015) (Figure A1.1). Noteworthy, expansion of Proteobacteria/Enterobacteriaceae abundance is observed in IBD patients and in preclinical models (Winter and Bäumler, 2014). Whether this bloom of microorganisms is related to microbe-mediated fecal IgA levels is unknown.

The microbiota continues to affect immune function well after development. Studies of Paneth cells using organoids generated from mice reveal that degranulation (release of antimicrobial products) is controlled by immune cell-derived interferon-γ, which may be induced *in vivo* during viral or bacterial challenge (Farin et al., 2014). Thymic and induced T regulatory lymphocytes (Treg) prevent autoimmunity and maintain tolerance to the microbiota, and a recent study suggests most colonic Tregs are thymic Tregs that recognize bacterial antigens, including antigens from *Clostridiales*, *Bacteroides*, and *Lactobacillus*. Importantly, antibiotic-induced alterations in microbiota, which decrease *Clostridiales* members among others, reduce intestinal Tregs and alter colonic thymic Tregs TCR repertoire,

suggesting that microbial composition influences the dynamic response of Tregs (Cebula et al., 2013).

One of the most studied immunomodulatory microbes are segmented filamentous bacteria (SFB), which colonize the terminal ileum in mice, induce IgA production and increase effector T cells, particularly T helper (Th) 17 cells (Belkaid and Hand, 2014). Work from several groups suggest that SFB-induction of Th17 cells occurs in the intestinal lamina propria rather than Peyer's patches or the mesenteric lymph nodes (Geem et al., 2014; Goto et al., 2014a; Lécuyer et al., 2014). Further studies revealed that major histocompatibility complex class II (MHCII)-dependent antigen presentation by intestinal dendritic cells is essential for SFB-induced Th17 cells (Geem et al., 2014; Goto et al., 2014a). Additionally, Goto et al. provide evidence that MHCII presentation by innate lymphoid cells (ILCs) may constrain Th17 cell differentiation (Goto et al., 2014a). SFB also stimulate expansion of germinal centers and induce IgA-secreting cells in Peyer's patches, isolated lymphoid follicles, and tertiary lymphoid tissue (Lécuyer et al., 2014). Recently, Schnupf et al. cultured SFB *in vitro* and provided evidence that SFB attachment *in vivo* is required to elicit ileal epithelial responses (Schnupf et al., 2015). Future studies examining the specific structural component(s) of SFB responsible for stimulating IgA production and inducing Th17 cells could be aided by the *in vitro* culture system. Although SFB have not yet been isolated from the human gastrointestinal tract, human cell lines support SFB growth (Schnupf et al., 2015) and SFB specific 16S rRNA has been detected within human stool samples (Yin et al., 2013). Gram-stained human ileal-cecal biopsies from a small set of IBD and non-

inflamed patients suggest SFB is present in ulcerative colitis patients but absent in Crohn's disease patients (Caselli et al., 2013). Thus, future studies may reveal a role for SFB or SFB-related bacteria in human immune development and IBD.

In addition to interactions with the immune system, microbes interact with other microorganisms. While many symbionts have beneficial immune properties, some bacteria facilitate host infection by viruses. For example, human norovirus likely binds to histo-blood group antigen-expressing bacteria such as *Enterobacter cloacae*, which promotes attachment and infection of B cells (Jones et al., 2014). Conversely, treating mice with the bacterial product, flagellin, prevents rotavirus infection by modulating host innate immune signaling (Zhang et al., 2014). Thus, microbiota composition can promote or inhibit viral infection depending on the type of virus.

Microbiota effects on extraintestinal immunity

The effects of the microbiota on host immunity extend beyond the intestine. GF zebrafish have fewer and less active neutrophils compared to zebrafish colonized with a normal microbiota, as well as impaired neutrophil recruitment in a tail fin injury model; a phenomenon linked to microbial induction of serum amyloid A (Kanther et al., 2014). Neonatal mice that are GF or born from antibiotic-treated dams have fewer circulating and bone marrow neutrophils and are more susceptible to *E. coli* K1 and *Klebsiella pneumoniae* sepsis, likely through microbiota induction of granulocytosis (Deshmukh et al., 2014). Thus, the microbiota contributes to

neutrophil development, homeostasis, and function in both mice and zebrafish (Deshmukh et al., 2014; Kanther et al., 2014). GF mice have increased invariant natural killer T (iNKT) cells in the lung and colon due to enhanced CXCL16 expression, making them more susceptible to an ovalbumin-driven model of allergic asthma (Olszak et al., 2012). These studies also suggest that early exposure to microbes is important, as the iNKT cell levels returned to low levels when GF mice were exposed to specific-pathogen-free (SPF) conditions upon birth but not as adults (Olszak et al., 2012). Consequently, the intestinal microbiota has both local and systemic effects on innate and adaptive immunity (Figure A1.1).

