Hypoxia, Neutrophils, and the Colon Tumor Inflammatory Response

by

Daniel Augustus Triner

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular and Integrative Physiology)
in the University of Michigan
2018

Doctoral Committee:

Associate Professor Yatrik M. Shah, Chair
Professor Eric R. Fearon
Professor Juanita L. Merchant
Professor Martin G. Myers Jr.
Professor Linda C. Samuelson

Daniel Augustus Triner

trinerda@med.umich.edu

ORCID iD: 0000-0003-2661-6295

I dedicate this thesis to my wife, family, lab, and mentor.

Acknowledgements

I would like to begin by thanking my wife. Your continuous encouragement, love, and support has helped me continue to push through hard times and rough patches. I would also like to thank my family, my parents, and my in-laws. All your love and support has helped me realize my dreams and continue to push myself every day. I owe a tremendous amount of gratitude to the members of the Shah Lab, Sadeesh Ramakrishnan, Xiang Xue, Nupur Das, Xiaoya Ma, Andrew Schwartz, Sammi Devenport, Sumeet Solanki, and Cristina Castillo. Without your continuous help on my projects this thesis would not have been possible. I would also like to thank the Medical Scientist Training Program and Molecular and Integrative Physiology Department. I would also like to thank my thesis committee, Eric Fearon, Linda Samuelson, Juanita Merchant, and Martin Myers. Your comments and critiques of my projects have been instrumental in the success of my PhD. Most importantly I'd like to thank my mentor Dr. Yatrik Shah. You have helped me to fully realize my potential as a scientist and truly showed me the value of hard work. You have taught me how to ask important scientific questions and devise novel ways to address them. Thanks to your dedication and persistence, I feel well prepared to pursue my own career as an independent scientist.

Table of Contents

List of Figures

List of Tables

Abstract

In 1863, Rudolf Virchow proposed that tumors arise from sites of chronic inflammation. This concept was largely ignored throughout the majority of the next century as the genetic basis of cancer was explored. However, it has become well appreciated that inflammation and cancer are intimately linked. For example, chronic inflammation of the intestine in the form of Inflammatory Bowel Disease (IBD) predisposes to the development of colon cancer. Although the vast majority of colon tumors do not arise from sites of chronic inflammation, sporadic colon tumors elicit an inflammatory response that is essential for tumor growth, progression, and evasion of anti-tumor immunity.

Hypoxia is a well-characterized feature of nearly all solid tumors and promotes stabilization of the hypoxia inducible transcription factors (HIF-1α and HIF-2α) with known roles in modulating tumor-associated inflammation. We have previously reported that intestinal epithelial HIF-2α is an important driver of the acute inflammatory response in colitis. The present work describes a novel axis by which intestinal epithelial HIF-2α serves as a critical link between inflammation and cancer of the colon. Mechanistically, our work shows a crucial role for intestinal epithelial HIF-2α in regulation of the immune microenvironment of colon tumors through recruitment of intra-tumoral neutrophils. Neutrophils are granulocytic myeloid cells of the innate immune system that are the first responders to sites of infection to limit microbes.

Neutrophils are highly infiltrated in nearly all solid tumors including colon cancer. We showed that neutrophil influx was due to direct HIF-2α-dependent regulation of the potent neutrophil chemokine CXCL1. These data identify a novel role for HIF-2α in modulation of the tumor immune microenvironment of inflammation-driven colon tumors and suggest therapeutic potential.

Our data suggested an important role for neutrophils in the maintenance of colon tumors. However, the importance of neutrophils in the initiation of colon tumorigenesis is largely unknown. Using mice with constitutive genetic depletion of neutrophils, the present work demonstrates an essential role for neutrophils in restricting colon tumor growth and progression in both inflammation-driven and sporadic colon tumor models. Neutrophil depletion correlated with robust expansion of colon-tumor associated microbiota and tumor-associated B-cells, both of which had important roles in neutrophil-deficient colon tumorigenesis. Together, our data suggest divergent roles for neutrophils in the initiation and maintenance of colon tumors.

The work presented in this thesis also shows an important role for the transcription factor, myc-associated zinc finger (MAZ) in colitis and colon cancer. MAZ is an inflammation induced transcription factor that has a previously identified role as a HIF-2α transcriptional cofactor. We show that MAZ is highly active in human colitis and colon cancer. The present work delineates a critical function for MAZ in the inflammatory progression of colitis and colon cancer through regulation of oncogenic STAT3 signaling. Collectively, these studies shed new light onto the inflammatory progression of colon cancer and propose potential therapeutic targets.

Chapter 1[1]

Introduction

The tumor microenvironment is similar to an inflammatory focus as it consists of a complex milieu of both innate and adaptive immune cells (1). Hypoxia is a characteristic feature of both tumors and inflammatory foci. Increased metabolic demand from rapid cell turnover, immune cell infiltration, and vascular disruption cause local O_2 tension to decline. The decreased O_2 tension of tumors or inflamed tissue promotes activation of HIFs. HIFs are basic helix-loop-helix-per-arnt-sim (bHLH-PAS) containing transcription factors consisting of a heterodimer of an oxygen-sensitive α subunit (HIF-1α, HIF-2α, and HIF-3α) and a constitutively expressed β subunit (ARNT) (2). HIF-1α is ubiquitously expressed whereas HIF-2α and HIF-3α expression is more tissue restricted (3-5). HIF-α subunits are regulated by O_2-dependent post-translational hydroxylation of two specific proline residues by prolyl hydroxylase domain-containing (PHD-containing) enzymes. In normoxia, HIF hydroxylation leads to association with the von-Hippel Lindau (VHL) tumor suppressor/E3 ubiquitin ligase complex, ubiquitin conjugation, and 26s proteasomal degradation. As O_2 homeostasis is disrupted and O_2 concentration declines under inflammatory conditions or in tumors, HIFs are stabilized, dimerize with ARNT, and translocate to the nucleus to regulate transcription by binding to hypoxia response elements (HREs) in promoters of target genes (Fig. 1.1A). In addition to O_2-dependent regulation, inflammation and direct HIF regulation are intimately linked. NF-κB, a master transcription factor in the

[1] This chapter represents published manuscript: Triner D and Shah YM. "Hypoxia-inducible factors: a central link between inflammation and cancer." Journal of Clinical Investigation 2016.

inflammatory response, is a direct transcriptional regulator of *Hif1a*. In response to NF-κB-activating stimuli, such as bacterial lipopolysaccharide (LPS), NF-κB directly increases *Hif1a* mRNA in macrophages (6). LPS-induced NF-κB can also increase HIF-1α protein stability by increasing intracellular ferritin, which sequesters the labile iron pool leading to decrease PHD activity (7). Independent of NF-κB, several cytokines and intermediate metabolites such as succinate can lead to HIF activation (8, 9). In macrophages, IL-4 and IL-13 selectively induce *Hif2a* mRNA (10). Several studies demonstrate that cytokine-induced ROS and specifically mitochondrial ROS directly activate HIF (11-13), and recently it was shown that mitochondrial membrane potential increases mitochondrial ROS to modulate HIF activation (14) (Fig. 1.1B).

