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INFLAMMATION AND HOST-MICROBE SIGNALING IN THE DEVELOPMENT AND PROGRESSION OF COLORECTAL CARCINOMA

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ABSTRACT

Gut microbiota play an integral role in the postnatal development and maturation of the intestinal epithelium as well as the innate and adaptive immune system. Gut microbes communicate to the host via pattern recognition receptors (PRRs) which regulate intestinal homeostasis during health and disease. My thesis has elucidated the role of gut microflora and PRR-mediated signaling during inflammation, infection and tumor development. I have examined the relevant contributions of host-microbe crosstalk in the regulation of intestinal tumorigenesis (Paper I and II) and innate immune responses to enteric pathogens (Paper III), as well as the transcriptional regulation of gene expression during inflammation and cancer development (Paper IV).

In Paper I, the role of microbiota-derived signals in promoting tumor growth in $APC^{Min/+}$ mice, a mouse model of colorectal cancer (CRC) was examined. Our data showed that germ-free $APC^{Min/+}$ mice have a reduced tumor load compared to that observed in $APC^{Min/+}$ mice harboring gut microbiota. Further in-depth characterization studies suggested a role for c-Jun/JNK and myeloid cell-dependent STAT3 activation pathways in the acceleration of tumor growth. Thus, gut microbiota can accelerate tumor growth.

In Paper II, the role of PRR-mediated signaling in intestinal tumorigenesis was studied. By introduction of a constitutively active Toll-like-receptor 4 transgene (CD4-TLR4) to the intestinal epithelium of $APC^{Min/+}$ mice, we found a marked reduction of intestinal tumor burden in CD4-TLR4-$APC^{Min/+}$ mice. This tumor suppression was likely due to the observed Cox-2 down-regulation and IFNβ induction which resulted in increased apoptosis of tumor cells. These results unravel a previously unrecognized role of TLR4 signaling in modulating the balance between proliferative and apoptotic signals.

In Paper III, the regulation of host innate immune responses during *Salmonella* Typhimurium induced colitis was studied. Our data demonstrated an aggravated colitis in infected mice lacking the innate immune regulator gene - PPARγ in the intestinal epithelium. This increased tissue damage correlated with the elevation of lipocalin-2 (Lcn2) expression, which promoted the stabilization of tissue degrading enzyme, matrix metalloproteinase 9 (MMP-9). Interestingly, Lcn2-deficient mice were markedly protected from *S.* Typhimurium induced colitis. These findings therefore illustrate how enteric pathogens can exploit the host's mucosal defense mechanisms to disrupt normal host-microbe homeostasis, in order to ensure colonization and survival in the host.

In Paper IV, I have examined the significance of histone modifications and chromatin-binding proteins in the transcriptional regulation of T lymphocytes. Our results demonstrate that the bromodomain-containing protein, BRD4, is important in regulating Pol II Ser2-mediated transcriptional elongation in human CD4+ T cells.

In conclusion, my thesis work further underscores the significant impact of gut microbiota mediated signaling in the regulation of intestinal homeostasis and tumorigenesis.

LIST OF PUBLICATIONS

I. **Li Y**, Kundu P, Seow SW, de Matos CT, Aronsson L, Chin KC, Kärre K, Pettersson S, Greicius G.
Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in $APC^{Min/+}$ mice.
Carcinogenesis. 2012 Jun; 33(6): 1231-8. Epub 2012 Mar 29.

II. **Li Y**, Teo WL, Low MJ, Meijer L, Sanderson I, Pettersson S, Greicius G.
Constitutive TLR4 signalling in intestinal epithelium reduces tumor load by increasing apoptosis in $APC^{Min/+}$ mice.
Oncogene advance online publication, 14 January 2013.

III. Kundu P, Teo WL, **Li Y**, Arienzo RD, Korecka A, Arulampalam V, Chambon P, Mak TW, Wahli W, Pettersson S.
Absence of intestinal PPARγ aggravates acute infectious colitis in mice through a Lipocalin-2 dependent pathway.
Manuscript

IV. Zhang WS, Prakash C, Sum C, Gong Y, **Li Y**, Kwok JJ, Thiessen N, Pettersson S, Jones SJ, Knapp S, Yang H, Chin KC.
Bromodomain-Containing-Protein 4 (BRD4) Regulates RNA Polymerase II Serine 2 Phosphorylation in Human CD4+ T Cells
J Biol Chem. 2012 Oct 30. [Epub ahead of print]

CONTENTS

LIST OF ABBREVIATIONS

AKT	Protein kinase B
AOM	Azoxymethane
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
BET	Bromodomains and extraterminal
BRD4	Bromodomain-containing protein 4
CAC	Colitis-associated cancer
CBC	Crypt base columnar cell
CD	Crohn's disease
CK1	Casein kinase 1
COX	Cyclooxygenase
CRC	Colorectal carcinoma
DMEM	Dulbecco's Modified Eagle medium
DSS	Dextran sulphate sodium
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
GF	Germ-free
GSK3	Glycogen synthase kinase 3
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFNβ	Interferon β
IgA	Immunoglobulin A
IKKβ	IκB kinase
IL-	Interleukin-
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Lcn2	Lipocalin-2
LEF	Lymphoid enhancing factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
MMP-9	Matrix metalloproteinase 9
Mom1/2	Modifier of Min1/2
MyD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor κB
NLR	Nod-like receptor
NSAIDS	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
Pol II	RNA polymerase II
PPARγ	Peroxisome proliferator-activated receptor γ

PRR	Pattern recognition receptor
P-TEFb	Positive transcription elongation factor
Ptgs-2/Cox-2	Prostaglandin-endoperoxide synthase 2
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor associated macrophage
TCF	T-cell factor
T_H17	T helper 17 cell
TLR	Toll-like receptor
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
WT	Wild-type

1 INTRODUCTION

1.1 OVERVIEW OF COLORECTAL CARCINOMA

Colorectal carcinoma (CRC) is the third most frequently diagnosed malignancy in the world and one of the leading causes of cancer mortality in many developed countries (http://globocan.iarc.fr/). While treatment for early stages of CRC, entailing the surgical removal of noninvasive adenomas, has been highly effective, treatment options for patients in advanced, debilitating stages of the disease are often limited and less efficient, thus leading to poor prognosis. Unfortunately, most CRC cases are usually diagnosed at a stage where surgical excision cannot eradicate the lesions completely and hence the high mortality rates. As such, a better understanding of the mechanisms underlying the disease pathogenesis is crucial to improve treatment outcomes.

In this thesis, I define carcinogenesis as a multi-step process involving a series of genetic, epigenetic and environmental events that drive tumor initiation, progression to carcinoma, and ultimately the development of malignancy, which is the ability of the carcinoma to metastasize and cause death. Thus, the process of colorectal carcinogenesis requires multiple genetic events that lead to the inactivation of tumor suppressor genes and activation of oncogenes, thereby conferring neoplastic cells with a survival advantage and ability to escape normal regulation of growth and apoptosis.
Here, the term 'adenoma' is used to describe a benign tumor that arises from epithelial tissues and it is usually referred to as an adenomatous polyp when it develops in the colon or small intestine. In contrast, a 'carcinoma' arises from an adenoma that has progressed into an invasive, malignant tumor.

Current understanding of the genetic basis of CRC converges largely on mutations in the tumor suppressor gene, *adenomatous polyposis coli (APC)*, as the key initiating event in colorectal carcinogenesis (Figure 1).[1] This gene was first discovered in familial adenomatous polyposis (FAP), a hereditary colorectal cancer syndrome that is characterized by hundreds to thousands of adenomas in the colon and rectum.[2,3,4] Most FAP patients have been demonstrated to carry germline *APC* mutations, majority of which comprise of nonsense or frameshift mutations that lead to a truncated APC protein with abnormal function.[5] Consistent with Knudson's "two-hit" hypothesis, FAP patients are predisposed but also require a 'second hit' to develop CRC.[6] This 'second hit' is usually a somatic mutation of the wild-type *APC* allele or loss of heterozygosity at the *APC* locus and appears to be dependent on the nature of the first hit.[7,8]

While germline mutations in the *APC* gene accounts for ~1% of all CRC cases, a vast majority of sporadic colorectal tumors also acquire somatic *APC* mutations (Table 1).[9,10] These studies thus provide evidence that somatic *APC* mutations, resulting in loss of APC function, form a critical initiating step in the development of colorectal tumors. Additional mutations in genes such as K-RAS, p53, and mismatch repair (MMR) genes contribute to drive the progression of these adenomas to malignancy (Figure 1).[1,11,12] Meanwhile, there exists a small minority of CRC cases that are caused by mutations distinct from the *APC* locus. One example is hereditary non-polyposis colorectal cancer (HNPCC), which accounts for 2-4% of CRC cases and is caused by mutations in DNA

mismatch repair genes.[1] In addition, mutations in the β-catenin gene, CTNNB1, have been observed in a large proportion of CRCs that do not harbor APC mutations.[13,14]

Table 1. APC Mutations in Colorectal Neoplasia

	FAP	Sporadic Adenomas	Sporadic Cancers
Population incidence	1 in 7000	1 in 2	1 in 20
APC mutation prevalence	>85%[b] (Germline Mutations)	>80%[c] (Somatic Mutations)	>80%[d] (Somatic Mutations)
Nature of mutations[a]			
Truncating	96%[e]	89%[f]	98%[g]
Missense	4%[e]	11%[f]	2%[g]

[a] Based on APC mutations that could be precisely defined at the nucleotide level. For the purposes of this table, frameshift, nonsense, and splice site mutations were considered "truncating".
[b] Based on 62 kindreds (Powell et al., 1993).
[c] Based on analysis of 12 colorectal polyps (Jen et al., 1994).
[d] Based on analysis of 23 colorectal cancer cell lines (Smith et al., 1993).
[e] Based on 174 mutations (summarized in Nagase and Nakamura, 1993).
[f] Based on 19 mutations (Miyoshi et al., 1992; Powell et al., 1992).
[g] Based on 56 mutations (Miyoshi et al., 1992; Powell et al., 1992).

Table 1. Incidence and types of APC mutations in CRC. Source:[1]

Besides a well-defined genetic etiology, it is also widely recognized that an elevated risk of colon carcinogenesis is associated with chronic colonic inflammation (i.e. colitis) such as the inflammatory bowel disease (IBD) syndromes, ulcerative colitis (UC) and Crohn's disease (CD).[15,16] More than 20% of IBD patients develop colitis-associated cancer (CAC), a CRC subtype that is difficult to treat and is associated with high mortality.[17] In majority of such IBD cases, the affected individuals progress to a relapsing and chronic disease characterized by persistent inflammation of the gastrointestinal mucosa, rectal bleeding and diarrhea.

Chronic inflammation can promote the development of CAC through the production of oxidative stress, which increases the risk of DNA damage and accumulation of mutations in genes involved in carcinogenic pathways such as p53, k-ras and DNA mismatch repair.[18] The intestinal microbiota and inflammatory mediators such as growth and survival promoting cytokines have also been strongly implicated in the pathogenesis of CAC.[17,19] In contrast to the 'adenoma-carcinoma' transition found in FAP and sporadic CRCs, CRCs that occur in IBD typically develop in an 'inflammation-dysplasia-carcinoma' sequence and the neoplastic lesions usually manifest within mucosal regions with colonic inflammation (Figure 2).[18] Although loss of APC function is a major predisposing event in FAP and sporadic CRCs, it is rarely detected in colitic mucosa with null to low-grade dysplasia and usually occurs later in the development of CAC.[15] On the contrary, loss of p53 function appears to be an instrumental step in the progression of CAC, with allelic deletion of p53 detected in ~50-80% of colitis-associated tumors and frequent p53 mutations found in colitic, nondysplastic mucosa.[15]

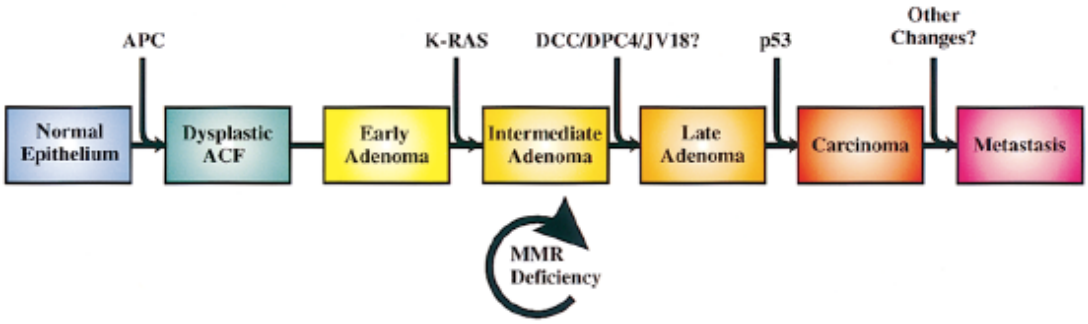

Figure 1. Model of the major genetic alterations linked to the initiation and progression of CRC. Source:[1]

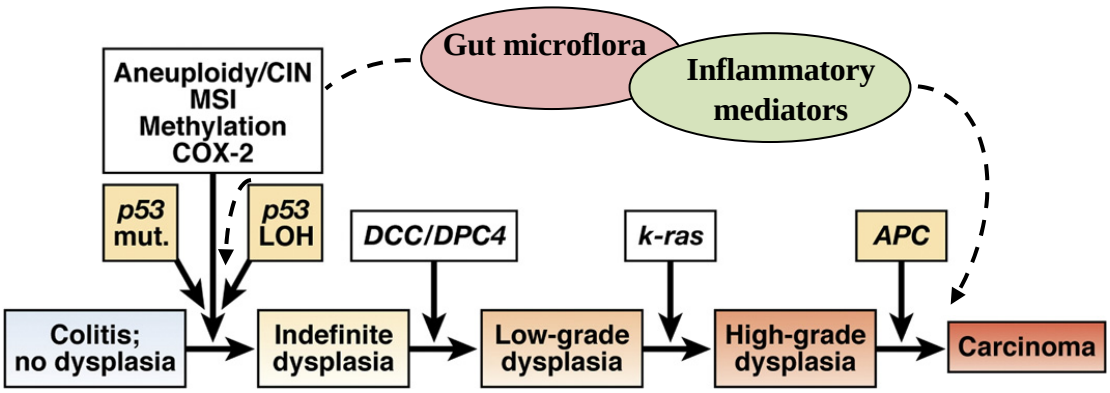

Figure 2. Molecular pathogenesis of colitis-associated colon cancer. Adapted and modified from:[15]

1.2 BIOLOGICAL FUNCTIONS OF APC IN REGULATION OF WNT/B-CATENIN PATHWAY

The APC protein is a multifunctional protein that has been extensively studied for its role in colorectal tumorigenesis. Besides its well-recognized role in the regulation of the Wnt/β-catenin signaling pathway, APC also has important functions in cell migration and adhesion, chromosome segregation, microtubule binding and apoptosis.[20] In this summary, I focus mainly on the role of APC in the context of CRC.