Although the majority of the microbiota resides within the intestine, the microbial communities located in extraintestinal regions also influence local host immunity (Surana and Kasper, 2014). Studies comparing GF mice to conventionally raised mice suggest that the skin microbiota regulates expression of complement component C5a receptor (C5aR), which regulates innate immune defense genes, thereby impacting microbiota diversity and composition (Chehoud et al., 2013). Certain skin microbiota community members, particularly *Staphylococcus epidermidis* interactions with $CD103^+$ dendritic cells, can induce $CD8^+$ T cell migration to the epidermis, which enhances barrier function and limits epicutaneous *Candida albicans* infection via induction of IL-17 (Naik et al., 2015). In a skin wound healing model, wound closure rate was restored in GF mice conventionalized with microbiota, a phenomenon associated with increased neutrophil accumulation and lower macrophage infiltration into the injured region (Canesso et al., 2014). Microbial dysbiosis has also been implicated in extraintestinal diseases, such as increased

Staphylococcus aureus which is associated with inflammatory skin conditions (Kobayashi et al., 2015). Thus, microbes occupying extraintestinal niches also influence host immunity, although the specific organisms and mechanisms responsible for these responses (Table A1.1) can differ between regions.

Bacterial components that effect innate and adaptive immunity

Structures detected by pattern recognition receptors

The innate immune system detects microbial components or products through several different families of pattern recognition receptors (PRRs), found on numerous cell types including macrophages, dendritic cells and epithelial cells (Iwasaki and Medzhitov, 2015). Toll-like receptors (TLRs) are a class of transmembrane PRRs located on either the cell surface or in endosomes (Maynard et al., 2012). One of the most characterized bacterial immunomodulators is *Bacteroides fragilis* polysaccharide A (PSA), which is recognized by TLR2 and capable of influencing T cell development and homeostasis (Troy and Kasper, 2010). Recent studies reveal that PSA activates TLR2 on plasmacytoid dendritic cells (pDCs) rather than conventional dendritic cells, leading to the induction of IL-10 secretion by $CD4^+$ T cells and mucosal protection during a 2,4,6-trinitrobenzene sulfonic acid (TNBS) model of colitis (Dasgupta et al., 2014). PSA-TLR2 activation of pDCs and Treg induction can also mediate protection in extraintestinal inflammatory diseases such as experimental autoimmune encephalomyelitis, a multiple sclerosis animal model (Dasgupta et al., 2014; Wang et al., 2014). Additional work

characterizing PSA-induced Treg activation via MHCII mediated antigen presentation suggests that the interaction depends on the zwitterionic (carries positive and negative charges) properties of PSA and induces a specific clonal expansion of Tregs (Johnson et al., 2015b). *Lactobacillus plantarum* teichoic acid D-alanylation (component of Gram-positive bacterial envelope) also signals through TLR2 and promotes a pro-inflammatory cytokine response in dendritic cells, which modulates effector and regulatory T cell populations (Smelt et al., 2013) (Figure A1.2).

Bacteria flagellin is recognized by TLR5 expressed on various cells including intestinal epithelial cells (IECs) and dendritic cells. IEC-derived TLR5 signaling appears to influence microbiota composition and host response because $TLR5^{\Delta IEC}$ mice have an altered microbiota compared to cohoused sibling wild-type controls, develop low grade inflammation and metabolic syndrome, and have delayed clearance of adherent invasive *Escherichia coli* (AIEC) (Chassaing et al., 2014). How TLR5 activation controls microbiota composition is unclear but could involve immune cell recruitment to clear pathogens in close proximity to the epithelium, stimulation of epithelial antimicrobial peptide production, or induction of flagellin-specific IgA (Chassaing et al., 2014). The microbiota also impacts vaccine immunity through TLR5 signaling in B cells and macrophages, which is critical for mounting an antibody response to trivalent inactivated influenza vaccine and the inactivated polio vaccine (Oh et al., 2014).

Most of the downstream signaling from TLRs occurs through either myeloid differentiation primary response protein 88 (MyD88) or Toll-interleukin receptor

domain-containing adaptor protein inducing interferon-β (TRIF) adaptor proteins, resulting in activation of NF-κB or interferon regulatory factors, respectively (Gay et al., 2014). Luminal bacteria promote mucus secretion and movement of monocytes closer to epithelial stem cells through an epithelial MyD88-signaling pathway. Increased proximity of monocytes to epithelial stem cells results in increased crypt cell proliferation and intestinal stem cell numbers (Skoczek et al., 2014), which could be beneficial during intestinal injury response. Studies comparing GF mice to mice colonized with 3 strains of bacteria (*E. coli* K-12, *Staphylococcus xylosus*, and *Enterococcus faecalis*) reveal that GF mice have delayed microbial clearance, reduced inflammatory responses to intravenous *E. coli* K12 infection and a decreased myeloid cell pool size (Balmer et al., 2014). Heat-stable microbial antigens in the serum are able to restore bone marrow myeloid cell numbers through MyD88/TICAM-dependent TLR signaling (Balmer et al., 2014). MyD88-dependent TLR signaling also plays a role in microbiota-mediated tolerance to a non invasive strain of *Salmonella enterica* serovar Typhimurium by preventing CX_3CR1^{hi} mononuclear phagocytes-mediated transport of luminal bacteria to the mesenteric lymph nodes (Diehl et al., 2013). Furthermore, tumor necrosis factor receptor associated factor 6 (TRAF6), a component of TLR signal transduction, has MyD88-independent effects on immune and microbiota homeostasis. Mice with TRAF6-deficient dendritic cells develop Th2-driven small intestine inflammation and have decreased Treg cells, both of which are microbiota-dependent (Han et al., 2013).