HIFs are critical drivers of cancer and regulate a wide variety of cellular processes including metabolism, cell cycle progression, angiogenesis, invasion/metastasis, and chemoresistance (15). HIF-1α and HIF-2α are highly expressed in a wide variety of solid tumors, including those of the colon, breast, lung, and pancreas (16). Although HIF-1α and HIF-2α have several overlapping functions, distinct target genes and functions for HIF-1α and HIF-2α are well-characterized (17) and highlighted in several tumor models (18). In renal cell carcinoma (RCC) that is deficient in *VHL*, which stabilizes HIF-1α and HIF-2α in normoxic conditions, HIF-1α has an anti-tumor role and decreases tumor growth by increasing expression of proapoptotic genes (19). HIF-2α is essential for RCC tumor growth and promotes tumor cell proliferation through augmented c-Myc activity (20). It is well known that hypoxia and HIF signaling play an important role in inflammatory responses and regulating the immune environment (21). However, the intersection of hypoxia, inflammation, and cancer is not well

understood. Hypoxia can modulate the pro-tumor inflammatory response or anti-tumor-immune response in cancer.

1.1 Inflammation and cancer

It is widely recognized that inflammation plays an essential role in tumorigenesis and tumor progression. This paradigm dates back to Virchow, a 19th century pathologist whose observation of leukocytes in tumors led him to hypothesize that tumors originate in sites of chronic inflammation. The discovery of the first oncogene *v-src* in Rous sarcoma virus over a century ago led to decades of in-depth cancer genetic analysis and the discovery of numerous oncogenes and tumor-suppressors; however, studies of tumor-associated inflammation lagged behind. A renaissance over the last twenty years has uncovered a critical role for inflammation in the pathogenesis and progression of nearly all solid tumors and this knowledge has greatly changed the approach to current cancer treatments. The advent of immune-modulating therapies, such as immune checkpoint blockers for the treatment of advanced cancers, underscores the importance of understanding the specific function of inflammatory cells in cancer and is an exciting avenue for the discovery of novel therapeutic targets (22).

Chronic inflammation-associated cancers

As Virchow postulated, several chronic inflammatory diseases predispose the development of cancer. For example, *Helicobacter pylori* (*H. pylori*), a gram-negative bacterium, infects nearly 50% of the world's population and is the major causative agent of chronic gastritis (23). Chronic gastritis associated with *H. pylori* is asymptomatic in the majority of infected individuals; however, this chronic gastritis represents a significant risk factor for the

development of gastric cancer, which occurs in 1-3% of *H. pylori* infections and is the third leading cause of cancer death worldwide (23). Similarly, chronic viral infections predispose to the development of cancer. Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections are key risk factors for development of hepatocellular carcinoma (HCC). For example, HCV infects more than 100 million people worldwide and HCC due to chronic hepatitis induced by HCV infection occurs in approximately 1-5% of infected individuals (24).

One of the best-studied cancers from a genetic and inflammatory perspective is colon cancer. Chronic intestinal inflammation associated with inflammatory bowel disease (IBD), including Crohn's Disease and ulcerative colitis, represents a significant risk factor for the development of colon cancer, termed colitis-associated colon cancers (CAC). More than 1 million Americans suffer from IBD and 12-20% of patients will develop CAC within 30 years of developing IBD (25). The vast majority of colon cancers develop sporadically and proceed through a step-wise process from adenoma to invasive carcinoma (26). This process includes the acquisition of sequential mutations leading to the loss of adenomatous-polyposis coli (*APC*) tumor suppressor, activation of the oncogene *KRAS*, and loss of the *TP53* tumor-suppressor (27). Interestingly, in colon cancers preceded by chronic inflammation due to IBD, the kinetics of the genetic alterations are different, as *TP53* is lost early in disease progression and *APC* mutations are not as frequently observed compared to sporadic colon cancer (28, 29). These data suggest inflammation may induce novel mechanisms to drive cellular proliferation and survival, resulting in tumorigenesis.

Tumor-derived inflammation

All solid tumors elicit an inflammatory response that is critical in the tumor microenvironment. The tumor-derived inflammatory response is essential for the recruitment of immune cells, tumor cell proliferation, survival, and angiogenesis (30). Tumors initiate these responses through several mechanisms including transcriptional regulation of inflammatory genes by proto-oncogenes (31, 32). It was recently demonstrated that defects in epithelial permeability elicited an inflammatory response through a microbiota-mediated mechanism in colon cancer (33).

Pro- and anti-tumor immune responses

In tumors, there is a mix of both innate and adaptive immune cells with anti- and pro-tumor functions (1). Precise identification of immune cells found in tumor biopsies can serve as prognostic markers for clinical outcomes. Adaptive immune responses have been correlated with positive prognosis. Several distinct T-cell subsets reside within the tumor microenvironment, including cytotoxic anti-tumor $CD8^+$ T-cells and subsets of $CD4^+$ T-cells with both pro- and anti-tumor functions. In colon cancer, decreased expression of CD8 as well as cytotoxic T-cell markers GZMB and CD45RO all correlated with disease recurrence (34). Furthermore, patients with high intratumoral expression of T-cell marker CD3 had improved disease-free survival compared to patients with low levels of CD3 (34). Recent meta-analysis studies have mapped the prognostic impact of twenty-two immune cell types on recurrence-free survival by analyzing expression signatures across more than 5,000 tumor samples (35). In general, these studies found a positive correlation of T-cells with recurrence-free survival and a negative correlation of several myeloid cell types such as neutrophils and macrophages (35). In ovarian cancer, increased $CD8^+/CD4^+$ T-cell ratio was associated with improved survival, whereas increased

$CD4^+$ T-cells portend poor survival (36). Expression profiles associated with anti-tumor T-cell cytokines (IFNγ) and effector molecules (granulysin and granzyme B) are also independent prognosticators of decreased early metastasis in colon cancer (37). These associations, however, do not highlight the complexity of the tumor immune environment. Many specific cell types have been associated with better or worse prognosis and the plasticity of immune cells can confer both pro- and anti-tumor functions, which we will review in depth.

1.2 Hypoxia and epithelial-elicited tumor inflammatory response

Historically it was believed that the major function of epithelial surfaces, such as the skin or the intestinal epithelium, was to serve as a physical barrier separating the external environment from the underlying immune cells. It is now evident that epithelial surfaces play an active role in innate immunity and shape the underlying immune environment and inflammatory response (38, 39). This is also the case in epithelial-derived cancers. Colon tumor cell secretion of the C-C family chemokine, CCL2, was essential for tumorigenesis through recruitment and activation of pro-tumorigenic myeloid cells (40). PTX3 is a tumor suppressor that activates Complement-mediated anti-tumor immunity and is epigenetically silenced due to methylation in colon tumors (41). Recent studies in colon cancer have further highlighted the contribution of epithelial and stromal gene expression in patient-derived colon cancer xenograft models (42). In these studies the epithelial and stromal gene expression was readily delineated by analyzing tumor expression of human or mouse transcripts. Analysis of genes with a greater than 50% difference in expression between the epithelium and the stroma showed that several cytokines and chemokines are directly expressed by the tumor epithelium (42). These results demonstrate

that tumor epithelial cells play an active role in regulating the inflammatory response, which may impact tumorigenesis.