The connection between APC and Wnt signaling emerged soon after the discovery of the interaction between the APC protein and β-catenin.[21,22] Although its specific molecular activity still remains unresolved, APC is known from studies on CRC to be essential for the proper functioning of the destruction complex that regulates Wnt/β-catenin signaling. This signaling cascade is a key regulator of embryonic development and adult homeostasis, being one of the fundamental mechanisms governing cell proliferation, cell polarity and cell fate determination.[23] In the gut, the canonical Wnt/β-catenin pathway plays an essential role in regulating intestinal homeostasis and stem cell self-renewal. As such, it is not surprising that this signaling pathway is often

exploited during intestinal tumorigenesis as well as in other cancers, where Wnt pathway mutations leading to the inappropriate stabilization of β-catenin are frequently observed.[24] Notably, inactivating mutations or truncations in the *APC* gene are detected in a large proportion of tumors from FAP and sporadic CRC cases as discussed earlier.

This high frequency of *APC* mutations in CRC progression arises predominantly from the 'gatekeeper' function of the APC protein in controlling intestinal epithelial cell proliferation, through its regulation of β-catenin-mediated transcriptional activation. In the absence of Wnt ligands, cytoplasmic β-catenin is constantly phosphorylated and targeted for degradation by a complex comprising of APC, axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3).[24,25] Upon binding of Wnt ligand to its Frizzled receptor, the canonical Wnt signaling cascade is activated, resulting in inhibition of the degradation complex and thus stabilization of β-catenin. Subsequently, the accumulation of newly synthesized, unphosphorylated β-catenin triggers its translocation to the nucleus, where it acts as a co-activator for transcription factors of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family through displacement of the Groucho transcriptional repressors from Tcf/Lef (Figure 3).[24,25] Thus, in the setting of intestinal tumorigenesis, the dysfunction of APC facilitates constitutive activation of the TCF/β-catenin transcriptional complex, thereby inducing the expression of various cell-cycle regulatory genes such as *c-Myc* and *cyclin D1* that lead to aberrant cell proliferation.[26,27]

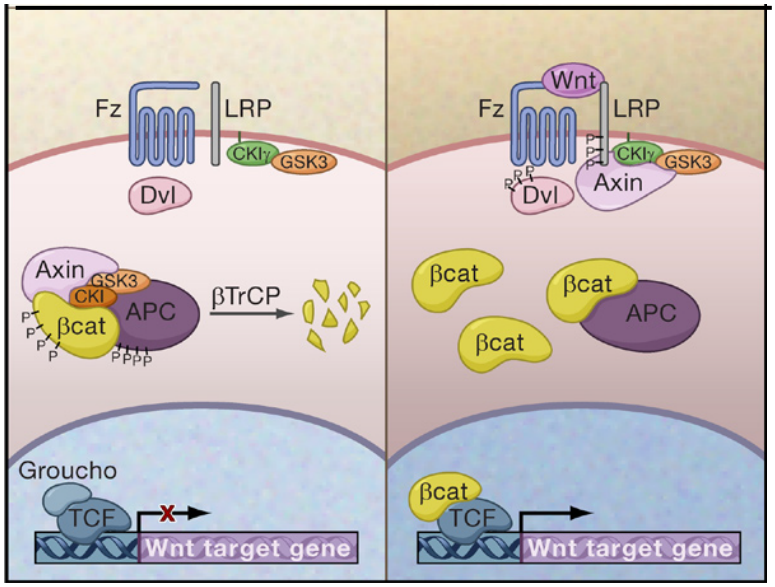

Figure 3. The canonical Wnt/β-catenin pathway. When the Frizzled (Fz)/LRP coreceptor complex is not bound by Wnt ligands, CK1 and GSK3 phosphorylate β-catenin. Phosphorylated β-catenin is targeted for proteasomal degradation by β-TrCP, a component of an E3 ubiquitin ligase. Thus, TCF remains bound to Groucho in the nucleus and the transcription of Wnt target genes is inhibited. Upon Wnt binding to Frizzled/LRP, the kinase activity of the destruction complex is inhibited by interaction of axin with LRP and/or Dishevelled (Dvl). As a result, β-catenin is stabilized and it accumulates and travels into the nucleus where it displaces Groucho from TCF to activate transcription of Wnt target genes. Source:[28]

1.3 BENEFITS AND LIMITATIONS OF THE APC$^{MIN/+}$ MOUSE MODEL

In my study, I used the APC$^{Min/+}$ mouse as a model of CRC. The APC$^{Min/+}$ mouse model carries a germline truncation at codon 850 of the *APC* gene, closely resembling inactivating *APC* mutations found in FAP patients. Heterozygous APC$^{Min/+}$ mice develop numerous adenomatous polyps in the small intestine, and hence the term 'Min' for multiple intestinal neoplasia, following a somatic event (the 'second hit') involving loss of the wild-type allele (i.e. loss of heterozygosity).[29,30] Thus, the APC$^{Min/+}$ mouse serves as a valuable experimental tool for gaining insights into the molecular pathways of intestinal tumorigenesis, for identifying environmental factors and the manipulation of genes influencing tumor progression, and for evaluating novel therapeutic strategies of human CRC.

Despite its broad utility, this mouse model also has limitations for modeling human CRC. In particular, these mice typically manifest small intestinal lesions whereas sporadic and inherited intestinal tumors in humans are predominantly found in the colon and rectum.[29,31] Moreover, APC$^{Min/+}$ mice rarely develop carcinomas due to death arising from the progressive onset of anemia and malnourishment as a consequence of multiple intestinal lesions, by 140 days of age. As such, the APC$^{Min/+}$ model can only partially recapitulate some of the key features observed in advanced forms of human CRC.

Deregulation of the Wnt/β-catenin pathway has generally been considered to be one of the key events underlying the initiation and progression of CRC as well as APC$^{Min/+}$ intestinal tumorigenesis. However till date, several studies have identified various modifiers to this canonical signaling cascade. Two notable examples are *Mom1* (Modifier of *Min 1*) and *Mom2* (Modifier of *Min 2*), two modifier loci which have been found to influence tumor size and multiplicity in APC$^{Min/+}$ mice.[32,33] Through backcrosses of APC$^{Min/+}$ mice from different strains, *Mom1* and *Mom2* were identified to strongly suppress *Min*-induced tumorigenesis, providing evidence that genetic background can significantly impact on the penetrance of the APC$^{Min/+}$ mutation. [32,33] Besides the two genetic modifiers, various non-canonical protein complexes such as the transcriptional corepressor C-terminal binding protein-1 (CtBP1), AP-1 transcription factor c-Jun and KRAS have also been demonstrated to influence the oncogenic activity of stabilized β-catenin.[34,35]

1.4 INFLAMMATION AND COLON CANCER

The connection between inflammation and cancer is well recognized and a plethora of supporting evidence from genetic, pharmacological and epidemiological studies has been generated over the past decade. Epidemiological studies illustrate a predisposition to cancer when tissues are chronically inflamed and the long-term administration of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce the risk of various cancers.[36] Moreover, the critical contributions of major inflammatory pathways and tumor-infiltrating immune cells in tumor promotion have been extensively

investigated by numerous studies, using a range of genetic and pharmacological manipulations.

There are various triggers of chronic inflammation that can promote cancer development and progression. Microbial infection may be one such trigger that precedes tumor development and the inflammatory response arises from normal host defense mechanisms of pathogen elimination. Immune deregulation and autoimmune disorders such as IBD is another type of chronic inflammation that elevates the risk of developing cancer.[37] Environmental exposure to carcinogenic substances and irritants can also induce persistent inflammatory mechanisms that have a tumor-promoting effect.[38] The connection between inflammation and cancer can be broadly classified into two major pathways: (1) an extrinsic pathway which is driven by non-resolving, inflammatory conditions (such as IBD) that accelerate cancer development and (2) an intrinsic pathway which is driven by genetic alterations in oncogenic or tumor suppressor pathways that activate expression of inflammatory mediators (i.e. chemokines and cytokines).[37]

Inflammation can contribute to carcinogenesis via the generation of reactive oxygen and nitrogen species which can induce DNA base lesions, activate signal transduction pathways leading to the transcriptional induction of proto-oncogenes, or modify proteins involved in DNA repair and apoptotic regulation.[39,40] In addition, the production of pro-inflammatory cytokines, chemokines and growth factors by infiltrating immune cells in the tumor microenvironment enhances cell proliferation, survival and migration, as well as angiogenesis, which can thereby promote tumor growth and progression.[41]

In the context of CRC, chronic inflammation is intimately linked to colon carcinogenesis. IBD patients have an elevated risk for the development of CAC and cancer susceptibility increases with the duration and extent of mucosal inflammation.[15,42] Moreover, a robust inflammatory infiltrate and heightened pro-inflammatory cytokine expression profile can also be detected in colorectal tumors from sporadic CRCs.[17,43] In the $APC^{Min/+}$ setting of spontaneous intestinal tumorigenesis, E. Huang and colleagues have demonstrated the strong connection between intestinal inflammation and colon carcinogenesis through use of the interleukin-10 (IL-10)-deficient mouse model of IBD.[44] IL-10 deficient mice develop colitis spontaneously and the breeding of $APC^{Min/+}$ mice into the IL-10 null background resulted in an increased incidence of colonic tumors.[44] Furthermore, the development of colonic dysplasia or carcinoma correlated with the severity of colonic inflammation,[44] thereby reaffirming the crucial role of inflammation in the acceleration of adenoma formation and progression to carcinomas.

1.4.1 Key signaling pathways connecting inflammation and colon cancer

1.4.1.1 The canonical IKKβ- NF-κB pathway

One major signaling pathway that links inflammation and CRC is orchestrated through the activation of nuclear factor κB, NF-κB. Being a central transcriptional regulator of innate immune and inflammatory responses, NF-κB regulates the expression of various cytokines, cell cycle and anti-apoptotic genes. Thus, NF-κB activation can drive the proliferation of premalignant cells and enhance their survival via induction of anti-apoptotic responses. In addition, NF-κB can induce the expression of genes encoding adhesion molecules, angiogenic factors, inducible nitric oxide synthase and key enzymes of the prostaglandin synthesis pathway, which have a promoting effect in tumor progression. As such, activation of NF-κB is one of the key hallmarks of various inflammation-associated cancers.[37,45]

In an elegant study by F. Greten and colleagues, they examined the role of the canonical NF-κB activation pathway in both intestinal epithelial cells and myeloid cells during colitis-associated colon carcinogenesis. Using enterocyte-specific ablation of the IκB kinase (IKKβ) complex, which facilitates NF-κB nuclear translocation via phosphorylation and thus ubiquitin-targeted degradation of NF-κB bound IκBs, the investigators showed a markedly reduced incidence of CAC tumors without an effect on tumor size.[46] However, the inactivation of IKKβ in enterocytes did not reduce intestinal inflammation during acute or chronic colitis and the reduced tumor load was instead found to be associated with enhanced epithelial apoptosis during early tumor promotion.[46] These findings thus suggest that NF-κB activation in enterocytes contributes to tumor initiation and promotion through apoptotic inhibition rather than inflammation.

The investigators subsequently proceeded to address the role of the IKKβ-dependent NF-κB activation pathway in myeloid cells through specific ablation of IKKβ in myeloid cells. Interestingly, they found that this deletion led to a significant reduction in both tumor incidence and size without affecting apoptosis.[46] The decreased tumor growth following myeloid IKKβ deletion was linked to the diminished expression of proinflammatory cytokines rather than increased apoptosis, thereby suggesting that IKKβ-dependent NF-κB signaling in myeloid cells promotes tumor growth through the production of tumor-promoting paracrine factors.[46] This study thus illustrates the critical involvement of the canonical IKKβ-NF-κB signaling cascade in connecting inflammation and colon carcinogenesis, through its distinct tumor-promoting effects on the epithelial and myeloid compartments of the intestine.