Nod-like receptors (NLRs) are a class of cytosolic PRRs that act as intracellular sensors (Iwasaki and Medzhitov, 2015). Nod2 recognizes bacterial peptidoglycan through muramyl dipeptide (MDP). Studies using *Nod2*$^{-/-}$ mice reveal reduced intraepithelial lymphocytes (IELS) and administering MDP to antibiotic-treated mice restored IEL numbers through upregulation of IL-15, suggesting Nod2-mediated recognition of the microbiota affects IEL homeostasis (Jiang et al., 2013). Nod2 has also been implicated in preventing goblet cell dysfunction and restricting expansion of *Bacteroides vulgatus* in the small intestine, which prevents piroxicam-induced intestinal inflammation in mice (Ramanan et al., 2014). NLRs also have extraintestinal effects, as NLR ligands have been implicated in innate immunity in the lung, which is important for *K. pneumonia* clearance (Clarke, 2014).

Inflammation or injury can cause members of the microbiota to become pathogenic and stimulate the immune system to induce inflammation. In the context of a dextran sulfate sodium (DSS) mouse model, *Proteus mirabilis* can induce IL-1β production via NLRP3 inflammasome activation in recruited inflammatory monocytes, promoting intestinal inflammation (Seo et al., 2015). After comparing different strains, the authors determined that *P. mirabilis* HpmA hemolysin induces K^+ efflux, which is required for NLRP3-induced inflammasome activation (Muñoz-Planillo et al., 2013; Seo et al., 2015). *In vitro* studies show AIEC isolated from IBD patients are also able to induce IL-1β through NLRP3 activation in macrophages (la Fuente et al., 2014).

Outer membrane vesicles

Outer membrane vesicles (OMVs) are produced by Gram-negative bacteria and contain various bacterial components, many of which activate PRRs (Kaparakis-Liaskos and Ferrero, 2015). OMVs can promote immune homeostasis or enhance bacterial pathogenesis; effects that likely depend on the type of bacteria, OMV content and the host environment (Kaparakis-Liaskos and Ferrero, 2015). For example, *Bacteroides thetaiotaomicron* (*B. theta*) OMVs containing homologs of mammalian inositol phosphatase interact with IECs *in vitro* to promote intracellular calcium signaling (Stentz et al., 2014). This signaling confers nutritional benefits and potentially anticarcinogenic properties, as dietary inositol hexaphosphate administration reduces tumorigenesis in carcinogen (1,2-dimethylhydrazine or azoxymethane)–induced cancers in rats and mice (Stentz et al., 2014). On the other hand, spontaneous colitis prone CD4-*dnTgfb*2;*IL10rb*$^{-/-}$ mice exposed to *B. theta* develop inflammation due to OMVs containing sulfatase activity, which degrades mucin glycans and allows *B. theta* to interact with host macrophages (Hickey et al., 2015). *B. fragilis* PSA is also released in OMVs which can be detected by TLR2 (Deng et al., 2015; Shen et al., 2012). Some bacteria produce OMVs that have adverse effects on host immunity. For example, enterotoxigenic *B. fragilis* (ETBF) secrete *B. fragilis* toxin-dependent particles that can induce host IECs to secrete sphingolipids (specifically, sphingosine-1-phosphate) in exosome-like particles which induce Th17 cells and enhance tumorigenesis in multiple colon cancer mouse models (Deng et al., 2015).

Metabolites

Besides structural components, bacteria also generate a wide spectrum of metabolites that have the capacity to engage and trigger numerous host responses (Figure A1.2). Multiple intestinal *Bacteroides* species are able to synthesize sphingolipids, which are structurally similar to host lipid agonists of iNKTs. *B. fragilis* sphingolipids have been shown to modulate cellular homeostasis by both promoting iNKT activation (Wieland Brown et al., 2013) and inhibiting activation and expansion of iNKT cells during mouse neonatal development, which protects against oxazolone-induced colitis in adulthood (An et al., 2014).