Several lines of evidence suggest that intra-tumoral hypoxia and HIFs plays an essential role in sculpting the tumor immune environment in several epithelial-derived tumors. For example, in a *Kras*-driven mouse model of non-small cell lung cancer, loss of HIF-2α increased tumor burden and tumor cell proliferation; however, loss of HIF-1α had no effect on tumorigenesis (43). Tumors lacking HIF-2α also displayed increased infiltration of CD45+ immune cells, specifically Gr-1+ granulocytic cells, suggesting that HIF-2α repression of granulocytic cell infiltration is in part responsible for its anti-tumor effects (43). Hypoxia and HIF stabilization is also a key feature of pancreatic ductal adenocarcinoma (PDAC) (16). PDAC development occurs in a step-wise manner and is preceded by precursor lesions termed pancreatic intraepithelial neoplasias (PanINs) (44). In contrast to studies showing decreased PanIN progression following pancreatic-specific disruption of HIF-2α (45), deletion of HIF-1α in a murine model of *Kras*-initiated PDAC significantly enhanced PanIN progression and increased tumor cell proliferation (46). Loss of HIF-1α correlated with increased pancreata B cell infiltration and antibody-mediated depletion of B cells reversed the increased PanIN progression (46). Hypoxic inflammation is also important in colon tumorigenesis and both HIF-1α and HIF-2α are overexpressed in colon tumors (16). Using the $Apc^{min/+}$ model of intestinal tumorigenesis, intestine-specific disruption of the tumor suppressor *Vhl* significantly increased colon tumorigenesis and adenoma-to-carcinoma progression (47). The increase in colon tumorigenesis was HIF-2α-dependent, as double disruption of *Vhl* as well as *Hif2a* ameliorated the effect (47). HIF-2α-mediated inflammatory responses are essential in colon tumorigenesis. Epithelial HIF-2α regulates expression of the pro-inflammatory meditator *Tnfa* (48). TNFα has

a crucial role in the progression of cancer and inhibiting TNFα decreases growth in several mouse models of cancer (49, 50). HIF-2α-induced inflammation was found to be critical to tumor progression, as treatment with the anti-inflammatory drug nimesulide significantly reduced HIF-2α-driven colon tumorigenesis. (48). These previous studies of HIF-2α in colon cancer have been examined in sporadic colon tumor models. Although HIF-2α has a prominent role in acute colitis, its function in colitis-associated colon cancer has yet to be investigated.

Intestine-specific overexpression of HIF-1α does not enhance tumorigenesis in colon cancer (51). The precise factors which govern the divergent functions of HIF-1α and HIF-2α in colon cancer are currently not well understood. It has been proposed that HIF target gene specificity are dependent upon interactions with cofactors. Interestingly, we have identified a critical transcription factor, Myc-associated zinc finger (MAZ), as an essential cofactor interacting with HIF-2α and driving HIF-2α-dependent transcription of inflammatory target genes *Tnf*α in colitis (48). The work in this thesis expands upon these previous studies to suggest that MAZ is essential in the regulation of HIF-2α-dependent *Cxcl1* expression in inflammation-induced colon cancer (52). These studies suggest that tumor HIFs have the potential to modulate tumor-associated inflammation to regulate tumor growth and progression.

1.4 Hypoxia and immune cell recruitment

Tumors are highly infiltrated by cells of both the innate and adaptive immune systems. This infiltration is in part mediated by tumor-derived secretion of a host of cytokines and chemokines (Fig. 1.2A). HIF-induced secretion of tumor-derived factors can modulate immune cell recruitment to aid in tumor growth, which we will review in detail by immune cell subtype (Table 1.1).

T-cells

$CD4^+$ T-cells can be differentiated into several different helper T (Th) cell types. Th1 and Th2 $CD4^+$ T-cells are the classical types of Th cells that play an important role in the inflammatory response to infection and cancer (53). Th1 and Th2 cells promote anti-tumor immunity by cytotoxic lymphocyte activation and humoral-mediated immune responses, respectively (53). In addition to the classic Th1 and Th2 T-cell effector populations, other subsets of T-cells have been found to have an important role in cancer. For example, Th17 cells are a recently identified subset of IL-17-expressing $CD4^+$ T-cells that are highly prevalent in tumors and have a controversial role in tumor progression. IL-6 and TGF-β collaboratively promote Th17 differentiation (54) and Th17 differentiation by these cytokines is dependent on HIF-1α (55). Th17 cells have both anti- and pro-tumor functions. In a melanoma model, Th17 cells showed more potent tumor eradication than Th1 cells (56). However, in other models, Th17 cells promote tumor growth through angiogenic and immune suppressive effector functions (57, 58).

Immunosuppressive regulatory T-cells (Tregs) are frequently increased in cancers (59). Tregs are $CD4^+$ and defined by expression of forkhead box transcription factor 3 (FoxP3). Tregs have been largely shown to promote tumorigenesis through suppression of anti-tumor $CD4^+$ and $CD8^+$ T-cell-mediated immune responses by secreting immunosuppressive molecules such as IL-10 and TGF-β; removal of Tregs improves anti-tumor immunity (60-62). Primary tumor hypoxia regulated recruitment of $CCR10^+CD4^+FoxP3^+$ immunosuppressive Tregs in a model of ovarian cancer, which increased immune tolerance and angiogenesis through VEGF secretion (63). This effect was regulated by hypoxia-induced excretion of the chemokine CCL28, which was dependent upon HIF-1α and to a lesser extent HIF-2α (63).

B-cells

The precise role for B-cells in tumor progression is controversial and they may function to enhance or inhibit anti-tumor immune responses. B-cells can be directly cytotoxic to tumors and promote anti-tumor T-cell responses (64). However, B-cells have also been shown to inhibit the function of anti-tumor T-cells, as depletion of B-cells increases anti-tumor immunity, suggesting context specific roles for B-cells in anti-tumor immunity (65). In pancreatic cancer, as detailed above, HIF-1α in epithelial cells decreased tumor growth by attenuating expression of B-cell-recruiting chemokines, resulting in decreased B-cell infiltration into the tumors (46).

Myeloid Derived Suppressor Cells

Hypoxia also regulates tumor recruitment of immune suppressive myeloid cells. Myeloid cells include monocyte/macrophages, neutrophils, eosinophils, basophils, mast cells, and dendritic cells. During infection or in cancers, immature myeloid cells closely related to neutrophils and monocytes can be detected in circulation. These cells, termed myeloid derived suppressor cells (MDSCs), dampen immune responses to infection and are an important suppressor of anti-tumor immunity. (66). MDSCs promote tumor growth through suppression of both NK- and T-cell-mediated anti-tumor immune responses (66). This is through multiple mechanisms including arginine metabolism via increased expression of arginase 1 (ARG1), which converts the available L-arginine pool to urea and L-ornithine. Additionally, MDSCs mediate nitration of tyrosine residues in the T-cell receptor (TCR) and CD8, which decreases the function of these proteins (67-69). The function of MDSCs is well described in the azoxymethane (AOM) and dextran sulfate sodium (DSS) model of CAC. Mice with CXCR2-deficient bone marrow have significantly reduced MDSC homing to colon tumors and decreased colon tumorigenesis (70). Tumor-specific hypoxia increased recruitment of MDSCs

(CD11b+/Ly6C-/Ly6G+) to the lung premetastatic niche to promote metastasis. This effect was partly regulated through inhibition of NK cell-mediated cytoxicity (71). In head and neck squamous cell carcinoma, hypoxia increased recruitment of MDSCs in a process that was dependent upon both HIF-1α and HIF-2α induction of the potent chemoattractant migration inhibitory factor (MIF) (72).