More recently, two studies further analyzed the effect of chronic NF-κB activation in the development of intestinal tumorigenesis using gut-specific transgenic activation of IKKβ.[47,48] Notably, the persistent IKKβ-driven activation of NF-κB in intestinal epithelial cells led to the spontaneous development of intestinal adenomas in aged mice despite the lack of destructive colonic inflammation.[47,48] In one study, the investigators showed enhanced chemical- and APC mutation-mediated tumorigenesis following

constitutive NF-κB activation in the intestinal epithelium.[47] This was found to be associated with increased β-catenin activation, hyperproliferation and elevated expression of stem cell markers, thus illustrating the synergy of IKKβ-NF-κB signaling with Wnt signaling in tumor initiation and promotion.[47] Moreover, the persistent activation of NF-κB in intestinal epithelial cells resulted in the production of cytokines and chemokines that triggered increased recruitment of myeloid cells as well as activation of stromal fibroblasts, both of which contributed to a tumor-promoting microenvironment.[47] In the second study, chronic epithelial NF-κB activation led to accelerated intestinal tumorigenesis in mice carrying a gut-specific allelic deletion of APC.[48] This elevated tumor initiation rate in the gut was found to be linked to increased oxidative DNA lesions,[48] thereby illustrating another mechanism by which chronic inflammation can contribute to intestinal tumorigenesis. As such, there is accumulating evidence from various genetic knockout or constitutively active transgenic models that support the unequivocal role of IKKβ-NF-κB signaling in driving colon cancer.

1.4.1.2 The gp130-JAK/STAT3 signaling pathway

Similar to NF-κB, the signal transducer and activator of transcription 3 (STAT3) also represents a point of convergence for various pro-oncogenic signaling pathways. Notably, STAT3 is a major transcription factor regulating genes involved in cell proliferation and survival pathways, as well as immunosuppressive and anti-apoptotic processes,[49,50] which can intersect with multiple carcinogenic pathways. It is frequently found to be aberrantly activated in various epithelial tumors and tumor-associated myeloid cells.[51-54] Moreover, there appears to be a significant correlation between constitutive STAT3 activation and poor clinical outcome or pathological features of various cancers including CRC.[55-59]

STAT3 is a major transcription factor that can promote cell proliferation and survival through regulating genes involved in cell cycle progression, such as *c-Myc* and *cyclin D1*,and inhibition of apoptosis, such as *Bcl-2* and *Bcl-xL*.[36,49] Thus, the deregulated or persistent activation of STAT3 is often connected to the pathogenesis of many human cancers. In the context of CRC, constitutively active STAT3 was found to be abundant in transformed, dedifferentiated cells as well as infiltrating lymphocytes of CRC biopsies.[60] Furthermore, the induction of STAT3 activity in colon carcinoma cells resulted in accelerated proliferation while blockade of STAT3 activation in colon carcinoma xenograft tumors led to a significant reduction of tumor growth.[60] Hence, persistently activated STAT3 is a positive regulator of cell proliferation and tumor growth in CRC.

Besides its effect on tumor cell proliferation and survival, the persistent activation of STAT3 also mediates tumor-promoting inflammation through its dual role in driving pro-oncogenic inflammatory pathways and suppression of anti-tumor immunity. Notably, STAT3 signaling is highly interconnected with the canonical NF-κB activation pathway as well as the interleukin-6 (IL-6)-gp130-Janus kinase (JAK) signaling cascade. This is well illustrated in a study by Hua Yu's group, who showed that constitutively activated STAT3 is necessary for the maintenance of constitutive NF-κB activity in tumors and tumor-associated myeloid cells.[61] Importantly, STAT3

interacts directly with NF-κB (RelA) and prolongs its nuclear retention through mediating RelA acetylation.[61] These findings thereby reveal the cooperativity between the two major transcription factors in stimulating a repertoire of proliferative and prosurvival genes which are essential for tumor promotion.

Interestingly, several inflammatory factors encoded by NF-κB target genes, such as interleukin-6 (IL-6), are major activators of STAT3. IL-6 is a multifunctional cytokine that acts on both epithelial and immune cells. In particular, it is known to drive STAT3 activation through interaction with the membrane-associated gp130 subunit, thereby activating Janus kinases (JAKs) and one of the downstream effectors - STAT3. In the aspect of colitis-associated tumorigenesis, the role of IL-6 as a critical tumor promoter, acting via stimulation of gp130-mediated STAT3 activation, was well illustrated by two elegant studies. Using mice deficient for STAT3 in intestinal epithelial cells, both studies showed the remarkable reduction in CAC tumor size and incidence following STAT3 ablation in the intestinal epithelium.[62,63]

Notably, one study by S. Grivennikov and coworkers demonstrated that IL-6 knockout mice were similarly protected from CAC tumorigenesis and bone marrow-derived myeloid cells were one of the main producers of IL-6.[63] Consistent with the stimulatory effect of IL-6 on epithelial cell proliferation and survival, the exogenous administration of IL-6 to these mice during early CAC induction triggered an increase in tumor multiplicity while IL-6 administration during the late stages of CAC growth led to an increase in tumor burden.[63] Moreover, IL-6 deficient mice displayed reduced levels of activated STAT3 in intestinal epithelial cells during colitis and CAC induction.[63]

In the second study, J. Bollrath and colleagues further showed that the hyperactivation of STAT3 in mice carrying mutant gp130 receptor resulted in the acceleration of CAC tumor incidence and growth.[62] In contrast, the reduction of CAC tumors in mice lacking STAT3 in intestinal epithelial cells correlated with increased epithelial apoptosis during early CAC induction.[62] Mice deficient in epithelial STAT3 also exhibited more profound mucosal damage and apoptosis during colitis induction,[62,63] thereby implying the important role of STAT3 in epithelial proliferation and apoptotic inhibition which can be utilized for tumor formation and growth. As such, the two studies reveal the critical role of epithelial STAT3 activation in the transduction of tumor-promoting signals from IL-6 in the tumor microenvironment during CAC tumorigenesis.

STAT3 also has important functions in myeloid cells, most notably in the suppression of anti-tumor immunity, thereby attesting to its relevance in tumor promotion. In particular, the ablation of STAT3 in hematopoietic cells has been demonstrated to inhibit tumor growth and progression via the induction of an intrinsic tumor immune surveillance response.[53] STAT3 activity in tumors is known to mediate immune suppression through the downregulation of proinflammatory cytokines that are crucial for dendritic cell (DC) activation, which in turn modulates T-cell immunity.[64] Furthermore, STAT3 signaling can stimulate the production of several immunosuppressive factors such as IL-10 and vascular endothelial growth factor (VEGF), which are also activators of STAT3.[65] Thus, targeting STAT3 in

hematopoietic cells, via genetic ablation or use of a specific inhibitor, resulted in enhanced antitumor immune responses leading to tumor regression.[53]

Interestingly, the oncogenic functions of STAT3 can also be mediated through its cooperation with c-Jun, a proto-oncoprotein which forms a component of the activator protein 1 (AP-1) transcription factor complex. In a study by V. Ivanov and colleagues, the combined activity of STAT3 and c-Jun in melanoma cells was found to suppress the transcription of Fas, a receptor that participates in the induction of apoptosis.[66] This downregulation of Fas surface expression was inversely correlated with the sensitivity of the melanoma tumors to Fas-ligand mediated apoptosis, thereby implicating the cooperative role of STAT3 and c-Jun in mediating tumor resistance to therapy-targeted cell death.[66]

1.4.1.3 The JNK-c-Jun/AP-1 pathway

c-Jun is a member of the AP-1 family of basic leucine-zipper proteins or transcription factors that regulates the expression of genes involved in cell cycle progression, apoptosis and tumorigenesis.[67,68] It can form functional transcription factors via heterodimerization with other members of the AP-1 group. The c-Jun/AP-1 signaling pathway is triggered by various stimuli such as cytokines, growth factors and extracellular stresses, and is mediated by c-Jun N-terminal kinases (JNKs).[69] Notably, JNKs activate AP-1 signaling by phosphorylating critical serine and threonine residues found within the transactivation domain of c-Jun, thereby stimulating the transcription of various target genes including *c-jun* itself.[69]

The oncogenic ability of c-Jun is well established more than a decade ago from numerous studies demonstrating its cooperation with Ras, another known oncoprotein, in driving cell transformation, a process that is also dependent on JNK-mediated N-terminal phosphorylation of c-Jun.[70-74] Meanwhile, the significant contribution of JNK-dependent c-Jun phosphorylation in the promotion of intestinal tumorigenesis has been implicated in at least two studies by Axel Behrens's group. Using $APC^{Min/+}$ mice which carry either a genetic abrogation of c-Jun N-terminal phosphorylation or a gut-specific conditional inactivation of c-Jun, the investigators revealed a significant reduction in tumor incidence and burden as well as prolonged lifespan in these mice with impaired c-Jun phosphorylation or c-Jun deficiency.[35] This reduced tumor load was correlated with the decreased proliferation index in adenoma cells lacking c-Jun function,[35] thus highlighting the critical role of JNK-mediated c-Jun N-terminal phosphorylation in oncogenic transformation and development. Importantly, the group showed a phosphorylation-dependent interaction between c-Jun and the transcription factor TCF4 as a potential molecular mechanism regulating $APC^{Min/+}$ intestinal tumorigenesis.[35] This transcriptional cooperation between TCF4 and c-Jun was also dependent on β-catenin activation, thereby providing evidence for the integration of the JNK-c-Jun/AP-1 and TCF/β-catenin pathways, which can both be activated independently by Wnt signaling.[35]

The investigators then proceeded to further characterize the significance of JNK activation in the gut by generating transgenic mice overexpressing constitutively active

JNK proteins in intestinal epithelial cells. Intriguingly, augmented JNK signaling resulted in increased intestinal cell proliferation and villus length.[75] This was linked to the increased JNK activity and hence phosphorylated c-Jun in crypt base columnar (CBC) progenitor cells, as well as the concomitant stimulation of Wnt signaling in progenitor cells.[75] Moreover, *tcf4* was found to be a direct c-Jun target gene and its expression could be augmented by JNK signaling, henceforth illustrating a synergistic interaction between JNK and Wnt pathways.[75] Interestingly, hyperactivation of JNK signaling in the gut accelerated colitis-triggered tumorigenesis but did not affect $APC^{Min/+}$ tumor development.[75] These differential effects were attributed by the authors to the possible saturation in endogenous levels of phosphorylated c-Jun in $APC^{Min/+}$ adenomas whilst colitis-induced tumors had much lower amounts of c-Jun phosphorylation prior to transgenic JNK activation.[75] The two studies thus emphasize the critical role of JNK-dependent c-Jun/AP-1 signaling in the promotion of both spontaneous and colitis-associated intestinal tumorigenesis, that represents a relevant therapeutic target for human CRCs.

1.4.2 Role of tumor-infiltrating immune cells in CRC progression

It is well accepted that inflammation promotes the progression of epithelial-derived tumors indirectly via activation of inflammatory cells within the tumor microenvironment.[76] Emerging evidence from various animal models targeting myeloid cells has suggested that the cross-talk between epithelial cells and inflammatory cells is crucial for inflammation-dependent tumor development. The role of activated NF-κB in myeloid cells during colitis-associated tumor initiation and progression in the gut for instance, has been addressed in the earlier described study.[46]

Besides cancer cells and their surrounding stroma such as fibroblasts, endothelial cells and mesenchymal cells, the tumor microenvironment contains various types of immune cells from the innate and adaptive immunity. These immune cells include macrophages, neutrophils, myeloid-derived suppressor cells, dendritic cells, natural killer cells, T and B lymphocytes, and they can produce cytokines and chemokines that either promote or modulate tumor growth. Tumor associated macrophages (TAMs) are the most common type of immune cells found within the tumor microenvironment. Although TAMs can produce cytokines that induce tumor cell killing, they are mostly known for their tumor growth promoting functions.[77] These myeloid cells secrete various cytokines, chemokines and reactive oxygen species which can promote mutagenesis, proliferation and survival of premalignant cells, as well as tumor angiogenesis. The production of IL-6 by activated myeloid cells in the tumor infiltrate for instance, has been implicated in the initiation and progression of colitis-associated tumors as discussed previously.[63] As such, understanding the origin, mechanisms of recruitment and immunosuppressive functions of this immune cell subset is relevant for the future development of therapeutic targets for human CRC.

1.5 HOST-MICROBE INTERACTIONS IN NORMAL INTESTINAL HOMEOSTASIS AND DISEASE

The mammalian gastrointestinal tract harbors a diverse and dynamic community of microorganisms, collectively termed the microbiota or microflora, which interact symbiotically with their host.[78,79] Several important functions of gut microbiota in host physiology have been described, namely the regulation of nutrient acquisition,[80] gastrointestinal maturation,[81] mucosal barrier fortification,[82] angiogenesis,[83] and the development of innate and adaptive immunity.[84-86]

Despite the beneficial contributions of commensal microbiota to intestinal homeostasis and host immunity, they pose a major threat to the host during pathological states where the integrity of the mucosal barrier is breached. In such scenarios, the disease pathogenesis is usually promoted by an elevated immune response to the intestinal microflora such as that observed in IBD. The aberrant epithelial barrier facilitates the increased translocation of both pathogenic and commensal microbes to the lamina propria, leading to the persistent activation of resident inflammatory cells. Thus, even though the host normally develops a variety of tolerogenic mechanisms to maintain a symbiotic coexistence with the commensal microbiota, this delicate balance is disrupted during chronic intestinal inflammation arising from the loss of epithelial barrier integrity. This often leads to the development of a dysbiotic microbiotal community, which further perpetuates the inflammation, immune deregulation and pro-carcinogenic events that facilitate CRC development (Figure 4).