Short chain fatty acids (SCFAs) are bacterial metabolites generated as byproducts of dietary fiber fermentation. Butyrate, propionate, and acetate are the most common intestinal SCFAs and are normally present in the millimolar range in the gut. The mechanisms by which SCFAs impact immunity include activation of G protein-coupled receptors (GPRs), inhibition of histone deacetylases, and regulation of autophagy (Brestoff and Artis, 2013). Levels of SCFAs depend on two interdependent factors: dietary fiber and microbiota composition. SCFAs may modulate protection against chemically induced DSS colitis through GPR43 and GPR109A receptor interactions that are dependent on the NLRP3 inflammasome in non-hematopoietic cells (Macia et al., 2015). Butyrate exerts anti-inflammatory effects on bone marrow derived and colonic macrophages via histone deacetylase inhibition. However, gavaging mice with butyrate does not impact the outcome of DSS colitis, suggesting butyrate promotes bacterial tolerance rather than tissue

repair (Chang et al., 2014). Additionally, butyrate has been shown to increase barrier function by stimulating epithelial metabolism in the colon to promote oxygen depletion, stabilizing hypoxia-inducible factor (HIF) and inducing HIF-dependent target genes that promote barrier function (Kelly et al., 2015). SCFAs from the microbiota can also have systemic effects. In a mouse model of allergic inflammation in the lung, high levels of propionate are protective, likely through GPR41 signaling which results in dendritic cells with high phagocytic capability and an impaired capacity to induce Th2 differentiation (Trompette et al., 2014).

In addition, microbial metabolites can influence host immune response through an indirect route. For example, SCFAs augment 5-hydroxytryptamine (serotonin) production from intestinal enterochromaffin cells through upregulation of the rate-limiting biosynthetic enzyme tryptophan hydroxylase (Yano et al., 2015). The wide impact of serotonin on host biological response including immunity (Baganz and Blakely, 2013) suggests that microbes could shape immune responses through complex mechanisms. SCFAs also directly impact innate immune cells in the brain and central nervous system. GF mice have defects in microglial (tissue macrophages of the brain) maturation, differentiation and function with a diminished response to LPS and viral challenges, while administering a mixture of propionate, butyrate and acetate to the drinking water restores microglial maturation (Erny et al., 2015). Indigenous bacteria metabolites may have key roles in inhibiting colonization of specific pathogens. For instance, *Clostridium scindens* inhibition of *Clostridium difficile* is associated with secondary bile acid synthesis (Buffie et al., 2015).

Tryptophan catabolites from the microbiota expand lactobacilli that produce an aryl hydrocarbon receptor (AHR) ligand, indole-3-aldehyde. AHR activation results in IL-22 transcription that promotes antimicrobial peptide expression and mucosal homeostasis, providing colonization resistance against gastrointestinal or vaginal *Candida albicans* infection and DSS colitis models (Zelante et al., 2013). Group 3 ILCs also rely on AHR signaling to inhibit Th17 cell expansion and regulate SFB levels, which could play a role in IBD because ~40% of mice that lack AHR signaling abilities in ILCs develop spontaneous colitis between 12 and 20 weeks of age and have exacerbated inflammation in a CD45RB*hi* T-cell transfer model of colitis (Qiu et al., 2013). In summary, bacterial components and metabolites affect both innate and adaptive immunity in the intestine (Figure A1.2).

Viral, archaeal, and eukaryotic microbiota members that influence immunity

Part of how the microbiota impacts host immunity is by limiting pathogen colonization through niche occupation and resource utilization. These indirect protective effects may extend to the viral members of the microbiota, of which there are an estimated 10^9 viruses per gram feces. Some of these viruses target mammalian cells but bacteriophages, which exclusively infect bacteria, make up the majority of the viral community (Cadwell, 2015). Bacteriophages displaying Ig-like domains on phage capsid proteins adhere to host intestinal mucus and are able reduce microbial colonization in the mucosal niche by infecting and lysing bacteria (Barr et al., 2013). Recently, metagenomic sequencing of the human fecal virome

from healthy and IBD patients revealed an expansion of *Caudovirales* bacteriophages associated with IBD (Norman et al., 2015). Thus, microbiota-associated bacteriophages may impact the pathogenesis of IBD by targeting microbial members with protective or deleterious function.

When it comes to immune system development and function, viral members of the microbiota may be able to confer some of the same immune benefits as bacteria. Murine norovirus infection of GF or antibiotic-treated mice restores intestinal morphology, lymphocyte function, and suppresses ILC2 expansion; additionally RNA-seq revealed transcriptional changes in the intestine associated with immune development and type I interferon signaling (Kernbauer et al., 2014). Whether viruses contribute to human immune system development and homeostasis remains to be determined.

Archaeal members of the microbiota can also activate host immune cells. Specifically *Methanosphaera* and *Methanorevibacter* have differential capacities to induce pro-inflammatory cytokine release from human dendritic cells. Activation requires phagocytosis of the archaea, but whether induction involves PRRs that recognizes components of the archaeal cell envelope is still unknown (Bang et al., 2014).