Tumor–associated macrophages

The best-characterized immune cell type in the tumor microenvironment is tumor-associated macrophages (TAMs). TAMs are highly prevalent in the tumor microenvironment and can be polarized into anti-tumor M1 or pro-tumor/immunosuppressive M2 phenotypes (73). M2 TAMs regulate tumor angiogenesis and are an important source of VEGF (74). TAMs directly promote tumor growth via direct secretion of cytokines such as IL-6, which induces tumor-cell STAT3 signaling to promote growth and stem cell expansion (75), and have been directly linked to tumor invasion and metastasis (76, 77). TAMs inhibit anti-tumor immune responses through secretion of immune suppressive cytokines such as IL-10 and TGF-β (78). ARG1 expression in human monocytes and macrophages is controversial and has not been definitively shown in tumors (79). In rodents, however, there is clear evidence showing TAM expression of immunesuppressive ARG1 (80). TAMs reside in largely avascular and hypoxic regions of tumors (81). Tumor hypoxia is a potent driver of TAM recruitment, and induces the secretion of chemoattracants such as oncostatin M and eotaxin (82). Moreover, tumor HIF-1α directly induces recruitment of monocyte/macrophage cells through regulation of stromal-derived factor 1α (SDF1α/CXCL12) expression (83). Expression of the SDF1α/CXCL12 receptor, CXCR4, is also regulated by hypoxia in TAMs (84). TAM polarization into a pro-tumorigenic M2 phenotype can be directly regulated by tumor hypoxia. Tumor-derived lactic acid is induced by

hypoxia in a HIF-1α-dependent manner and promotes M2 macrophage polarization and regulates expression of M2 TAM markers ARG1 and VEGF (85). Importantly, blockade of TAM recruitment to hypoxic tumor areas and trapping TAMs in normoxic tumor microenvironments through loss of the Semaphorin 3A receptor Nrp1 decreased tumor growth through blunted angiogenesis and increased anti-tumor T-cell responses showing that hypoxia-induced localization of macrophages causes a switch from anti- to pro-tumor phenotypes. (86).

Neutrophils

A close relationship of neutrophils (polymorphonuclear neutrophils [PMNs]) and tissue hypoxia has been recently shown. The reactive oxygen burst that is critical for neutrophil function can affect local tissue oxygenation (87). Although PMNs are highly prevalent in most solid tumor types, the specific role for PMNs is not completely understood. PMNs are highly plastic and can be differentiated into anti-tumor (N1) and pro-tumor (N2) phenotypes (88). Pro-tumor PMNs regulate tumor growth through secretion of cytokines, ROS production, generation of matrix degrading enzymes, and angiogenesis (89). PMNs have also been shown to play an essential role in promoting metastasis in a murine breast cancer model (90). The specific role for PMNs in colon cancer has largely suggested a pro-tumorigenic role. However, PMNs expressing the hepatocyte growth factor (HGF) receptor, c-MET, were found to be anti-tumorigenic in mouse models of colon cancer (91). It is not currently well understood the contribution of PMNs to the initiation of most cancers, particularly colon cancer. Interestingly, PMNs have recently been demonstrated to reside in hypoxic tumor regions in epithelial uterine tumors and this effect was regulated by hypoxic tumor cell expression of PMN chemoattractants such as CXCL5 (92).

1.5 Hypoxic regulation of tumor immune cell function.

Hypoxia is a hallmark of tumors and most infiltrating immune cells function in the hypoxic tumor environment. Immune cell expression of HIF-1α and HIF-2α regulates effector function (Table 1.1) (93). Tumor hypoxia has an essential role in regulating tumor inflammatory cell functions in addition to regulating immune cell recruitment (Fig. 1.2B). TAMs express both HIF-1α and HIF-2α (16, 94). TAM HIF-2α expression is highly correlated with tumor vascularity and tumor grade (95). Macrophage HIF-2α is critical in regulating macrophage inflammatory cytokine expression following LPS and IFNγ challenge (96). Importantly, macrophage loss of HIF-2α impaired TAM infiltration of tumors and decreased tumor burden in murine models of HCC and CAC (96). TAM HIF-1α has also been shown to play an important role in TAM-mediated suppression of tumor-associated T-cells (97). TAMs cultured under hypoxic conditions exhibited increased suppression of T-cells in a HIF-1α-dependent manner without affecting TAM recruitment or polarization (97). Furthermore, tumor hypoxia regulates TAM expression of VEGF, suggesting a role for hypoxic TAMs in angiogenesis (98). Hypoxia and HIF-1α have an important role in regulating tumor-associated MDSC function. MDSCs cultured with the hypoxia-mimetic desferroxamine (DFO) robustly suppress T-cell proliferation and loss of HIF-1α decreases MDSC-mediated T-cell suppression (99). Hypoxia induces MDSC expression of miR-210 in a HIF-1α-dependent manner and miR-210 promotes MDSC-mediated T-cell suppression by increasing ARG1 expression and NO synthesis (100). Notably, HIF-1α increases mRNA expression of the immune checkpoint receptor programmed death ligand-1 (*Pdl1*) in MDSCs, which is essential for their T-cell immunosuppressive ability (101). MDSCs also have the capacity to differentiate into TAMs and HIF-1α is a critical mediator of this plasticity (99).

The role of PMN HIF-1α and HIF-2α in the tumor microenvironment are not well understood. However, it has been shown that PMN HIF-1α is an essential PMN survival factor through an NF-κB-dependent signaling loop (102). Constitutive HIF-2α activation increases PMN inflammatory responses and loss of HIF-2α increases susceptibility to apoptosis (103). Dendritic cells (DCs) are antigen-presenting cells that are central regulators of the adaptive immune response. DCs can sample tumor antigens and activate $CD8^+$ T-cell responses and are currently in clinical trials as a vaccination strategy to prime anti-tumor immune responses (104). DCs function is directly regulated by hypoxia and HIFs. Activated dendritic cells increase expression of costimulatory molecules and T-cell activation in response to TLR stimulation when cultured in hypoxia in a HIF-1α-dependent manner (105). Alternatively, it has been suggested that hypoxic treated immature DCs have impaired antigen uptake and T-cell activation (106, 107). Moreover, hypoxia inhibits DC maturation and T-cell activation but simultaneously increases DC inflammatory cytokine secretion (108). Although it is unclear, this dichotomy may be due to maturation state of DCs. Further work is needed to address more precisely DC HIF-1α and HIF-2α in the progression of cancer and anti-tumor immune responses.