Interestingly, the correlation between gut microflora and human diseases has been implicated in a variety of ailments including metabolic disorders,[87-90] IBD,[91] and CRC,[15,92] whereby alterations in microbial composition are linked to disease. Although an altered microbiotal community may be a consequence rather than a cause of disease, the ability to transmit the colitogenic activity of an altered microbiota in a variety of cross-fostering and co-housing studies have implicated the active role of a dysbiotic microbial community in disease pathogenesis.[93] Moreover, the composition of commensal microbiota can regulate the differentiation of lamina propria immune cells, thereby impacting on intestinal tolerance, immune responses and susceptibility to IBD.[94] As such, there exists a strong causal link between a dysbiotic microbiota and gastrointestinal diseases, particularly IBD and CRC.

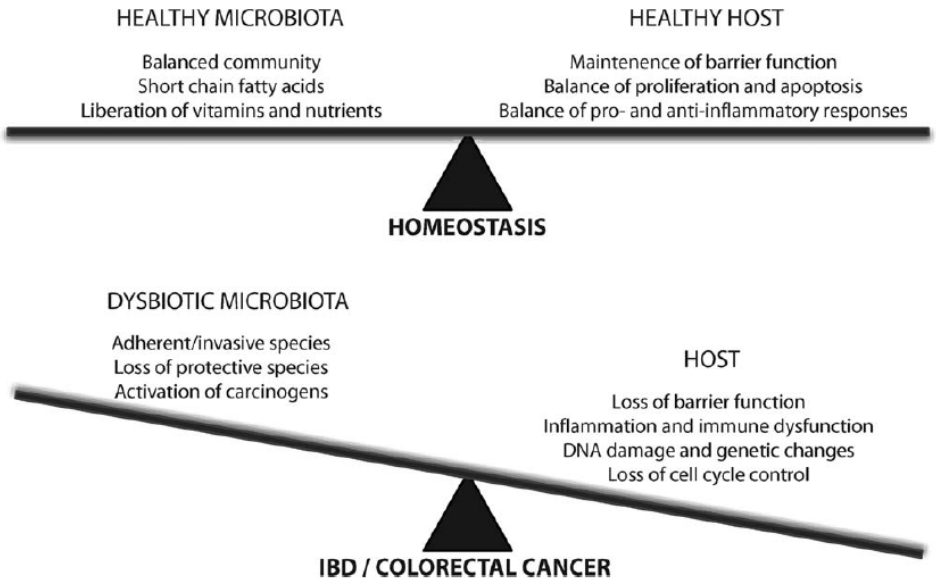

Figure 4. A balanced microbiota community supports proper mucosal barrier function and maintenance of intestinal homeostasis in a healthy host. In contrast, microbial dysbiosis leads to the prevalence of adherent/invasive species over protective commensals, thus fostering dysregulated immune responses and inflammation, loss of barrier function and cell cycle control, as well as increased genetic alterations in a susceptible host. Thus a dysbiotic microbiota promotes IBD and the development of CRC. Source:[95]

1.5.1 Impact of gut microflora on intestinal inflammation and tumorigenesis

Disruption of the homeostasis of intestinal microflora can promote gastrointestinal diseases such as IBD and CRC. Microbial dysbiosis, characterized by changes in the abundance, diversity and stability of commensal bacteria, can impact significantly on the innate and adaptive immune responses of the host, thus leading to disease. For instance, one of the multifactorial mechanisms underlying the pathogenesis of IBD, which can be triggered by an altered mucosal barrier function, is the loss of tolerance to commensal microbes and enhanced immune response to bacterial antigens.[16] In addition, in a genetically susceptible or immunocompromised host, the presence of gut microflora can promote intestinal inflammation. This is observed in many mouse models of IBD whereby the administration of antibiotics or rederivation of mice into germ-free conditions ameliorates disease severity.[96,97]

Several enteric microbes have been demonstrated to contribute to IBD and CRC, using a variety of mechanisms including the activation of inflammatory pathways, induction of oxidative stress and shift in diversity of commensal species.[17] Notably, at least three distinct bacterial pathogens have been found to promote colon tumorigenesis in the genetically susceptible, $APC^{Min/+}$mouse model. In a study by Cynthia Sears's group, the human colonic bacterium, enterotoxigenic *Bacteriodes fragili*s (ETBF), was found to chronically colonize $APC^{Min/+}$ mice, triggering colitis and accelerating colon

tumorigenesis via a STAT3- and T_H17-dependent pathway in these mice.[98] In contrast, non-toxigenic B. fragilis colonized $APC^{Min/+}$ mice similarly but did not induce intestinal inflammation nor affect colonic tumor incidence.[98] In another study by David Schauer's group, *Citrobacter rodentium* infection was shown to promote colon tumor formation in $APC^{Min/+}$ mice.[99] In the third study, the enterohepatic bacterial pathogen, *Helicobacter hepaticus*, was demonstrated to increase colonic tumor incidence in BALB/c-$Rag2^{-/-}$ $APC^{Min/+}$ mice which have an altered immune function.[100]

1.5.2 Pattern recognition receptors: Key mediators of host-microbe signaling that influence cancer and inflammation in the gut

Two major classes of pattern recognition receptors (PRRs) that play a central role in host-microbial signaling are the Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs). They recognize a variety of broadly conserved microbial components and mediate various signal transduction processes in the host, thus impacting on host physiology and disease. In this thesis, I shall focus mainly on the role of TLRs in intestinal homeostasis and disease.

1.5.2.1 Role of TLRs in the regulation of intestinal epithelial homeostasis, mucosal barrier fortification and inflammation

Commensal microflora has been long implicated to play a significant role in regulating mucosal barrier functions. This was illustrated by Kitajima S. and co-workers who showed the increased susceptibility of germ-free mice to colonic epithelial injury induced by dextran sulphate sodium (DSS) as compared to conventionalized mice.[101] In another study by Jeffrey Gordon's group, colonization of a single commensal bacterium, *Bacteroides thetaiotaomicron*, in germ-free mice for less than two weeks was sufficient to trigger IgA responses and the expression of genes involved in mucosal barrier fortification.[102] These findings thus support the critical contribution of intestinal microbiota in the regulation of epithelial homeostasis and mucosal barrier functions.

In particular, the recognition of microbiota by TLRs has been well documented to be critical for the maintenance of intestinal epithelial integrity and homeostasis. Mice deficient in TLRs and the major signaling adaptor protein of TLRs, MyD88, have increased susceptibility to DSS-induced colitis.[103-105] This augmented severity to DSS-mediated colonic epithelial injury observed in TLR2-, TLR4- and MyD88- deficient mice was mainly attributed to the defective production of cytoprotective and reparative factors, as well as tight-junction defects in the intestinal epithelial barrier.[103,105] These findings illustrate the crucial dependence on TLR/MyD88-mediated microbial signaling in the maintenance of intestinal epithelial barrier integrity against injury.

In further support of the protective role of microbiota, the depletion of microflora in wild-type (WT) and TLR-deficient mice via broad spectrum antibiotic treatment not only rendered WT mice more sensitive to DSS-induced colitis, but also further

aggravated DSS-induced colonic epithelial damage in TLR-deficient mice leading to increased mortality.[103] Notably, oral administration of lipopolysaccharide (LPS), a gram-negative bacterial component also known to be a TLR4 agonist, to wild-type and TLR2-deficient DSS-treated mice ameliorated the severity of mucosal injury.[103] Oral treatment of WT DSS-mice with a TLR2 ligand also restored tight-junction associated integrity of the intestinal epithelium, thus mitigating mucosal injury and inflammation.[105] Taken together, these studies highlight the importance of microbiota and MyD88-dependent TLR signaling in maintaining mucosal barrier integrity and triggering epithelial cell proliferation or regenerative processes upon injury.

However, one major limitation of using these animal knockout models is the inability to define the precise contributions of various cell types in the intestinal compartment in injury-triggered epithelial proliferation. A number of more recent studies have since progressed to identify the role of microbial signaling in both the epithelial and stromal compartments of the intestine. In particular, TLR signaling via MyD88 in lamina propria macrophages was found to be crucial for stimulating the regenerative responses of colonic epithelial progenitors during epithelial injury in the gut.[106] The reconstitution of irradiated WT mice with MyD88-deficient bone marrow resulted in a hypoproliferative response of these colonic epithelial progenitors to DSS-mediated injury whilst reconstitution of MyD88-deficient mice with WT bone marrow restored their epithelial regenerative capabilities.[106]

These findings thus suggest that microbial signals from the intestinal lumen collaborate with mesenchymal-epithelial interactions to trigger proper regenerative/reparative responses during tissue injury in the gut. Several studies subsequently proceeded to further characterize the crosstalk between the mesenchymal and epithelial compartments that is regulated by TLR/MyD88 signaling in the intestine. Intriguingly, MyD88 signaling was found to be crucial for the distribution of a population of stromal cells expressing prostaglandin-endoperoxide synthase 2 (Ptgs2 or Cox-2), which is a major mediator of prostaglandin E2 (PGE_2) synthesis.[107] Notably, during injury, these Ptgs2-expressing stromal cells redistributed to enrich the crypt-base associated mesenchyme as well as the crypt base epithelium, where proliferative colonic epithelial progenitors reside. This alteration in their localization was demonstrated to require MyD88 signaling and was crucial for the epithelial proliferative response during DSS-mediated tissue injury.[107] These data thus reveal that MyD88-dependent signaling can intersect with other pathways such as Ptgs2-mediated PGE_2 synthesis, which has important growth-stimulatory and anti-apoptotic functions that promote epithelial restitution.

In addition to the Ptgs2-expressing stromal cells, myeloid cells have also been shown to be critical for the colonic epithelial response to injury.[108] Importantly, MyD88 signaling in colonic myeloid cells was necessary to drive the proliferative response,[108] thereby highlighting the significance of microbiotal stimulation in maintaining robustness of the host in mitigating perturbations in epithelial barrier integrity. Moreover, myeloid and non-myeloid cells in the stromal compartment play a major role in the orchestration of epithelial proliferative/reparative responses during barrier disruption and this is regulated by MyD88-mediated microbial signaling.

Meanwhile, the role of microbial signaling by TLRs in the epithelial compartment of the gut has been well addressed in studies examining regulation of the TLR4 pathway in intestinal inflammation, predominantly using the carcinogen, azoxymethane (AOM)-induced, in combination with DSS treatment model of CAC. This is mainly illustrated by a number of studies by Maria Abreu's group. Notably, they demonstrated that TLR4-deficient mice were markedly protected from colits-associated colon carcinogenesis as compared to WT mice, which display elevated TLR4 expression in their CAC tumors.[109] Moreover, TLR4-deficient mice displayed a reduced pro-inflammatory profile during DSS-induced colitis, which correlated with decreased severity of dysplasia and colitis-associated neoplasia.[109] The group then proceeded further to examine the role TLR4 signaling in colonic epithelial cells through bone marrow transfer experiments between TLR4-deficient and WT mice. In their study, WT mice reconstituted with TLR4-deficient bone marrow or WT bone marrow did not differ in the incidence of dysplastic lesions.[110] Furthermore, the size and extent of dysplasia were significantly greater in mice expressing TLR4 in colonic epithelial cells, in contrast to TLR4-deficient mice receiving WT bone marrow.[110] TLR4 expression in colonic epithelial cells was also found to be critical for the recruitment of mucosal neutrophils and macrophages during AOM/DSS treatment, consistent with an increased inflammatory status in the intestinal mucosa that can promote colitis-associated tumorigenesis.[110] These observations thus implicate the role of epithelial TLR4 signaling in promoting intestinal inflammation and inflammation-associated neoplasia in the AOM/DSS setting.

More recently, using a transgenic mouse expressing constitutively active TLR4 specifically in the intestinal epithelium, the same group showed an augmented inflammatory response to DSS-induced mucosal injury in transgenic mice as compared to WT mice.[111] These transgenic mice displayed enhanced neutrophilic infiltration and expression of inflammatory mediators during DSS-mediated colitis.[111] Moreover, they also exhibited an increased susceptibility to inflammation-induced neoplasia in an AOM/DSS model, which was ameliorated by TLR4 antibody treatment.[111] Taken together, the data from Abreu's group support the role of epithelial TLR4 signaling in promoting intestinal inflammation during epithelial barrier disruption, especially in aberrant situations where it is persistently activated. While their findings of TLR4-deficient mice during DSS-induced colitis[109] appear to contradict earlier studies by Ruslan Medzhitov's group,[103] Abreu's group adopted a chronic colitis model comprising of two cycles of DSS treatment with a recovery phase after each cycle while the latter group used an acute colitis model involving a single cycle of DSS treatment only. As such, the mucosal damage mediated by chronic inflammation or repeated DSS administration may outweigh the epithelial regenerative/reparative responses triggered by TLR4 signaling, thereby accounting for the contrasting treatment outcomes. These important studies thus provide an intriguing, yet incomplete understanding of the complex regulation of microbiotal signaling by TLRs in intestinal homeostasis and disease, particularly in the context of TLR4 signaling.