Studies examining the immunomodulatory effects of the fungal microbiota have mostly focused on one of the most abundant members, *Candida albicans*, which can cause severe infections in immunocompromised people (Mukherjee et al., 2015). The host uses TLRs as well as C-type lectins, a class of PRRs, to recognize fungal cell wall components such as mannans, β-glucans and chitin (Mukherjee et al.,

2015; Wagener et al., 2012). *C. albicans* cell wall components are immunomodulators, with cell wall glycosylation playing a key role in inducing pro-inflammatory cytokine expression, proliferation and apoptosis in epithelial cells (Wagener et al., 2012). Chitin induces secretion of the anti-inflammatory cytokine IL-10 that is dependent on NOD2, TLR9, and mannose receptors. Anti-inflammatory cytokines induced by chitin may play a role in resolving immune homeostasis after pathogen clearance and eosinophilia, which is a feature of asthma (Wagener et al., 2014).

Helminths are parasitic worms that modulate host immunity by inducing a strong Th2 immune response, Tregs and regulatory cytokines such as IL-10 and transforming growth factor-β (Yang et al., 2014a). Epidemiological evidence and experimental studies suggest the helminth-induced immune response may be therapeutic for treating allergies and autoimmune diseases (Girgis et al., 2013). Recent research focused on identifying the helminth immunomodulatory products has revealed that administering excretory/secretory products from *Trichinella spiralis* adult worms protects mice from DSS-induced colitis through upregulation of Tregs and reduction of pro-inflammatory cytokines (Yang et al., 2014a). The *Acanthocheilonema viteae* product (AvCystatin) modulates MAPK, p38, and ERK pathways in macrophages to induce IL-10 secretion. Administration of AvCystatin-treated macrophages to mice with OVA-induced airway inflammation or DSS-induced colitis ameliorates disease by suppressing inflammation (Ziegler et al., 2015). However, in the context of viral infection, the helminth immune response may

be detrimental. *T. spiralis* infected mice induce alternative activation of macrophages, which upregulates genes that impair the T cell response to murine norovirus (Osborne et al., 2014).

Bacterial adaptations to host immune mechanisms

Previous sections have examined how microbes direct immune development and function. However, the immune system also impacts microbes, which includes influencing microbiota composition as well as virulence capacities. Mechanisms by which specific microbes have adapted to coexist with the host immune system have begun to emerge (Table A1.2). For example, *B. theta* utilizes lipid A dephosphorylation to resist host antimicrobial peptides that target the lipopolysaccharide portion of the bacterial outer membrane (Cullen et al., 2015).

Part of the immune response to infection can promote indigenous microbiota colonization through release of nutrients. TLR agonists induce dendritic cell IL-23 production in a MyD88-dependent manner which stimulates ILCs to produce IL-22, resulting in rapid fucosylation of small intestine epithelial cells (Pickard et al., 2014). Work by Goto et al. demonstrated that bacteria such as SFB, stimulate IL-22 production by ILC3s which mediates epithelial fucosylation in the ileum and protects the host from *Salmonella typhimurium* infection (Goto et al., 2014b). Resident bacteria, such as *Bacteroides acidifaciens*, have the capacity to cleave fucose, which can subsequently be used by other members of the microbiota; a process that may promote tolerance to intestinal pathogens (Pickard et al., 2014). Host IL-22RA1

signaling promotes intestinal fucosylation in colonic organoids and the mouse cecum, restoring anaerobic bacterial diversity in the colon to protect against opportunistic pathogens such as *E. faecalis* and *Citrobacter rodentium* (Pham et al., 2014). Thus, fucosylation seems to be a host response to specific members of the microbiota or infectious challenge, which bolsters the microbiota and protects the host from multiple enteric pathogens. Reinforcing the importance of IL-22 production in maintaining colonization resistance against enteric pathogens, ID2 (a transcriptional regulator of ILCs) promotes colonization resistance against *Citrobacter rodentium* by mediating IL-22 production by ILC3s through an AHR and IL-23 receptor pathway (Guo et al., 2015).

Some pathogenic bacteria have evolved virulence factors that allow them to better cope with host immune defense mechanisms compared to indigenous bacteria. IL-22 is part of the immune response to infection and leads to induction of lipocalin-2 and calprotectin, which sequester iron, zinc and manganese ions. *Salmonella enterica* serovar Typhimurium overcomes host iron sequestration with the siderophore salmochelin and zinc sequestration through a zinc transporter, giving *Salmonella* a colonization advantage over resident Enterobacteriaceae that lack additional siderophores (Behnsen et al., 2014). *Fusobacterium nucleatum,* which has previously been linked to colon cancer (Allen-Vercoe and Jobin, 2014) is able to bind to an inhibitory receptor (TIGIT) on NK and T cells, leading to inhibition of NK cell mediated cytotoxicity/T cell activities (Gur et al., 2015). Additionally, the authors demonstrate *F. nucleatum* OMVs bind TIGIT suggesting a pro-carcinogenic role for *F. nucleatum* OMVs by inhibiting host immune function (Gur et al., 2015).

This bilateral communication between microbes and the host clearly highlights the intricate and complex nature of microbe-host interactions.