Tumor hypoxia increases T-cell expression of FoxP3 through tumor secretion of TGF-β *in vitro*, suggesting hypoxic tumor cells can induce Treg differentiation (109). Tregs are more efficiently activated systemically than within the tumor microenvironment (110) and tumor secretion of cytokines such as TGF-β may be a critical source of systemic Treg activation and infiltration into tumors. $CD4^+$ T-cell HIF-1α directly targets the *Foxp3* promoter and increases immunosuppressive Treg cell production and function (111, 112). Loss of HIF-1α in Tregs decreased their immune-suppressive function (111). The specific role for hypoxia signaling in anti-tumor $CD4^+$ and $CD8^+$ T-cell responses is not completely clear. T-cell HIF-1α represses T-

cell inflammatory responses, as depletion of HIF-1α significantly enhanced IFNγ and IL-2 production (113). However, other studies have shown that hypoxia signaling has the capacity to increase $CD8^+$ T-cell function, as *Vhl* depletion in $CD8^+$ T-cells increased T-cell effector responses and decreased tumor growth in a model of melanoma (114). Additionally, HIF-1α regulates expression of T-cell CD137, which augments anti-tumor immunity upon antibody-mediated activation (115). More work is needed to further understand the specific roles of hypoxia and HIFs in anti-tumoral T-cell responses. In addition, hypoxia has cell intrinsic roles in CTLs and NK, and NKT cells, which have not been completely assessed in tumor biology.

1.6 Hypoxia, stromal cells, & inflammation

In addition to immune cells, the tumor microenvironment is also made up of vascular endothelial cells, fibroblasts, and pericytes, collectively known as the stroma. In tumor endothelial cells, depletion of HIF-1α reduced tumor metastasis, whereas depletion of endothelial HIF-2α increased tumor metastasis (116). This dichotomy was due to differential regulation of nitric oxide (NO) homeostasis by HIF-1α and HIF-2α. HIF-1α regulates expression of inducible nitric oxide synthase (iNOS), which catalyzes the conversion of l-arginine to NO. iNOS was also an essential regulator of VEGF expression to promote increased tumor vascularity (116). Myeloid cell and mesenchymal stem cell NO production has been implicated in decreasing T-cell cytotoxicity; thus, it is possible that endothelial cell NO has a similar function (117-119). On the other hand, HIF-2α regulates expression of ARG1, which metabolizes l-arginine. ARG1 expression in immune cells is a potent suppressor of anti-tumor T-cells. Future studies should determine if endothelial ARG1 also has an immune-suppressive role in the tumor microenvironment.

Fibroblasts are frequently recruited to tumors and make up a variable proportion of the tumor mass (120). Cancer-associated fibroblasts (CAFs) have a largely pro-tumor role and promote angiogenesis through VEGF secretion and increase invasion/metastasis through secretion of extracellular matrix-degrading matrix metalloproteinase enzymes (120-122). Conditional loss of CAF HIF-1α by *Fsp1*-Cre expression decreased TAMs in mouse mammary tumors suggesting that CAF HIF-1α is important for TAMs tumor infiltration (123). These results demonstrate that stromal cell HIF signaling can also modulate the microenvironment of tumors. However, the specific role for HIFs in the tumor endothelial cells and CAFs and the significance of this relationship to tumor inflammatory responses has not been fully elucidated.

1.7 Hypoxia and intratumor heterogeneity

Tumor growth is an evolving process that leads to an accumulation of genetic alterations. This evolutionary process in tumors leads to substantial spatial variation in which genetically distinct subclonal populations of cancer cells exist (124). Although not completely clear at present, a few studies have demonstrated that intratumor heterogeneity is an independent risk factor for poor survival in several tumor types (125). Mechanisms which drive regional heterogeneity in tumors are poorly described. Hypoxia-induced immune cell recruitment and modulation could be an important process in driving regional selective pressures in tumors. More work is needed to elucidate mechanisms by which hypoxia and immune cells establish microenvironments leading to intratumor heterogeneity. Although, through enhanced imaging it is clear that distinct subpopulation of hypoxic cells are observed in tumors (126-128). Through histopathologcal and molecular analysis it is clear that distinct tumor regions have different inflammatory infiltrates and this can be modulated by intratumoral hypoxia (129, 130).

1.8 Perspectives

Hypoxia is an important microenvironmental feature in solid tumors and is essential for tumor growth. HIF-1α and HIF-2α have been extensively studied in regulating tumor glucose metabolism, angiogenesis, cell survival, proliferation and migration. Research in the past two decades has established an essential role for pro-tumor inflammatory response or anti-tumor-immune response in the growth of most solid tumors and with this increased focus, Hanahan and Weinberg's "hallmarks of cancer" were recently updated to include tumor inflammation and immune evasion as a major enabling factor in cancer progression (131). It is becoming clear that hypoxia is central to regulating the inflammatory response in tumors. The work presented in this thesis clearly delineates a novel role for epithelial HIF-2α as a regulator of the colon tumor inflammatory microenvironment in colon cancer through recruitment of PMNs (Chapter 2). Our work also challenges the role for PMNs in colon cancer and suggest that PMNs while important for promoting growth and progression of established tumors, restrict the earliest stages of tumorigenesis (Chapter 3). Lastly, we have identified Myc-associated zinc finger (MAZ), a previously described HIF-2α interacting protein, as having a novel role in the inflammatory progression in colitis and colon cancer and regulation of oncogenic signaling pathways (Chapter 4). Future studies should be directed towards a better understanding of the precise molecular mechanisms by which hypoxia and PMNs alters the balance between growth-promoting inflammation and the anti-tumor immune response, which may lead to better use of existing drugs that alter the HIF response as stand-alone therapies and improve the efficacy of standard chemotherapeutics.