1.5.2.2 TLR4/MyD88 signaling and tumorigenesis in the gut

As described in the earlier section, microbiota-derived signals mediated through TLR4 can influence tumorigenesis in the gut. Till date, animal models mimicking microbiota regulation of tumor development using various treatment approaches or genetic manipulations reveal that there are at least two distinct facets of intestinal tumorigenesis that is regulated by TLR/MyD88 signaling. The first model involves spontaneous tumorigenesis whereby the host is genetically predisposed to the development of tumors, which arise in an inflammation-independent manner. In contrast, the second model entails intestinal tumorigenesis in an inflammation-dependent context such as colitis.

Using the genetically susceptible, $APC^{Min/+}$ mouse model of intestinal tumorigenesis, Medzhitov's group investigated the role of MyD88 signaling in spontaneous tumor development. Interestingly, genetic ablation of MyD88 in these mice resulted in a dramatic reduction of intestinal tumor load.[112] Subsequent gene expression profiling of intestinal adenomas from $APC^{Min/+}$ and MyD88-deficient $APC^{Min/+}$ mice revealed a distinct set of modifier genes of intestinal tumorigenesis, as well as genes critical for intestinal tissue repair, that was MyD88-dependent.[112] These findings thus led the group to conclude that MyD88 signaling pathway is essential for intestinal tumor progression while induction of a tissue-repair program supports the current notion of tumor growth as "an abnormal form of a continuous and unregulated state of tissue repair".[112]

Intriguingly, while MyD88 ablation attenuates spontaneous intestinal tumorigenesis, MyD88-knockout mice exhibit enhanced adenoma formation and progression to adenocarcinomas in a chronic inflammatory setting.[113] Using the AOM/DSS model of CAC, Salcedo R and colleagues demonstrated the protective role of MyD88 signaling in the development of colitis-induced colon carcinogenesis. In their study, the impaired mucosal healing ability of MyD88-deficient mice led to an altered inflammatory environment that triggered various expression changes in genes associated with cell proliferation, apoptosis and DNA repair. Consistent with an altered expression profile that increases frequency of β-catenin mutations and promotes tumor development, AOM/DSS treatment resulted in an elevated incidence of colonic adenocarcinomas in MyD88-/- mice as compared to wild-type mice.[113] Thus, in contrast to the spontaneous tumorigenesis model, MyD88-deficiency appears to promote colitis-associated tumorigenesis as a result of defective epithelial healing functions that supports increased intestinal inflammation during injury.

In addition, these findings are further complicated by Abreu and colleagues' data from TLR4-deficient mice and transgenic mice carrying constitutively active epithelial TLR4 as elaborated earlier. This suggests that additional pathways exist between TLR4 and the downstream MyD88 adaptor and they can be independent of MyD88 signaling. This can in turn lead to very distinct functional consequences during tumor progression. Hence, taken together, these studies implicate the multi-faceted as well as controversial roles of microbial signaling mediated via PRRs in tumorigenesis.

As such, interfering with host-microbiota interactions through PRRs can influence colon carcinogenesis, likely via the microbiota's ability to drive proinflammatory responses or epithelial reparative processes. While targeted ablation of the signaling pathways mediated by some PRRs, such as TLR4, has markedly reduced the occurrence of colitis-associated colon tumorigenesis, perturbing the interactions by downstream signaling effectors such as MyD88 can exacerbate intestinal inflammation and CAC instead. Meanwhile, perturbing such interactions in the setting of spontaneous tumorigenesis can result in very contrasting effects. Thus, the regulation of gut microflora in host health and disease via PRRs is complex and manipulations of PRR-mediated recognition of microbiota need to be interpreted with care. Despite their potential detrimental effects to the host during dysregulated PRR signaling, host-microbe interactions mediated through PRRs are integral for the proper development of host innate and adaptive immunity in the gut, as well as intestinal homeostasis and injury repair responses.

1.6 ROLE OF COX-2 AND PROSTAGLANDINS IN COLON CANCER

Besides the inflammatory mediators and TLRs-mediated microbial signaling discussed earlier, biologically active lipids such as prostaglandins and the enzymes that catalyze their production have also been implicated in the pathogenesis of CRC during chronic intestinal inflammation. Notably, pro-inflammatory prostaglandin E2 (PGE_2) is well recognized for its role in promoting tumor growth and is frequently linked to a poor prognosis of CRC.[114] PGE_2, including other prostaglandins, is synthesized from arachidonic acid by cyclooxgenases (COX), which exist in at least three known isoforms – Cox-1, Cox-2 and Cox-3.[115] While Cox-3 has been implicated as a splice variant of Cox-1 that lacks enzymatic activity,[116] Cox-1 is believed to play a housekeeping role in the maintenance of basal prostaglandin levels that are essential for tissue homeostasis.[114,115] In contrast to the constitutive expression of Cox-1 in a wide variety of human tissues, Cox-2 is an immediate-early response gene that is highly induced during inflammatory and tumorigenic settings.[114,115,117]

Cox-2 is best known for its significant involvement in colorectal tumorigenesis, as illustrated by its aberrant up-regulation in a vast majority of colorectal adenomas and adenocarcinomas.[118-121] Furthermore, elevated levels of PGE_2, the downstream metabolite of Cox-2, have also been detected in the adenomatous polyps and carcinomas of FAP patients, relative to colorectal neoplasia-associated mucosa as well as mucosa of control subjects.[122] These observations thus strongly implicate the critical contribution of Cox-2 in the initiation, promotion and progression of CRCs, which is likely to be mediated through PGE_2 signaling. Consequently, a plethora of studies have progressed over the last decade to elucidate the causal link between Cox-2/PGE_2 signaling and intestinal tumorigenesis.

Notably, the administration of exogenous PGE_2 to rats resulted in an augmented incidence and multiplicity of AOM-induced colorectal tumors that corresponded to enhanced tumor cell proliferation and reduced apoptotic index.[123] In $APC^{Min/+}$ mice, PGE_2 treatment was found to stimulate epithelial cell proliferation and Cox-2

expression in intestinal adenomas via activation of an oncogenic pathway –Ras-mitogen–activated protein kinase (MAPK) signalling.[124] These findings support the tumorigenic role of Cox-2 derived PGE_2, which is further substantiated by the efficacy of Cox-2 selective NSAID, celecoxib, in reducing the occurrence of sporadic colorectal adenomas[125] as well as the induction of colorectal tumor regression in FAP patients.[126] The suppression of intestinal tumorigenesis was also observed in $APC^{Min/+}$ mice following celecoxib treatment as well as the genetic disruption of Cox-2.[127,128] Moreover, the genetic ablation of hydroxyprostaglandin dehydrogenase-15 (15-PGDH), a prostaglandin-degrading enzyme regulating endogenous PGE_2 levels, markedly increased colon tumorigenesis in $APC^{Min/+}$ mice and a carcinogen-induced model.[129] As such, there exists a large body of evidence converging on the critical contribution of Cox-2/PGE_2 pathway in the neoplastic progression of CRC.

During chronic inflammation or the initiation of epithelial tumors, Cox-2 induced PGE_2 biosynthesis can be triggered in transformed or normal epithelial cells as well as tissue-resident immune cells.[114] In the context of CRC, Cox-2/PGE_2 signaling is known to promote colon carcinogenesis through a variety of mechanisms. Besides its interaction with the Ras-MAPK cascade, the Cox-2/PGE2 pathway can also stimulate colorectal tumor growth through activation of the epidermal growth factor receptor (EGFR) and Wnt/β-catenin pathways.[130,131] In addition, Cox-2 derived PGE_2 can promote the survival of colorectal cancer cells via activation of apoptosis inhibitory pathways such as PI3K/AKT signaling, as well as inducing the expression of anti-apoptotic genes including Bcl-2.[132] The overexpression of Cox-2 in colon cancer cells is also associated with angiogenesis-promoting effects such as the increased production of angiogenic factors,[133] consistent with the role of Cox-2 in promoting CRC progression through stimulation of tumor vascularization. Moreover, Cox-2 and PGE_2 have been reported to enhance the metastatic potential of colorectal tumor cells through increasing cancer cell migration and invasiveness.[134,135] More recently, a novel role for PGE_2 in the promotion of intestinal tumor growth and progression was uncovered, whereby PGE_2 was found to silence certain tumor suppressor and DNA repair genes via regulation of DNA methylation.[136]

In spite of its pro-carcinogenic functions, Cox-2-dependent PGE_2 is also a critical mediator of epithelial repair responses during intestinal injury. This is due to its pro-proliferative and anti-apoptotic effects on intestinal epithelial cells as highlighted earlier. This mucosal healing role of PGE_2 was addressed in at least three studies (one of which was discussed in the previous section), whereby LPS administration or the TLR4- and MyD88- dependent pathways were found to be crucial for the induction of epithelial restitution following intestinal injury.[107,137,138] Notably, the decreased epithelial proliferation and increased apoptosis observed in Cox-2-, TLR4- and MyD88- deficient mice treated with DSS was rescued by exogenous PGE_2 administration.[107,138] However, while Cox-2-dependent PGE_2 signaling was found to be important for the induction of epithelial regeneration following acute colitis, the induction of Cox-2 appeared to promote the development of CAC during chronic intestinal inflammation.[109] Taken together, these studies reveal that although the stimulatory effects of Cox-2/PGE_2 signaling on epithelial proliferation is critical for the maintenance of a healthy intestinal epithelial barrier, they can also be diverted into tumor promoting pathways in a genetically predisposed environment.

2 AIMS OF THESIS

The main objective of this thesis is to examine the role of gut microflora and inflammation in driving intestinal tumorigenesis and the regulation of gut homeostasis.

2.1 SPECIFIC AIMS

To achieve this, we investigated the role of commensal microflora and explored the contribution of underlying inflammatory mediators in the tumor progression of $APC^{Min/+}$ mice (Paper I). We then ventured into the signaling mechanisms of luminal microbes and focused on the Toll-like receptor 4 (TLR4) pathway, a major component of microbial signaling mediated via gram negative bacteria. Here, we examined the role of constitutive epithelial TLR4 signaling in the regulation of intestinal homeostasis and $APC^{Min/+}$-driven tumorigenesis (Paper II). We further investigated the host mechanisms driving intestinal inflammation during pathogenic bacterial infection (Paper III). Finally, we attempted to understand the transcriptional regulation of inflammatory and oncogenic responses through evaluation of the role of bromodomain-containing protein 4 (BRD4) in transcriptional elongation (Paper IV).

2.2 SIGNIFICANCE OF STUDY

The primary significance of all four studies is the eventual hope to:

- ➔ Identify critical inflammatory cascades and mediators driving the disease progression of human CRC.
- ➔ Enhance our understanding of how pathogenic microbes interact with the host during enteric infections.
- ➔ Provide targets for the therapeutic intervention of intestinal inflammation and cancer through understanding of the signalling mechanisms by which gut microbes regulate mucosal inflammation, tumor growth and apoptosis.
- ➔ Selectively limit the activation of pro-inflammatory or tumor-promoting responses through targeting histone modifications and/or transcriptional co-regulators.

3 METHODOLOGY

In this section, I describe some of the unique systems which we developed during the course of my PhD studies while majority of the well-established scientific methods used in my work are elaborated in the constituent papers.

3.1 PRIMARY CULTURE OF SPLENIC MACROPHAGES, COLONIC EPITHELIAL CELLS AND TUMORS

In an attempt to understand the underlying mechanisms governing inflammation-associated $APC^{Min/+}$ tumorigenesis, we analyzed for two major inflammatory pathways connected to CRC – STAT3 and c-Jun activation, using a series of *ex vivo* culture assays of purified splenic macrophages, colonic epithelial cells and intestinal tumors from $APC^{Min/+}$ mice.

3.1.1 Purification of splenic macrophages

Although the most relevant site of analysis for the role of myeloid cells would be from the tumor microenvironment itself, we have attempted and recognized the technical difficulty in obtaining sufficient purified CD11b+ cells from colonic tumors for different treatment groups. This is due to the much lower incidence of colonic tumors as compared to small intestinal tumors in $APC^{Min/+}$ mice. Moreover, the long digestion and purification times of several pooled tumors may have contributed to the increased cell death or change in phenotype of this population. As such, we chose to purify mature macrophages from the spleens of $APC^{Min/+}$ mice which developed colonic tumors instead, based on the assumption that they would be mobilized to the tumor site eventually.

Spleens from colon tumor-bearing $APC^{Min/+}$ mice were dissociated mechanically into single cell suspensions in RPMI-1640 medium, using blunt-end forceps. The cell suspensions were then washed with PBS before incubation with erythrocyte lysis buffer (155mM NH_4Cl, 12mM $NaHCO_3$, and 0.1mM EDTA) for 5-10min at RT to remove erythrocytes. Cells were subsequently washed with ice-cold PBS, passed through 45µm cell strainers and counted. For isolation of CD11b+ macrophages, splenocytes were resuspended in 90µl of buffer (cold PBS containing 0.5% BSA and 5mM EDTA to prevent cell clumping) per 10^7 cells and 10µl of CD11b-specific microbeads (Miltenyi Biotec) were added. Incubation with gentle rotation was performed for 30min at 4°C and cell purification was conducted using MS columns (Miltenyi Biotec) in accordance with manufacturer's recommendations.