Conclusions/Perspective

The impact of the microbiota on myriad components of innate and adaptive immunity has been well established, especially in the intestine. Recent studies have moved on to characterizing how members of the microbiota and the host immune system communicate with one another. It is likely that the dialogue between microbes and host is a dynamic phenomenon, taking place from birth and evolving over time, even though the phylogenic microbial composition is quite stable for the majority of life. Therefore, microbial bioactivities rather than composition may account for most of the host response, including immunity. Clearly, more information is needed on the specific nature of this dialogue and how communication breakdowns result in disrupted host immune homeostasis. It will be important to continue detailing the relationship between microbial-derived metabolites and host immune homeostasis. These studies should include transcriptomic and metabolomics approaches to better understand the impact of microbial activities on the host immune response. The continuous characterization of microbial communities at the genomic and proteomic level, in conjunction with specific culture methods, will contribute to understanding how specific microbes shape the immune response. Ahern et al. offer a possible approach for identifying members of the human microbiota that impact the immune system using gnotobiotic mice (Ahern et

al., 2014). The persistent mapping and annotation of microbial genes in conjunction with the establishment of tools to genetically modify these genes will enable molecular dissection of the contributions of bacteria to host immunity. Additionally, it is likely that more interactions occur between members of the microbiota (viruses, prokaryotes, and eukaryotes) that influence host immune health than are currently known. Continued research focused on the signaling that occurs between microbiota components and the immune system may lead to the development of new or improved strategies to restore or reset altered communication networks between the host and microbes.

Figures and Tables

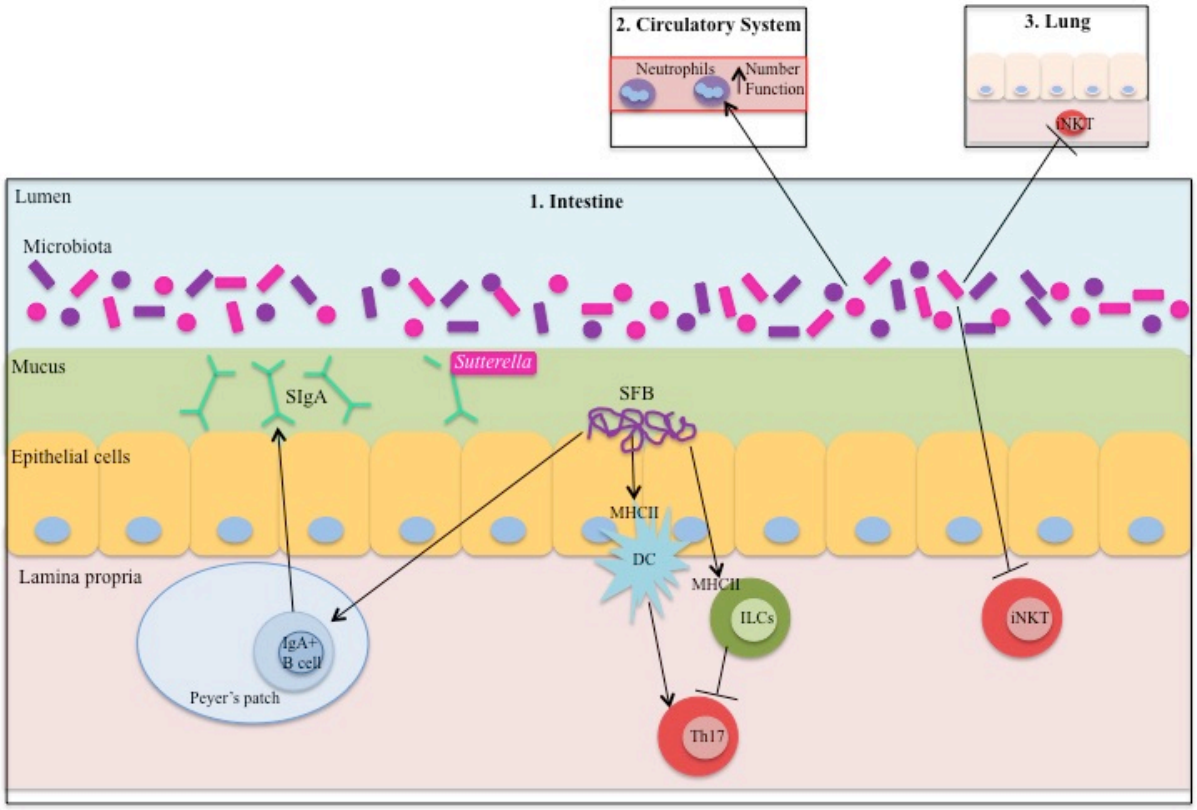

Figure A1.1. The microbiota affects local and systemic immunity. The intestine (1) contains the greatest number and diversity of microbiota members. Proteobacteria, specifically *Sutterella*, alter fecal IgA levels, likely through degradation of SIgA. SFB also alter IgA levels by promoting expansion of germinal centers and inducing IgA-secreting cells in Peyer's patches, isolated lymphoid follicles, and tertiary lymphoid tissue. MHCII-dependent SFB antigen presentation on intestinal dendritic cells induces Th17 cell differentiation, while MHCII-dependent SFB antigen presentation by ILCs constrains Th17 cell differentiation. The intestinal microbiota also influences systemic immunity, including the number and function of circulating neutrophils (2) as well as constraining iNKT levels in the lung (3) and colon (1). ILCs, innate lymphoid cells; iNKT, invariant natural killer T cell; SFB, segmented filamentous bacteria; SIgA, secretory immunoglobulin A; Th17, T helper 17 lymphocyte; Treg, T regulatory lymphocyte.