References

1. Gajewski TF, Schreiber H, and Fu Y-X. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol.* 2013;14(10):1014-22.
2. Semenza GL. Hypoxia-Inducible Factors in Physiology and Medicine. *Cell.* 2012;148(3):399-408.
3. Wiesener MS, Jürgensen JS, Rosenberger C, Scholze C, Hörstrup JH, Warnecke C, et al. Widespread, hypoxia-inducible expression of HIF-2α in distinct cell populations of different organs. *The FASEB Journal.* 2002.
4. Wiener CM, Booth G, and Semenza GL. In VivoExpression of mRNAs Encoding Hypoxia-Inducible Factor 1. *Biochemical and Biophysical Research Communications.* 1996;225(2):485-8.
5. Gu YZ, Moran SM, Hogenesch JB, Wartman L, and Bradfield CA. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr.* 1998;7(3):205-13.
6. Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, et al. NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. *Nature.* 2008;453(7196):807-11.
7. Siegert I, Schödel J, Nairz M, Schatz V, Dettmer K, Dick C, et al. Ferritin-Mediated Iron Sequestration Stabilizes Hypoxia-Inducible Factor-1α upon LPS Activation in the Presence of Ample Oxygen. *Cell Reports.* 2015;13(10):2048-55.
8. Haddad JJ, and Harb HL. Cytokines and the regulation of hypoxia-inducible factor (HIF)-1α. *International Immunopharmacology.* 2005;5(3):461-83.
9. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is a danger signal that induces IL-1β via HIF-1α. *Nature.* 2013;496(7444):238-42.
10. Takeda N, O'Dea EL, Doedens A, Kim J-w, Weidemann A, Stockmann C, et al. Differential activation and antagonistic function of HIF-α isoforms in macrophages are essential for NO homeostasis. *Genes & Development.* 2010;24(5):491-501.
11. Hellwig-Bürgel T, Rutkowski K, Metzen E, Fandrey J, and Jelkmann W. Interleukin-1β and Tumor Necrosis Factor- Stimulate DNA Binding of Hypoxia-Inducible Factor-1. *Blood.* 1999;94(5):1561-7.
12. Haddad JJ, and Land SC. A non-hypoxic, ROS-sensitive pathway mediates TNF-α-dependent regulation of HIF-1α. *FEBS Letters.* 2001;505(2):269-74.
13. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, and Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences.* 1998;95(20):11715-20.
14. Martínez-Reyes I, Diebold Lauren P, Kong H, Schieber M, Huang H, Hensley Christopher T, et al. TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions. *Molecular Cell.* 2016;61(2):199-209.
15. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends in pharmacological sciences.* 2012;33(4):207-14.
16. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, et al. The Expression and Distribution of the Hypoxia-Inducible Factors HIF-1α and HIF-2α in Normal Human Tissues, Cancers, and Tumor-Associated Macrophages. *The American Journal of Pathology.* 2000;157(2):411-21.

17. Hu C-J, Wang L-Y, Chodosh LA, Keith B, and Simon MC. Differential Roles of Hypoxia-Inducible Factor 1α (HIF-1α) and HIF-2α in Hypoxic Gene Regulation. *Molecular and Cellular Biology.* 2003;23(24):9361-74.
18. Keith B, Johnson RS, and Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumor growth and progression. *Nature reviews Cancer.* 2011;12(1):9-22.
19. Raval RR, Lau KW, Tran MGB, Sowter HM, Mandriota SJ, Li J-L, et al. Contrasting Properties of Hypoxia-Inducible Factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-Associated Renal Cell Carcinoma. *Molecular and Cellular Biology.* 2005;25(13):5675-86.
20. Gordan JD, Bertovrt JA, Hu C-J, Diehl JA, and Simon MC. HIF-2α promotes hypoxic cell proliferation by enhancing c-Myc transcriptional activity. *Cancer cell.* 2007;11(4):335-47.
21. Palazon A, Goldrath Ananda W, Nizet V, and Johnson Randall S. HIF Transcription Factors, Inflammation, and Immunity. *Immunity.*41(4):518-28.
22. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* 2012;12(4):252-64.
23. Ahn HJ, and Lee DS. Helicobacter pylori in gastric carcinogenesis. *World Journal of Gastrointestinal Oncology.* 2015;7(12):455-65.
24. Mitra AK. Hepatitis C-related Hepatocellular Carcinoma: Prevalence Around the World, Factors Interacting, and Role of Genotypes. *Epidemiologic Reviews.* 1999;21(2):180-7.
25. Munkholm P. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics.* 2003;18:1-5.
26. Fearon ER. Molecular Genetics of Colorectal Cancer. *Annual Review of Pathology: Mechanisms of Disease.* 2011;6(1):479-507.
27. Fearon ER, and Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990;61(5):759-67.
28. Kim ER, and Chang DK. Colorectal cancer in inflammatory bowel disease: The risk, pathogenesis, prevention and diagnosis. *World Journal of Gastroenterology : WJG.* 2014;20(29):9872-81.
29. Robles AI, Traverso G, Zhang M, Roberts NJ, Khan MA, Joseph C, et al. Whole-exome Sequencing analyses of Inflammatory Bowel Disease-associated Colorectal Cancers. *Gastroenterology.*
30. Grivennikov SI, Greten FR, and Karin M. Immunity, Inflammation, and Cancer. *Cell.* 2010;140(6):883-99.
31. Schwitalla S, Fingerle Alexander A, Cammareri P, Nebelsiek T, Göktuna Serkan I, Ziegler Paul K, et al. Intestinal Tumorigenesis Initiated by Dedifferentiation and Acquisition of Stem-Cell-like Properties. *Cell.* 2013;152(1–2):25-38.
32. Sparmann A, and Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell.* 2004;6(5):447-58.
33. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature.* 2012;491(7423):254-8.
34. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science.* 2006;313(5795):1960-4.

35. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med.* 2015;21(8):938-45.
36. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8(+) tumor-infiltrating lymphocytes and a high CD8(+)/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(51):18538-43.
37. Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. *New England Journal of Medicine.* 2005;353(25):2654-66.
38. Shaykhiev R, and Bals R. Interactions between epithelial cells and leukocytes in immunity and tissue homeostasis. *Journal of Leukocyte Biology.* 2007;82(1):1-15.
39. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol.* 2008;8(6):411-20.
40. Chun E, Lavoie S, Michaud M, Gallini CA, Kim J, Soucy G, et al. CCL2 Promotes Colorectal Carcinogenesis by Enhancing Polymorphonuclear Myeloid-Derived Suppressor Cell Population and Function. *Cell reports.* 2015;12(2):244-57.
41. Bonavita E, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, et al. PTX3 Is an Extrinsic Oncosuppressor Regulating Complement-Dependent Inflammation in Cancer. *Cell.* 2015;160(4):700-14.
42. Isella C, Terrasi A, Bellomo SE, Petti C, Galatola G, Muratore A, et al. Stromal contribution to the colorectal cancer transcriptome. *Nat Genet.* 2015;47(4):312-9.
43. Mazumdar J, Hickey MM, Pant DK, Durham AC, Sweet-Cordero A, Vachani A, et al. HIF-2α deletion promotes Kras-driven lung tumor development. *Proceedings of the National Academy of Sciences.* 2010;107(32):14182-7.
44. Vincent A, Herman J, Schulick R, Hruban RH, and Goggins M. Pancreatic cancer. *The Lancet.*378(9791):607-20.
45. Criscimanna A, Duan L-J, Rhodes JA, Fendrich V, Wickline E, Hartman DJ, et al. PanIN-specific regulation of Wnt signaling by HIF2α during early pancreatic tumorigenesis. *Cancer research.* 2013;73(15):4781-90.
46. Lee KE, Spata M, Bayne LJ, Buza EL, Durham AC, Allman D, et al. Hif1α deletion reveals pro-neoplastic function of B cells in pancreatic neoplasia. *Cancer Discovery.* 2015.
47. Xue X, Taylor M, Anderson E, Hao C, Qu A, Greenson JK, et al. Hypoxia-inducible factor-2α activation promotes colorectal cancer progression by dysregulating iron homeostasis. *Cancer Research.* 2012;72(9):2285-93.
48. Xue X, Ramakrishnan S, Anderson E, Taylor M, Zimmermann EM, Spence JR, et al. Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice. *Gastroenterology.* 2013;145(4):831-41.
49. Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, et al. Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. *The Journal of Clinical Investigation.*118(2):560-70.
50. Zhao X, Rong L, Zhao X, Li X, Liu X, Deng J, et al. TNF signaling drives myeloid-derived suppressor cell accumulation. *The Journal of Clinical Investigation.*122(11):4094-104.