After purification, splenic CD11b+ cells were incubated in RPMI-1640 medium (supplemented with 1% FBS and antibiotics) at 37°C for 30min to allow them to adapt to culture conditions and minimize non-specific effects, before stimulation with EPO (600 U/ml) for 1h. Reduced serum conditions were used to increase the contact of added stimulants with their receptors on macrophages and minimize any possible interaction of stimulants with serum components. Even though we detected an

induction of STAT3 activation in the macrophages following 1h of erythropoietin (EPO) stimulation (Paper I, Figure 5Bi), we decided to collect conditioned media after 24h of stimulation in the bid to maximize the pool of secreted cytokines, chemokines or growth factors in the conditioned media, without causing cell death in the myeloid cells. Moreover, the macrophages retained elevated mRNA expression levels of STAT3 target genes following 24h of stimulation (Paper I, Figure 5Bii), thereby reaffirming suitability of our chosen treatment time point. This conditioned media was used for subsequent proliferation assays involving the CT26 murine colon carcinoma cell line.

3.1.2 Ex vivo treatment of colonic epithelial cells and tumors

As we observed an induction of c-Jun phosphorylation in the CT26 epithelial cell line following incubation with conditioned medium of LPS-treated macrophages, we were also interested to understand if gut microflora has a *direc*t effect on intestinal epithelial cells. In an attempt to mimic the *in vivo* situation as much as possible, we used LPS as a major gut microbiotal component to treat primary colonic epithelial cells *ex vivo*, from GF mice as they have not been colonized with any microorganisms since birth.

Colons from these mice were cut longitudinally, washed and minced into 5mm segments. The tissues were then digested with dispase I (1mg/ml) at 37^{o}C for 90min. Mild digestion methods such as using dispase I with gentle mechanical shaking were chosen to obtain live colonic epithelial cells with as few contaminating lamina propria cells as possible. Following digestion, tissues were flushed gently several times with DMEM medium to release cells and crypts. The cells were then plated at a density of ~1.5x10^{6} cells per well in 24-well plates that were coated with a 1:1 solution of DMEM: matrigel as a form of substratum for the primary epithelial cells to attach to. Subsequently, the colonic epithelial cells were allowed to settle for 30min before LPS exposure for 30min.

In order to correlate our tumor counts data from EPO treated APC$^{Min/+}$ mice with levels of STAT3 phosphorylation observed in colonic tumors of untreated APC$^{Min/+}$ mice, we decided to perform *ex vivo* treatment assays of colonic tumors isolated from APC$^{Min/+}$ mice. As better *ex vivo* culture techniques of primary intestinal tumors were not available at that time, we decided to adopt a similar method for our tumor cultures as per primary colonic epithelial cells. Although we were able to obtain an induction of STAT3 phosphorylation in pooled colonic tumors with EPO treatment and that was consistent for at least 3 experimental sets, it was a technical challenge to obtain similar levels of fold induction possibly owing to the variable basal state of tumors from different APC$^{Min/+}$ mice of varying health statuses. As such, we acknowledge that improved methods for the long term expansion of both colonic epithelia and tumors for human tissues are now published[139] and may be more appropriate for our *ex vivo* stimulation assays if such techniques can be optimized for mouse colonic tissues as well.

3.2 INTESTINAL CRYPT-VILLUS ORGANOID CULTURE SYSTEM

The isolation of intestinal crypts from murine small intestines for the long-term culture and expansion of crypt-villus organoids *ex vivo* was established by the Hans Clevers

group.[140] It is a novel approach to obtain long-term cultures of primary intestinal crypts that can expand and differentiate to form all other lineages of the intestinal epithelium. We have adapted this crypt isolation procedure in Paper II to examine how constitutive epithelial TLR4 activation regulated intestinal epithelial cell proliferation and differentiation, without interference from the underlying mesenchyme.

Mouse small intestines were opened longitudinally, washed thrice in PBS and cut into 1-3mm segments. Tissues were then incubated with 2mM EDTA/PBS at 4°C for 30min, before vigorous resuspension with the pipet for 40 times. Following 3min of tissue sedimentation, the supernatant was removed and the gut pieces were washed twice with ice-cold PBS. This resuspension step was subsequently repeated twice using fresh ice-cold EDTA/PBS and the supernatant collected at the last resuspension (i.e. the crypts-enriched fraction) was plated with 50µl of matrigel, at a density of $6x10^3$ crypts per well in 48-well plates. The plates were then incubated at 37°C for 30min before adding culture medium supplemented with growth factors and additives, in accordance with the published protocol.[140]

3.2.1 Sorting of Paneth cells

For the purification of Paneth cells, primary murine intestinal crypts were purified as described above and dissociated in culture medium containing 0.5X trypsin for 40min in a 37°C shaking incubator. Cells were resuspended by pipetting at 10min intervals to facilitate dissociation into single cells. The trypsin was then neutralized with medium containing 20% FBS before passing the cell suspension through a 40µm cell strainer. Cells were subsequently resuspended in PBS/2% FBS and incubated with anti-CD24 antibody (1µg antibody per million cells) (BD Pharmingen) for 50min on ice. Following primary antibody incubation, cells were washed with ice-cold PBS/2% FBS and stained with Alexa-fluor488 goat anti-rat IgG (Invitrogen) (1µl antibody per 300µl cell suspension) for 30min at 4°C. Stained cells were then washed once and counterstained with DAPI (4',6-diamidino-2-phenylindole) before sorting of viable $CD24^{hi}/SSC^{hi}$ (side scatter) Paneth cells by flow cytometry, in accordance with the parameters highlighted by Hans Clevers group.[141]

4 RESULTS AND DISCUSSION

This thesis consists of four papers which represent a concerted effort to understand how host-microbe interactions regulate intestinal homeostasis, inflammation and tumorigenesis, as well as the significance of histone modification patterns and chromatin-associated proteins in the regulation of gene expression.

4.1 PAPER I: GUT MICROBIOTA ACCELERATE TUMOR GROWTH VIA C-JUN AND STAT3 PHOSPHORYLATION IN APC$^{MIN/+}$ MICE

In our investigation of the role of gut microflora in the tumorigenesis of APC$^{Min/+}$ mice, we found that APC$^{Min/+}$ mice which have not been exposed to microflora since birth i.e. GF APC$^{Min/+}$ displayed a remarkable down-regulation of intestinal tumor incidence as compared to their age-matched specific pathogen free (SPF) counterparts (Figure 1). Moreover, we also observed a significant reduction of intestinal tumor load in SPF APC$^{Min/+}$ carrying a myeloid-specific deletion in IKKβ (Figure S5). These two observations support the tumor promoting role of commensal microbiota in a genetic susceptibility model, which most likely involves the activation of myeloid cells from lamina propria. We thus hypothesized that inflammation triggered by the invasion of microflora into lamina propria, following disruption of the epithelial lining during the formation of intestinal lesions, enhanced the tumor progression of APC$^{Min/+}$ mice.

As such, we further addressed the inflammatory pathways and cellular mediators that may be driving APC$^{Min/+}$ tumorigenesis through in-depth characterization of the intestinal tumors and various types of immune cells infiltrating these tumors, as well as a variety of ex-vivo treatment assays of primary tumors and myeloid cells. We found a distinct pro-inflammatory status in APC$^{Min/+}$ colonic tumors as compared to small intestinal tumors (Figure 2B-D), which correlated with the histological verification of epithelial lining disruption in regions surrounding colonic lesions but not small intestinal lesions (Figure 2A). This was also consistent with a significantly higher amount of infiltrating CD11b+ and GR1+ myeloid cells in colonic tumors (Figure S3-4), strongly suggesting the involvement of TAMs in the perpetuation of tumor-promoting inflammation as reported in various CAC models.[46,63]

Moreover, the significantly increased levels of phosphorylated c-Jun and p-STAT3 (Tyr-705) in colonic tumors (Figure 2C-D) demonstrated the up-regulation of two well established oncogenic pathways - JNK-c-Jun/AP-1 and JAK/STAT3 signaling, during APC$^{Min/+}$colonic tumorigenesis. Their critical involvement in mediating tumor cell proliferation and survival was further illustrated in our *ex vivo* stimulation assays of primary APC$^{Min/+}$ tumors and CD11b+ macrophages (Figure 5A-B and 6A-B). Notably, JNK-mediated c-Jun phosphorylation, which has been implicated in mediating APC$^{Min/+}$ intestinal tumorigenesis via cooperative interaction of the AP-1 complex with TCF4 and β-catenin,[35] could be induced in primary colonic epithelial cells by a major microbiotal component of gram-negative bacteria - lipopolysaccharide (LPS) (Figure

6C) and was also necessary for the induction of epithelial cell proliferation (Figure 6A-B).

In addition to the direct proliferative effect of gut microbiota on epithelial cells during tumorigenesis, soluble factors produced by activated myeloid cells infiltrating the colonic tumors also have a significant tumor-promoting role as suggested by our findings (Figure S5). One crucial observation in our study was the detection of high levels of activated STAT3 in tumor-associated macrophages of $APC^{Min/+}$ colonic adenomas (Figure 2E and S7). Importantly, erythropoietin (EPO) which triggered colon tumorigenesis in $APC^{Min/+}$ mice (Figure 4) was also found to induce STAT3 activation in $APC^{Min/+}$ colonic tumors and splenic macrophages *ex vivo* (Figure 5A-B). The critical connection between JAK/STAT3 signaling and $APC^{Min/+}$ tumorigenesis was further elucidated in our study through the administration of curcumin, a known STAT3 inhibitor,[142] in $APC^{Min/+}$ mice. In particular, we found that the short-term treatment of $APC^{Min/+}$ mice with curcumin, which has previously been shown to reduce intestinal tumorigenesis,[143] could induce the down-regulation of p-STAT3 (Tyr-705) in their colonic tumors (Figure 5C). As STAT3 phosphorylation has been associated with tumor-promoting functions such as the suppression of anti-tumor immunity in myeloid cells[53] and stimulation of intestinal epithelial cell proliferation and survival,[62,63] our findings thereby establish the causal role of STAT3 activation, most notably in tumor-associated myeloid cells, in $APC^{Min/+}$ colon tumorigenesis.

Furthermore, the finding that EPO administration can promote colon tumorigenesis in $APC^{Min/+}$ mice (Figure 4) has important clinical implications as EPO is traditionally used to treat anemia in cancer patients and is also popular amongst athletes as a performance enhancing drug. EPO can trigger JAK-mediated phosphorylation of all STATs through engagement with the EPO receptor (EPO-R),[144] which is known to be expressed on erythrocyte precursors and certain tumor cells.[145,146] Although EPO signaling via EPO-R activation is widely recognized to induce erythropoiesis in erythroid progenitor cells via JAK2/STAT5 signaling,[146] EPO has also been reported to act directly on macrophages, enhancing their pro-inflammatory activity and function.[147] Meanwhile, our finding that EPO can induce STAT3 activation in macrophages (Figure 5B) reaffirms that EPO-R may be found on myeloid cells as well and that its activation can mitigate other erythropoiesis-independent functions in non-erythroid progenitor cells. As EPO has also been demonstrated to exert tumor-promoting effects such as the paracrine stimulation of tumor angiogenesis,[148] our findings therefore provide further understanding to the multiple tumor-promoting mechanisms of EPO.

In conclusion, our data from this study illustrates the active role of gut microflora in promoting intestinal tumorigenesis during microbial dysbiosis-triggering events, such as the loss of epithelial barrier integrity. Thus, an aberrant mucosal barrier in the $APC^{Min/+}$ mouse model propagates a chronic inflammatory state in the gut which drives enhanced tumor growth and progression. This vicious feed forward cycle of augmented intestinal lining disruption and tumor-promoting inflammation is mediated by at least two distinct pathways – JAK/STAT3 activation in myeloid cells and JNK-dependent c-Jun phosphorylation in intestinal epithelial cells. Henceforth, targeting the two pathways sequentially or in combination, in the specific tissue compartments, may represent an effective therapeutic strategy for CRC patients harboring *APC* mutations.

4.2 PAPER II: CONSTITUTIVE TLR4 SIGNALING IN INTESTINAL EPITHELIUM REDUCES TUMOR LOAD BY INCREASING APOPTOSIS IN $APC^{MIN/+}$ MICE

After establishing the critical role of gut microbiota in fuelling inflammation-driven intestinal tumorigenesis in $APC^{Min/+}$ mice, we proceeded further to examine the host-microbial signalling pathways involved in the regulation of intestinal tumor growth and development. One major class of PRRs involved in microbial recognition and signalling through the host is TLRs, which play a critical role in regulating intestinal homeostasis and tumorigenesis as discussed in section 1.5.2. In the earlier described study by Medzhitov's group, the genetic ablation of MyD88, a major signalling adaptor downstream of TLRs, resulted in a dramatic reduction in the intestinal tumor burden of $APC^{Min/+}$ mice.[112] This finding resonated strongly with our data in the first study and thus led us to focus our attention on the TLR4 pathway, a major component of host-microbe interactions mediated by gram negative bacteria.

As we observed in the previous study that LPS, a known activator of TLR4 signaling, could stimulate epithelial cell proliferation (Paper I, Figure 6B), we thus hypothesized that constitutive TLR4 activation in the gut would stimulate tumor growth in $APC^{Min/+}$ mice. As such, we generated transgenic mice expressing constitutively active TLR4 (CD4-TLR4) specifically in the intestinal epithelium and crossed them with $APC^{Min/+}$ mice. Unexpectedly, we found that $APC^{Min/+}$ mice expressing intestinal epithelial cell-specific CD4-TLR4 displayed significantly reduced tumor load and size, relative to age-matched, wild-type $APC^{Min/+}$ mice (Figure 1). This observed suppression of spontaneous tumorigenesis in a genetically susceptible model was in stark contrast to a CAC model, whereby constitutive TLR4 signaling in the intestinal epithelium augmented inflammatory responses to colitis, which promoted carcinogen-induced tumorigenesis in an AOM/DSS treatment regimen.[111] The two apparently disparate facades of constitutive TLR4 activation in the regulation of intestinal tumorigenesis highlight the context dependent manner of this control, thereby prompting us to further probe the underlying mechanism in our CD4-TLR4-$APC^{Min/+}$ mice.