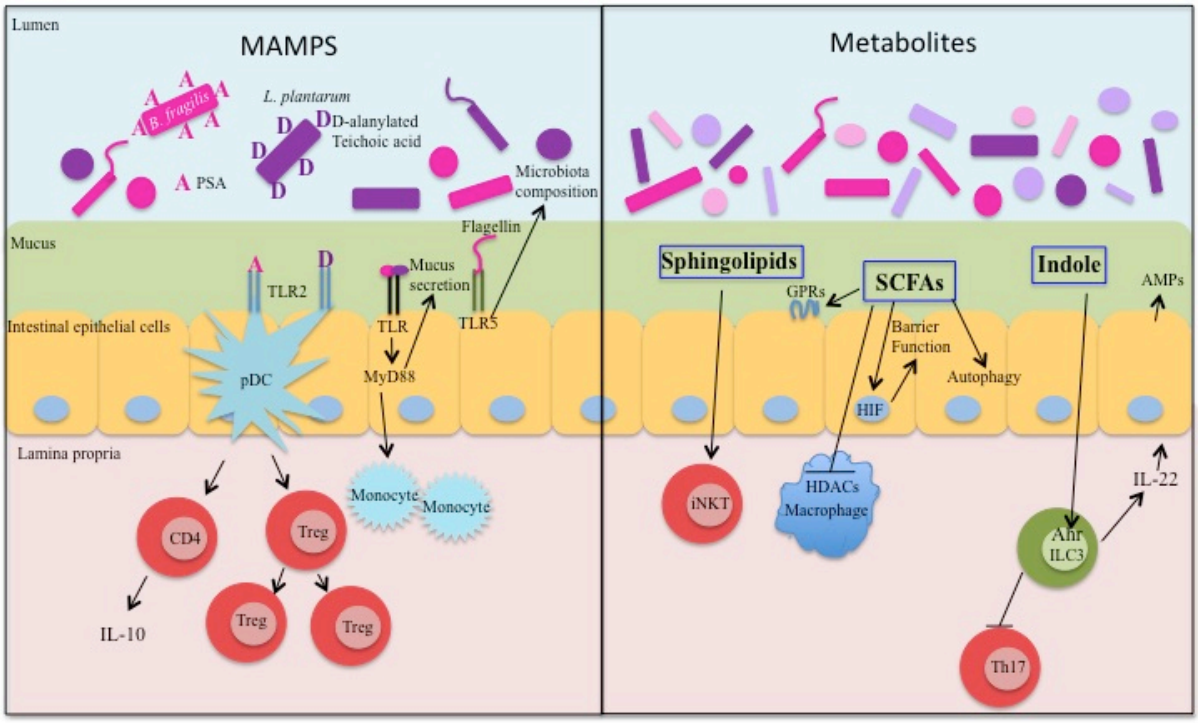

Figure A1.2: Bacterial components that affect innate and adaptive immunity in the intestine. Bacterial MAMPS signal through host PRRs. PSA (A) from *Bacteroides fragilis* interacts with TLR2 on pDCs to induce IL-10 production from CD4+ T cells and Treg clonal expansion. *Lactobacillus plantarum* D-alanylated teichoic acid (D) also signals through TLR2 on dendritic cells to modulate effector and regulatory T cell populations. Flagellin activation of TLR5 on epithelial cells alters microbiota composition. Luminal bacteria promote mucus secretion and movement of monocytes closer to epithelial stem cells through a MyD88-dependent signaling pathway. Sphingolipid metabolites from *B. fragilis* promote iNKT activation in adults. SCFA metabolites from bacteria impact immunity through multiple mechanisms: activation of GPRs, inhibition of HDACs, and regulation of autophagy. Butyrate exerts anti-inflammatory effects on macrophages through HDAC inhibition and promotes barrier function in IECs through stabilization of HIF. Lactobacilli produce an AHR ligand, indole-3-aldehyde, which induces IL-22, promoting AMP expression and mucosal homeostasis. AHR signaling on ILC3s has also inhibits Th17 cell expansion. AHR, aryl hydrocarbon receptor; AMPs, antimicrobial peptides; GPRs, G protein-coupled receptors; HIF, hypoxia-inducible factor; I, indole-3-aldehyde, an AHR ligand; IECs, intestinal epithelial cells, ILC3, group 3 innate lymphoid cell; MAMPS, microbe-associated molecular patterns; MyD88, myeloid

differentiation primary response protein 88; PRRs, pattern recognition receptors; pDCs, plasmacytoid dendritic cells; PSA, polysaccharide A from *B. fragilis*; SCFAs, short chain fatty acids; TLRs, toll-like receptors.