Interestingly, CD4-TLR4 transgenic mice exhibited a higher proliferative status in the intestinal epithelium, as illustrated by increased nuclear Ki-67 staining in epithelial cells residing above crypt bases (Figure 2A) as well as *ex vivo* intestinal crypt-villus organoids (Figure 3B). These findings were consistent with the data from our earlier study. Furthermore, persistent epithelial TLR4 activation had a significant impact on the functions of all secretory cell lineages, namely Paneth cells, goblet cells and enteroendocrine cells (Figure 3C). Notably, constitutive epithelial TLR4 stimulation enhanced Paneth cell activity, which was consistent with the increased expression of markers for Paneth cell functions and intestinal stem cells (Figure 3C). These observations correlated well with the increased proliferative potential of CD4-TLR4 organoids (Figure 3) and extend support to the current notion that Paneth cells provide essential niche signals for the survival and expansion of intestinal stem cells.[141] Importantly, the findings reveal that long-term TLR4 signaling in the intestinal epithelium can impact on both the stem cell and differentiated epithelial lineages,

which may have major functional consequences on intestinal homeostasis and regenerative responses.

Hence, we were intrigued by the reduced tumor burden of CD4-TLR4 expressing $APC^{Min/+}$ mice despite the increased proliferative capacity of the intestinal epithelium and searched for alternative pathways of TLR4 activation that can provide a plausible explanation for this phenomenon. Surprisingly, the persistent activation of TLR4 led to a down-regulation of Cox-2 in both CD4-TLR4 expressing intestinal organoids and tumors (Figure 3C and 4B). This down-regulation of Cox-2 expression was inversely correlated with levels of transgene expression as well as the expression of pro-apoptotic markers (Figure 4A and C), suggesting of a modulation of the pro-survival functions of Cox-2 by constitutive TLR4 signaling. Although we were unable to identify the precise regulator of Cox-2 in this study, we found that interferon β (IFNβ), a direct target of the TLR4 pathway was significantly up-regulated in CD4-TLR4 expressing tumors (Figure 4B). This cytokine, popularly known for its host defense function against microbial pathogens and tumor cells, has been previously demonstrated to inhibit the transcription of Cox-2 in intestinal epithelial cells.[149] Of note, the production of IFNβ by TLR4 activated tumors was also implicated to be critical in mediating anti-tumoral immunity.[150]

Indeed, the down-regulation of Cox-2 was consistent with an elevated apoptotic status in CD4-TLR4-$APC^{Min/+}$ tumors as compared to tumors from wild-type $APC^{Min/+}$ mice. This was elegantly visualized *in vivo* using the FAM-FLIVO apoptosis assay and verified by the enhanced protein levels of cleaved caspase 3 in CD4-TLR4-$APC^{Min/+}$ tumors (Figure 5B-D). While markers of tumor cell proliferation and autophagy remain unperturbed (Figure S6), the increased apoptosis in tumors of transgenic $APC^{Min/+}$ mice provided a plausible mechanism for their reduced tumor incidence and size. This was also consistent with studies demonstrating the amelioration of intestinal tumor burden in FAP patients and $APC^{Min/+}$ mice treated with Cox-2 inhibitors.[126,151] Furthermore, evidence of the improved efficacy of a treatment regimen involving Cox-2 inhibitor and IFNβ, in reducing the survival of xenograft tumors via induction of apoptosis,[152] lend further support to our data. Interestingly, the normal intestinal mucosa of CD4-TLR4 mice displayed similar levels of apoptotic cells as wild-type mice (Figure S7), implying that the enhanced apoptosis detected is only unique to a genetically predisposed setting.

Thus, our findings reveal a fine equilibrium between proliferation and programmed cell death in the intestinal epithelium that is regulated by microbial signaling. In particular, persistent TLR4 activation in the gut stimulates both epithelial cell proliferation and the functions of all secretory cell lineages. While normal homeostatic mechanisms are in place to prevent aberrant crypt-villus outgrowth, as depicted by the normal intestinal morphology of CD4-TLR4 mice (Figure S1D), our data show that in a genetically susceptible environment such as in the $APC^{Min/+}$ background, a higher extent of apoptosis ensues, resulting in a suppression of spontaneous tumorigenesis. Moreover, we further related our observations to the intriguing down-regulation of a critical survival factor, Cox-2, which is known to mediate tumor promoting effects (as discussed in section 1.6) and is frequently correlated with a poorer prognosis of CRC patients. Although Cox-2 expression has been shown to be induced in colorectal cancer

cells by short-term, acute stimulation of TLR4,[138] our data indicates that the persistent activation of TLR4 in intestinal epithelial cells results in the down-regulation of Cox-2 instead.

While additional bystander mechanisms may be triggered during constitutive TLR4 activation, that in turn regulate tumor growth and progression, we have so far identified IFNβ as a potential inducer of anti-tumoral immunity. This is an interesting aspect of microbial signaling that is not well understood till date. Moreover, the increased expression of a TLR4-inducible RNase and known immune response modifier - Zc3h12a,[153,154] in CD4-TLR4-expressing tumors (Figure S8C) further reinforces this concept. It remains to be determined whether Zc3h12a can also modulate Cox-2 expression. As such, although our findings from the previous study as well as Medzhitov's study[112] demonstrate the active role of host-microbial interactions in tumor promotion, the constitutive activation of a major aspect of microbial signaling resulted in tumor inhibition instead. Our findings thereby reveal the complex regulation of TLR4 signaling, which can trigger both MyD88- dependent and -independent mechanisms, as well as apoptotic pathways during constitutive activation in a genetically predisposed environment. This intriguing facade of host-microbe crosstalk adds on to the complexity highlighted in section 1.5.2 and has important functional implications in the use of microbial ligands or long-term antibiotics administration in the treatment of various gastrointestinal health ailments.

4.3 PAPER III: ABSENCE OF INTESTINAL PPARG AGGRAVATES ACUTE INFECTIOUS COLITIS IN MICE THROUGH A LIPOCALIN-2 DEPENDENT PATHWAY

The findings from our first two papers have provided an insight of how the complex interaction between the commensal microbiota and host can lead to diverse outcomes in intestinal tumorigenesis. Moving forward, we were interested to further investigate how pathogenic microbes interact with the host innate immune system to impact on intestinal homeostasis and mucosal defense. Thus in this study, we used a bacterial pathogen which causes enterocolitis -*Salmonella enterica* serotype Typhimurium in our model of infectious colitis to examine the host defense mechanisms during intestinal microbial infection. We decided to focus our attention on the nuclear receptor - peroxisome proliferator-activated receptor γ (PPARγ) because of its well established role in the regulation of inflammation and maintenance of gut homeostasis.

Belonging to the superfamily of ligand-dependent transcription factors, PPARγ is highly expressed in adipose and colonic tissues.[155] Its activation upon ligand recognition entails the heterodimerization with retinoid X receptor α (RXRα) in the nucleus, thereby facilitating the transcriptional regulation of specific genes via binding of the heterodimer to PPARγ response elements (PPREs).[156] In addition to its role in regulating adipocyte differentiation and carbohydrate metabolism,[155] PPARγ is well recognized as a critical immunomodulatory factor through its ability to down-regulate the expression of inflammatory cytokines, antagonize the activities of AP-1, STAT and

NF-κB, and polarize immune cell functions towards an anti-inflammatory phenotype.[157,158] Notably, PPARγ and its agonists have been implicated as crucial mediators of intestinal homeostasis and antimicrobial immunity, as well as potential therapeutic agents for the treatment of colitis.[159-162] Moreover, its strategic involvement in the host-microbe crosstalk, traversing between microflora-driven signals and the regulation of host inflammatory responses,[163,164] further fueled our interest in its regulation during *S. Typhimurium* induced colitis.

In our investigation, we observed that *S.* Typhimurium infection of mice pretreated with streptomycin resulted in the down-regulation of PPARγ expression in the colon after 24h (Figure 1A-C), suggesting of a perturbation in the homeostasis of the intestinal tract during infectious colitis. Consistently, mice carrying an intestinal epithelial cell-specific deletion of PPARγ (PPARγVillinCre+) displayed a more aggravated colitis than WT mice during *S.* Typhimurium infection (Figures 1D-E and 2). This increased severity of colitis in PPARγVillinCre+ mice and the concomitant induction of NF-κB and AP-1 activities in colon (Figures 3A-B, S2 and S3) corresponded with an elevated expression of inflammatory cytokines - TNF-α, IL-6, IL-17 and IL-22 (Figure 3C-F). Interestingly, the induction of IL-17 and IL-22 during infection correlated with an innate T helper type 17 (iT_H17) response, which was recently documented to be crucial for host defense against enteric pathogens including *S.* Typhimurium.[165] In particular, IL-22 was reported to stimulate the luminal release of antimicrobials such as regenerating islet-derived 3 gamma (Reg3γ) and lipocalin-2 (Lcn2) by epithelial cells.[166,167] Accordingly, the two antimicrobials were similarly induced during *S.* Typhimurium infection, with a corresponding pattern of augmented expression observed in mice lacking gut epithelial PPARγ as compared to WT mice (Figure 4). These findings therefore depict the significant role of epithelial PPARγ in the regulation of intestinal homeostasis and innate immune responses during exposure to bacterial pathogens.

We then sought to further examine the consequences of the heightened inflammatory and antimicrobial responses in infected PPARγVillinCre+ mice during disease pathogenesis. To our surprise, we found an elevated activity of both precursor and cleaved forms of matrix metalloproteinase 9 (proMMP-9 and MMP-9 respectively), which correlated with enhanced levels of Lcn2 bound proMMP-9 (proMMP-9/Lcn2) (Figure 5A-B). This finding disclosed a previously unexplored role of Lcn2 in promoting the stability of MMP-9 during infectious colitis, which can potentially lead to more severe tissue degradation arising from the increased enzymatic activity of MMP-9. Consistent with this notion, lipocalin-2 knockout mice ($Lcn2^{-/-}$) displayed a marked protection from epithelial denudation and tissue damage during *S.* Typhimurium infection (Figure 6), which corresponded to the reduced secretion and activity of proMMP-9 and MMP-9 in the intestinal milieu (Figure 7A-D) as compared to WT or PPARγVillinCre+ mice (Figure 5). Taken together, our results thereby illustrate how enteric pathogens such as *S.* Typhimurium can regulate and exploit the host innate immune and mucosal defense (antimicrobial) mechanisms to create an inflammatory environment that favors its survival and colonization in the host.

4.4 PAPER IV: BROMODOMAIN-CONTAINING-PROTEIN 4 (BRD4) REGULATES RNA POLYMERASE II SERINE 2 PHOSPHORYLATION IN HUMAN CD4+ T CELLS

In our previous three studies, we have demonstrated that changes in the expression of specific genes associated with cell proliferation, apoptosis, immune cell functions, and antimicrobial responses are important determinants of tumor survival and progression, inflammation as well as host responses to bacterial pathogens. One critical regulatory platform controlling host-microbial, inflammatory and/or oncogenic transcriptional responses is the post-translational modification of histones. These covalent modifications occur at the amino-terminal tails of histones and include acetylation, methylation, phosphorylation, ubiquitination and ribosylation, all of which are dynamically mediated by histone modifying enzymes.[168,169] The remarkable diversity of histone marks and their combinatorial complexity have led many investigators to favor the view that distinct histone modification patterns encode a 'language' that is read by other proteins to generate unique biological outcomes.[168] This concept was thus coined the 'histone code hypothesis'[168,170] and further proposed to impact on chromatin-related processes, thereby leading to distinct cell fate decisions and the development of both normal and pathological states.[171]

Indeed, the post-translational modification of histones impacts significantly on the regulation of gene expression through its ability to modulate chromatin structure and the recruitment of transcriptional regulators. Since the 'histone code hypothesis' was posited, emerging literature have revealed how alterations in covalent histone modifications and dysregulation in chromatin regulators are closely linked to the development and progression of cancer.[169,172,173] Moreover, chromatin remodeling processes and the transcriptional mediators that bind to specific chromatin modifications have also been implicated in the selective induction of inflammatory gene expression programs.[174-176] Thus in this study, we initially sought to understand the underlying epigenetic events regulating the transcriptional control of inflammatory or oncogenic responses globally.

We decided to focus our attention on a bromodomain-containing protein, BRD4, a member of the BET (bromodomains and extraterminal) family that recognizes acetylated lysine residues on histone tails.[177] Originally discovered as a ubiquitously expressed nuclear protein that binds to mitotic chromosomes, BRD4 was shown to mark select genes for transcriptional memory and regulate cell-cycle progression.[178] This double bromodomain-containing protein was later found to be critical for the recruitment of transcriptionally active P-TEFb (positive transcription elongation factor) to promoter, leading to stimulation of RNA polymerase II (Pol II)-dependent gene transcription.[179] Notably, BRD4 was identified as a novel co-activator of NF-κB through interaction with an acetylated lysine of RelA, further enhancing the transcription of NF-κB-dependent inflammatory genes via P-TEFb recruitment.[180] In addition, BRD4 recruitment to promoters was found to be critical for the transcription of genes involved in cell cycle progression[181] and this chromatin adaptor was also implicated in oncogenesis.[182] Taking into account its ability to bind acetylated

chromatin and significant role in transcription co-activation, BRD4 thus represented an interesting platform for us to examine the epigenetic mechanisms leading to inflammation as well as cancer.

In our genome-wide analysis of BRD4 binding sites using chromatin immunoprecipitation sequencing (ChIP-Seq), we found that BRD4 was predominantly associated with actively transcribed genes in human CD4+ T cells and its recruitment was positively correlated with levels of gene expression (Figure 1). Consistent with its co-regulatory role in transcriptional elongation,[179,180] the global distribution of BRD4 coincided mostly with known promoter and enhancer regions, with over 50% of binding sites occurring at intergenic and intragenic regions (Figure 2). Interestingly, BRD4 binding sites co-localized with that of Pol II and Ser2-phosphorylated Pol II (Pol II Ser2) at the promoters and enhancers of genes marked by active histone marks (Figure 3 and Figure S3). These observations therefore implicate the significant contribution of BRD4 in transcriptional elongation at a genome-wide level, which is potentially mediated through its recruitment of P-TEFb to Pol II bound sites.

As the signal-dependent recruitment of P-TEFb/BRD4 complex in the induction of gene-specific transcription elongation has been demonstrated previously to be dependent on the crosstalk with acetylated histones,[183,184] we thus examined the interaction of BRD4 with various acetylated histone marks. Through integration of global BRD4 binding sites with published databases of genome-wide acetylated histone sites, we found that majority of BRD4 binding sites was associated with histone 4 (H4) acetylated on lysine residues 5 and 8 (H4K5ac and H4K8ac respectively) (Figure 4). These interactions were further verified *in vitro* via histone peptide binding assays (Figure 4F and H), indicating that BRD4 can be recruited to promoters and enhancers through binding with acetylated histones. We thus proceeded further to assess the significance of this recruitment in the transcription of BRD4-bound genes by disrupting the binding of BRD4 to acetylated histones using a known BET inhibitor, JQ1.[185] As expected, JQ1 treatment resulted in the global reduction of both BRD4 and Pol II Ser2 gene occupancy, with a corresponding decrease in the expression of BRD4-bound lineage-specific genes in human CD4+ T cells (Figure 6). In addition, an enrichment of P-TEFb binding was also identified in a subset of enhancers where BRD4 and Pol II Ser2 were co-localized (Figure 8C-E) and the direct interaction of P-TEFb and BRD4 was verified in human T cells (Figure 8B). Thus taken together, our ChIP-seq data suggest the positive regulation of BRD4 in Pol II Ser2-mediated transcriptional elongation through P-TEFb recruitment at specific histone modifications, which is likely to drive the transcription of genes in a lineage-specific or signal-dependent manner.

Through the study of BRD4 in transcriptional regulation, we provide evidence for the significant role of histone modifications and chromatin-binding proteins in the regulation of specific gene expression. The dynamic interplay of a variety of histone modifiers as well as the ability of specific histone marks to modulate gene expression through interaction with transcriptional co-regulators allows for a gene to be expressed or silenced according to its function. This selective regulation of gene expression is not only important for the induction of an inflammatory response, but also the timely resolution of acute inflammation to restrict excessive tissue damage. The recent

characterization of BRD4 as a key determinant of various malignancies and inflammatory diseases[186-190] attests further to the significance of the 'histone code' and highlights the future promise of cancer epigenetic therapy via selective targeting of chromatin-associated regulators or chromatin remodeling.

5 CONCLUDING REMARKS AND PERSPECTIVES

The gut microbiota isn't simply a repository of microorganisms residing quiescently in our gastrointestinal tracts. These microbes, popularly referred to as 'our commensals', participate actively in several important biological processes that shape our immune system, intestinal homeostatic functions and development, as well as metabolism. As these microbes depend directly on their host for shelter and raw materials, it seems pretty intuitive that they would protect their host's well-being in order to ensure their own survival. However, this symbiotic relationship can be easily disrupted in the wake of physical lesions, pathogenic infections or deregulated inflammatory responses in the gut, often arising from certain genetic susceptibility event(s), and the commensals become a major threat to the host's health instead. Indeed, as we have learnt from past studies, microbial dysbiosis can trigger a cascade of events leading to various pathological conditions including obesity, diabetes, IBD and cancer. Therefore, understanding how the microbiota signals through the host, as well as its multifarious interactions with the host in human health and disease, is becoming increasingly relevant and crucial for yielding better and more tractable medical solutions.

One important ailment of the gut affecting the developed world today is colorectal cancer (CRC). The dysfunction of APC, which is observed in an overwhelming number of colorectal adenomas and adenocarcinomas, is widely accepted to play an instrumental role in CRC. As APC is a major scaffolding protein regulating the canonical Wnt/β-catenin signaling cascade, initial studies have proposed that the consequent constitutive activation of β-catenin (arising from APC inactivation) is the underlying mechanism driving early transformation of the colonic epithelium.[191,192] However, subsequent investigations have reported the lack of nuclear β-catenin accumulation in early adenomas,[193,194] suggesting that other 'modifiers' are required to fuel the canonical pathway during tumorigenesis. As such, it is highly pertinent to identify the potential 'modifiers' and assess their contributions to the development and progression of CRC.

From our recent investigations of the $APC^{Min/+}$ mouse model of CRC, we have learnt the integral role of gut microbiota in fueling a positive feedback channel that facilitates chronic inflammatory processes, thereby promoting intestinal tumor growth. While it may be interesting for future investigators to identify the relevant microbial species driving this dysbiosis-associated inflammation and examine their key regulatory elements, we have highlighted at least two signaling mechanisms that are aberrantly activated during disease pathogenesis in a genetically predisposed environment. These two major pro-oncogenic signaling nodes, the JNK-c-Jun/AP-1 and JAK/STAT3 pathways, represent potential targets for the therapeutic intervention of CRC and colitis-associated cancer (CAC) progression.

The phosphorylation of c-Jun, has been shown to interact with the canonical Wnt/β-catenin signaling cascade through transcriptional cooperation with TCF4.[35] Furthermore, our studies as well as others have implicated its causal contribution in intestinal tumorigenesis.[35,75] Thus, the inhibition of phospho-c-Jun-TCF4 interaction

appears to be a promising treatment strategy for human intestinal cancer. For instance, it would be interesting to determine the therapeutic effect of JNK inhibitors or small-molecule inhibitors of down-stream effectors of the c-Jun/AP-1 activation pathway on the disease progression of CRC. However, as JNK signaling and the activation of c-Jun have been suggested to affect progenitor cell proliferation and migration in intestinal crypts,[75] the long-term inhibition of JNK-c-Jun/AP-1 signaling is not recommended due to its possible impact on normal intestinal regeneration.

Meanwhile, the JAK/STAT3 signaling pathway is also a relevant target for the treatment of inflammation-associated colorectal cancers. As we have observed STAT3 activation predominantly in the infiltrating myeloid cells of colorectal adenomas and myeloid cells appear to be a major source of tumor-promoting IL-6, whose production is also dependent on STAT3 activation,[63] it would be useful to develop efficient methods of targeting STAT3 phosphorylation specifically in these TAMs. Furthermore, the inhibition of STAT3 signaling in these immune cells can also elicit anti-tumor immunity in the host through triggering an intrinsic immune-surveillance system as demonstrated in mouse studies.[53] This site-specific therapeutic strategy may be more beneficial to the patient as compared to the traditional 'bulldozer' treatment methods that destroy normal tissues and weaken the immune system in the process of tumor cell killing.

Although we did not address the role of constitutive STAT3 activation in tumor epithelial cells in our study, it appears to play a significant role in the development of CAC tumors[62,63] and thus should be considered as a potential target for the treatment of IBD-associated colon cancer. However, great caution should be taken in the long term use of STAT3 inhibitors, whether it is targeting the epithelial or myeloid compartments, in view of the important biological functions IL-6 and other STAT3 effectors have in the maintenance of normal intestinal epithelial homeostasis and reparative processes, as well as in the development of hematopoietic cells. For instance, the inactivation of STAT3 in immune cells of both the myeloid and lymphoid lineages resulted in the development of an IBD-like intestinal inflammation and malignant tumor formation in mice.[195] In addition, the conditional ablation of STAT3 in intestinal epithelial cells appeared to promote tumor progression at later stages in $APC^{Min/+}$ mice,[196] suggesting that STAT3 may negatively regulate intestinal cancer progression. As such, targeting the STAT3 or even the c-Jun/AP-1 pathways as a therapeutic approach for CRC may be context dependent and can lead to very distinct consequences at different stages of tumor development.

While we addressed the tumor promoting role of gut microbiota-associated interactions during inflammation-driven intestinal tumorigenesis, our studies also revealed an unrecognized role for a major host-microbe signaling component, the TLR4 pathway, in the suppression of spontaneous tumorigenesis. In particular, the unexpected modulation of Cox-2/PGE_2 signaling by constitutive activation of TLR4 in intestinal epithelial cells and the corresponding up-regulation of apoptosis in $APC^{Min/+}$ tumors illustrate the multi-faceted nature of microbial signaling in the regulation of tumor cell survival and death. Although the recognition of microbial ligands by TLRs can initiate inflammatory responses that are cancer promoting, the protective host defense or antimicrobial mechanisms launched by TLRs can in turn drive potent anti-tumor

responses as well. Through the manipulation of TLR4 signaling in an inflammation-independent fashion, our findings suggest a physiological role of TLR4 activation in the inhibition of aberrant cell growth in a genetically predisposed environment. These results are also consistent with the data from other groups demonstrating the tumor surveillance or elimination mechanisms elicited by TLR4 activation.[150,197]

Thus, the prospect of using TLR4 ligands or probiotics for reducing cancer risk in IBD patients or genetically susceptible individuals, and/or improving the disease outcome of CRC patients looks promising. In fact, the notion that gut microbiota can regulate health and disease was put forth by Élie Metchnikoff more than a century ago, where he hypothesized that beneficial microbes could replace harmful ones.[79] Accumulating evidence from animal models and clinical studies in the recent years has demonstrated the beneficial efficacy of probiotics in the treatment of various ailments including diarrhea, allergic disorders, gastroenteritis and IBD.[198] In addition, the use of fecal microbiota transplantation (or 'fecal bacteriotherapy') in the treatment of recurrent *Clostridium difficile* infection appeared to be effective in resolving symptoms such as diarrhea.[199] These studies suggest that gastrointestinal, immune or even metabolic disorders arising from microbial dysbiosis can be ameliorated through restoration of a healthy, stable gut microbiota. Moreover, using microbial ligands, beneficial microbial strains or fecal microbiota transplantation as a therapeutic strategy may eventually be more superior to antibiotics treatment, which can disrupt the native microbiota and promote colonization of resistant bacterial strains.

The therapeutic exploitation of the gut microbiota may also be applicable in the promotion of intestinal regeneration following injury. Although we did not address the role of constitutive TLR4 activation in intestinal regeneration in our studies, the increased proliferative potential of CD4-TLR4 intestinal crypt-villus organoids as well as their increased expression of markers of all secretory cell lineages of the gut strongly suggests the enhanced regenerative capacity of TLR4-active epithelia in the event of injury-related crypt loss. While host-microbe interactions play an integral role in the regulation of intestinal homeostasis and tumorigenesis, our infectious colitis model studies further reveal the significant impact of enteric microbial pathogens on the host's innate immune and antimicrobial defenses, which can elicit potent inflammatory responses in the gut that further potentiates disease severity. As such, better understanding and manipulation of the host mechanisms that are triggered by enteric pathogens may be a good strategy to limit disease pathogenesis and combat infections. In addition, the epigenetic regulation of specific patterns of gene expression represents yet another aspect of therapeutic targeting for the future management of cancer, inflammatory as well as infectious diseases.

Meanwhile, the recent advent of improved methods for the long-term *in vitro* culture and expansion of primary intestinal epithelial organoids and adenomas from the mouse and human,[139] as well as the feasibility of virally mediated gene transfer in some of these organoids systems,[200] offers a major advancement for translational research. For instance, it would now be possible to test the therapeutic efficacy of potential anti-cancer drugs on human CRC clinical samples. The identification of novel cancer targets through genetic manipulation studies of primary organoid systems also holds great promise in the near future. In retrospect, some of the *ex vivo* stimulation assays

described in the first paper would have been carried out more efficiently if some of these methods were available earlier.

The crucial importance of the gut microbiota in human health and disease has been rapidly emerging in the recent years. This is well illustrated by a number of reviews describing the interactions between the host immune system and gut microbiota, including how microbial composition and diversity can shape the adaptive immune system and influence the development of various human diseases such as IBD, autoimmune disorders and obesity.[201,202] Moreover, the discovery of a commensal microbial species that can promote intestinal tumorigenesis during colitis highlights how intestinal inflammation can alter microbial composition and activity, promoting a dysbiotic microbiota which in turn impacts on the host's health.[203] In light of the recent advances in the understanding host-microbiota interactions and their impact on human health, it has been extremely meaningful for me to put this thesis together and I hope that our studies will ultimately contribute to the biomedical field. Nonetheless, it has been an exciting and insightful learning experience to participate actively in all four studies and I am grateful for the valuable lessons and knowledge gained from this PhD journey.

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