Bacteria	Immunomodulatory effect	Mechanism	References
AIEC	Induce inflammatory cytokines *in vitro*	Activation of NLRP3 in macrophages, inducing IL-1β production	(la Fuente et al., 2014)
Bacteroides fragilis	Influence Treg cell development and homeostasis	Bacterial PSA/OMVs containing PSA interactions with TLR2 on pDCs	(Dasgupta et al., 2014; Johnson et al., 2015b; Shen et al., 2012; Troy and Kasper, 2010; Wang et al., 2014)
	Influence iNKT cell homeostasis	Bacterial sphingolipids modulate iNKT development and activation	(An et al., 2014; Wieland Brown et al., 2013)
Bacteroides thetaiotaomicron	Promote intracellular calcium signaling, nutritional benefits in IECs	Bacterial OMVs containing inositol phosphatase	(Stentz et al., 2014)
	Promote colitis in CD4-*dnTgfb2*;*IL10rb*$^{-/-}$ mice	Bacterial OMVs containing sulfatase, degrade mucin glycans	(Hickey et al., 2015)
Clostridium scindens	Inhibit *Clostridium difficile* infection	Bacterial secondary bile acid synthesis	(Buffie et al., 2015)
ETBF	Induce Th17 cells, enhance tumorigenesis in mouse models of CRC	Bacterial toxin-dependent OMVs induce host IECs to secrete sphingolipids	(Deng et al., 2015)

Lactobacillus plantarum	Alter distribution of pro- and anti-inflammatory T cell & dendritic cell populations	Bacterial teichoic acid D-alanylation signaling through TLR2	(Smelt et al., 2013)
Proteus mirabilis	Induce intestinal inflammation in DSS model	Dependent on bacterial hemolysin; activation of NLRP3 inflammasome, inducing IL-1β production	(Muñoz-Planillo et al., 2013; Seo et al., 2015)
SFB	Induce IgA & Th17	Stimulation of germinal centers & induction of IgA-secreting cells; MHCII presentation by dendritic cells & ILCs	(Geem et al., 2014; Goto et al., 2014a; Lécuyer et al., 2014)
Staphylococcus epidermidis	Induce CD8+ T cells to the epidermis, enhance barrier function	Interactions with CD103+ dendritic cells	(Naik et al., 2015)
Sutterella species	Alter fecal IgA levels	Degradation of the secretory component of IgA	(Moon et al., 2015)

Table A1.1. Examples of specific bacteria that modulate the host immune system. AIEC, adherent invasive *Escherichia coli*; ETBF, enterotoxigenic *B. fragilis*; IECs, intestinal epithelial cells; IgA, immunoglobulin A; ILCs, innate lymphoid cells; iNKT, invariant natural killer T cell; OMVs, outer membrane vesicles; pDCs, plasmacytoid dendritic cells; PSA, polysaccharide A; SFB, segmented filamentous bacteria; Th17, T helper 17

Bacteria	Host Immune component	Bacterial response	References
Bacteroides thetaiotaomicron	Antimicrobial peptides	Resistance through outer membrane lipid A dephosphorylation	(Cullen et al., 2015)
Indigenous bacteria	Fucosylation of IECs triggered by SFB or enteric infection	Metabolic capacity to cleave fucose, utilize fucose	(Goto et al., 2014b; Guo et al., 2015; Pham et al., 2014; Pickard et al., 2014)
Salmonella enterica serovar Typhimurium	Induction of lipocalin-2 & calprotectin	Additional siderophores & zinc transporters to overcome host metal ion sequestration	(Behnsen et al., 2014)
Fusobacterium nucleatum	NK cytotoxicity & T cell effector functions	Binds host inhibitory receptor (TIGIT) on NK & T cells	(Gur et al., 2015)

Table A1.2. Examples of bacterial adaptations to host immune mechanisms.

APPENDIX 2: CO-AUTHOR PUBLICATIONS RELATED TO DISSERTATION

Pope, J.L., **Tomkovich, S.**, Yang, Y., and Jobin, C. (2016). Microbiota as a mediator of cancer progression and therapy. Transl Res. Aug 3. pii: S1931-5244(16)30155-4

Goldsmith, J.R., **Tomkovich, S.**, and Jobin, C. (2016). A Rapid Screenable Assay for Compounds That Protect Against Intestinal Injury in Zebrafish Larva. Methods Mol Biol *1422*, 281–293.

Mousa, J.J., Yang, Y., **Tomkovich, S.**, Shima, A., Newsome, R.C., Tripathi, P., Oswald, E., Bruner, S.D., and Jobin, C. (2016). MATE transport of the E. coli-derived genotoxin colibactin. Nature Microbiology *1*, 15009.

Yang, Y., **Tomkovich, S.**, and Jobin, C. (2014). Could a swimming creature inform us on intestinal diseases? Lessons from zebrafish. Inflamm Bowel Dis *20*, 956–966.

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