51. Xue X, Ramakrishnan SK, and Shah YM. Activation of HIF-1α does not increase intestinal tumorigenesis. *American Journal of Physiology - Gastrointestinal and Liver Physiology.* 2014;307(2):G187-G95.
52. Triner D, Xue X, Schwartz AJ, Jung I, Colacino JA, and Shah YM. Epithelial Hypoxia-Inducible Factor 2α Facilitates the Progression of Colon Tumors through Recruiting Neutrophils. *Molecular and Cellular Biology.* 2017;37(5):e00481-16.
53. Knutson KL, and Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. *Cancer Immunology, Immunotherapy.* 2005;54(8):721-8.
54. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 2006;441(7090):235-8.
55. Dang Eric V, Barbi J, Yang H-Y, Jinasena D, Yu H, Zheng Y, et al. Control of TH17/Treg Balance by Hypoxia-Inducible Factor 1. *Cell.* 2011;146(5):772-84.
56. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood.* 2008;112(2):362-73.
57. Wang L, Yi T, Zhang W, Pardoll DM, and Yu H. IL-17 enhances tumor development in carcinogen-induced skin cancer. *Cancer research.* 2010;70(24):10112-20.
58. Chang SH, Mirabolfathinejad SG, Katta H, Cumpian AM, Gong L, Caetano MS, et al. T helper 17 cells play a critical pathogenic role in lung cancer. *Proceedings of the National Academy of Sciences of the United States of America.* 2014;111(15):5664-9.
59. Adeegbe DO, and Nishikawa H. Natural and induced T regulatory cells in cancer. *Frontiers in Immunology.* 2013;4.
60. Li X, Kostareli E, Suffner J, Garbi N, and Hämmerling GJ. Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. *European Journal of Immunology.* 2010;40(12):3325-35.
61. Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MWL, Ngiow SF, et al. Selective Depletion of Foxp3+ Regulatory T Cells Improves Effective Therapeutic Vaccination against Established Melanoma. *Cancer Research.* 2010;70(20):7788-99.
62. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, and Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *International Immunology.* 2009;21(10):1105-11.
63. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang L-P, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells. *Nature.* 2011;475(7355):226-30.
64. Li Q, Lao X, Pan Q, Ning N, Yet J, Xu Y, et al. Adoptive Transfer of Tumor Reactive B Cells Confers Host T-Cell Immunity and Tumor Regression. *Clinical Cancer Research.* 2011;17(15):4987-95.
65. Kim S, Fridlender ZG, Dunn R, Kehry MR, Kapoor V, Blouin A, et al. B-cell Depletion Using an Anti-CD20 Antibody Augments Antitumor Immune Responses and Immunotherapy in Nonhematopoetic Murine Tumor Models. *Journal of Immunotherapy.* 2008;31(5):446-57.
66. Gabrilovich DI, and Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009;9(3):162-74.
67. Lu T, Ramakrishnan R, Altiok S, Youn J-I, Cheng P, Celis E, et al. Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. *The Journal of Clinical Investigation.*121(10):4015-29.

68. Rodríguez PC, and Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunological reviews.* 2008;222:180-91.
69. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med.* 2007;13(7):828-35.
70. Katoh H, Wang D, Daikoku T, Sun H, Dey SK, and DuBois RN. CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis. *Cancer cell.* 2013;24(5):631-44.
71. Sceneay J, Chow MT, Chen A, Halse HM, Wong CSF, Andrews DM, et al. Primary Tumor Hypoxia Recruits CD11b+/Ly6Cmed/Ly6G+ Immune Suppressor Cells and Compromises NK Cell Cytotoxicity in the Premetastatic Niche. *Cancer Research.* 2012;72(16):3906-11.
72. Zhu G, Tang Y, Geng N, Zheng M, Jiang J, Li L, et al. HIF-α/MIF and NF-κB/IL-6 Axes Contribute to the Recruitment of CD11b+Gr-1+ Myeloid Cells in Hypoxic Microenvironment of HNSCC. *Neoplasia (New York, NY).* 2014;16(2):168-79.
73. Sica A, and Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *The Journal of Clinical Investigation.*122(3):787-95.
74. Lin EY, Li J-F, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages Regulate the Angiogenic Switch in a Mouse Model of Breast Cancer. *Cancer Research.* 2006;66(23):11238-46.
75. Wan S, Zhao E, Kryczek I, Vatan L, Sadovskaya A, Ludema G, et al. Tumor-Associated Macrophages Produce Interleukin 6 and Signal via STAT3 to Promote Expansion of Human Hepatocellular Carcinoma Stem Cells. *Gastroenterology.* 2014;147(6):1393-404.
76. Hagemann T, Wilson J, Kulbe H, Li NF, Leinster DA, Charles K, et al. Macrophages Induce Invasiveness of Epithelial Cancer Cells Via NF-κB and JNK. *The Journal of Immunology.* 2005;175(2):1197-205.
77. Gocheva V, Wang H-W, Gadea BB, Shree T, Hunter KE, Garfall AL, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes & Development.* 2010;24(3):241-55.
78. Hao N-B, Lü M-H, Fan Y-H, Cao Y-L, Zhang Z-R, and Yang S-M. Macrophages in Tumor Microenvironments and the Progression of Tumors. *Clinical and Developmental Immunology.* 2012;2012:11.
79. Thomas AC, and Mattila JT. "Of Mice and Men": Arginine Metabolism in Macrophages. *Frontiers in Immunology.* 2014;5:479.
80. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, et al. Arginase I Production in the Tumor Microenvironment by Mature Myeloid Cells Inhibits T-Cell Receptor Expression and Antigen-Specific T-Cell Responses. *Cancer Research.* 2004;64(16):5839-49.
81. Lewis C, and Murdoch C. Macrophage Responses to Hypoxia : Implications for Tumor Progression and Anti-Cancer Therapies. *The American Journal of Pathology.* 2005;167(3):627-35.
82. Tripathi C, Tewari BN, Kanchan RK, Baghel KS, Nautiyal N, Shrivastava R, et al. Macrophages are recruited to hypoxic tumor areas and acquire a Pro-Angiogenic M2-Polarized phenotype via hypoxic cancer cell derived cytokines Oncostatin M and Eotaxin. *Oncotarget.* 2014;5(14):5350-68.

83. Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegué E, et al. HIF1α Induces the Recruitment of Bone Marrow-Derived Vascular Modulatory Cells to Regulate Tumor Angiogenesis and Invasion. *Cancer cell.* 2008;13(3):206-20.
84. Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, et al. Regulation of the Chemokine Receptor CXCR4 by Hypoxia. *The Journal of Experimental Medicine.* 2003;198(9):1391-402.
85. Colegio OR, Chu N-Q, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature.* 2014;513(7519):559-63.
86. Casazza A, Laoui D, Wenes M, Rizzolio S, Bassani N, Mambretti M, et al. Impeding Macrophage Entry into Hypoxic Tumor Areas by Sema3A/Nrp1 Signaling Blockade Inhibits Angiogenesis and Restores Antitumor Immunity. *Cancer Cell.*24(6):695-709.
87. Campbell EL, Bruyninckx WJ, Kelly CJ, Glover LE, McNamee EN, Bowers BE, et al. Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. *Immunity.* 2014;40(1):66-77.
88. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of Tumor-Associated Neutrophil (TAN) Phenotype by TGF-β: "N1" versus "N2" TAN. *Cancer cell.* 2009;16(3):183-94.
89. Galdiero MR, Bonavita E, Barajon I, Garlanda C, Mantovani A, and Jaillon S. Tumor associated macrophages and neutrophils in cancer. *Immunobiology.* 2013;218(11):1402-10.
90. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau C-S, et al. IL17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature.* 2015;522(7556):345-8.
91. Finisguerra V, Di Conza G, Di Matteo M, Serneels J, Costa S, Thompson AAR, et al. MET is required for the recruitment of anti-tumoural neutrophils. *Nature.* 2015;522(7556):349-53.
92. Blaisdell A, Crequer A, Columbus D, Daikoku T, Mittal K, Dey Sudhansu K, et al. Neutrophils Oppose Uterine Epithelial Carcinogenesis via Debridement of Hypoxic Tumor Cells. *Cancer Cell.* 2015;28(6):785-99.
93. Cummins EP, Keogh CE, Crean D, and Taylor CT. The role of HIF in immunity and inflammation. *Molecular Aspects of Medicine.*
94. Burke B, Tang N, Corke KP, Tazzyman D, Ameri K, Wells M, et al. Expression of HIF-1α by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. *The Journal of Pathology.* 2002;196(2):204-12.
95. Leek RD, Talks KL, Pezzella F, Turley H, Campo L, Brown NS, et al. Relation of Hypoxia-inducible Factor-2α (HIF-2α) Expression in Tumor-infiltrative Macrophages to Tumor Angiogenesis and the Oxidative Thymidine Phosphorylase Pathway in Human Breast Cancer. *Cancer Research.* 2002;62(5):1326-9.
96. Imtiyaz HZ, Williams EP, Hickey MM, Patel SA, Durham AC, Yuan L-J, et al. Hypoxia-inducible factor 2α regulates macrophage function in mouse models of acute and tumor inflammation. *The Journal of Clinical Investigation.* 2010;120(8):2699-714.
97. Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, DeNardo DG, et al. Macrophage Expression of Hypoxia-Inducible Factor-1α Suppresses T-Cell Function and Promotes Tumor Progression. *Cancer Research.* 2010;70(19):7465-75.

98. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, and Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Frontiers in Physiology.* 2014;5:75.
99. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn J-I, Cheng P, et al. HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *The Journal of Experimental Medicine.* 2010;207(11):2439-53.
100. Noman MZ, Janji B, Hu S, Wu JC, Martelli F, Bronte V, et al. Tumor-Promoting Effects of Myeloid-Derived Suppressor Cells Are Potentiated by Hypoxia-Induced Expression of miR-210. *Cancer Research.* 2015;75(18):3771-87.
101. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, et al. PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *The Journal of Experimental Medicine.* 2014;211(5):781-90.
102. Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T, et al. Hypoxia-induced neutrophil survival is mediated by HIF-1α–dependent NF-κB activity. *The Journal of Experimental Medicine.* 2005;201(1):105-15.
103. Thompson AAR, Elks PM, Marriott HM, Eamsamarng S, Higgins KR, Lewis A, et al. Hypoxia-inducible factor 2α regulates key neutrophil functions in humans, mice, and zebrafish. *Blood.* 2014;123(3):366-76.
104. Palucka K, and Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer.* 2012;12(4):265-77.
105. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, et al. Hypoxia and Hypoxia-Inducible Factor-1α Modulate Lipopolysaccharide-Induced Dendritic Cell Activation and Function. *The Journal of Immunology.* 2008;180(7):4697-705.
106. Elia AR, Cappello P, Puppo M, Fraone T, Vanni C, Eva A, et al. Human dendritic cells differentiated in hypoxia down-modulate antigen uptake and change their chemokine expression profile. *Journal of Leukocyte Biology.* 2008;84(6):1472-82.
107. Yang M, Ma C, Liu S, Sun J, Shao Q, Gao W, et al. Hypoxia skews dendritic cells to a T helper type 2-stimulating phenotype and promotes tumour cell migration by dendritic cell-derived osteopontin. *Immunology.* 2009;128(1 Pt 2):e237-e49.
108. Mancino A, Schioppa T, Larghi P, Pasqualini F, Nebuloni M, Chen IH, et al. Divergent effects of hypoxia on dendritic cell functions. *Blood.* 2008;112(9):3723-34.
109. Deng B, Zhu J-M, Wang Y, Liu T-T, Ding Y-B, Xiao W-M, et al. Intratumor Hypoxia Promotes Immune Tolerance by Inducing Regulatory T Cells via TGF-β1 in Gastric Cancer. *PLoS ONE.* 2013;8(5):e63777.
110. Wang C, Lee JH, and Kim CH. Optimal Population of FoxP3(+) T Cells in Tumors Requires an Antigen Priming-Dependent Trafficking Receptor Switch. *PLoS ONE.* 2012;7(1):e30793.
111. Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, et al. Hypoxia-inducible factor-1 alpha–dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proceedings of the National Academy of Sciences of the United States of America.* 2012;109(41):E2784-E93.
112. Ben-Shoshan J, Maysel-Auslender S, Mor A, Keren G, and George J. Hypoxia controls CD4+CD25+ regulatory T-cell homeostasis via hypoxia-inducible factor-1α. *European Journal of Immunology.* 2008;38(9):2412-8.

113. Lukashev D, Klebanov B, Kojima H, Grinberg A, Ohta A, Berenfeld L, et al. Cutting Edge: Hypoxia-Inducible Factor 1α and Its Activation-Inducible Short Isoform I.1 Negatively Regulate Functions of CD4+ and CD8+ T Lymphocytes. *The Journal of Immunology.* 2006;177(8):4962-5.
114. Doedens AL, Phan AT, Stradner MH, Fujimoto JK, Nguyen JV, Yang E, et al. Hypoxia-inducible factors enhance the effector responses of CD8+ T cells to persistent antigen. *Nat Immunol.* 2013;14(11):1173-82.
115. Palazón A, Martínez-Forero I, Teijeira A, Morales-Kastresana A, Alfaro C, Sanmamed MF, et al. The HIF-1α Hypoxia Response in Tumor-Infiltrating T Lymphocytes Induces Functional CD137 (4-1BB) for Immunotherapy. *Cancer Discovery.* 2012;2(7):608-23.
116. Branco-Price C, Zhang N, Schnelle M, Evans C, Katschinski Dörthe M, Liao D, et al. Endothelial Cell HIF-1α and HIF-2α Differentially Regulate Metastatic Success. *Cancer Cell.* 2012;21(1):52-65.
117. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, et al. Myeloid Suppressor Lines Inhibit T Cell Responses by an NO-Dependent Mechanism. *The Journal of Immunology.* 2002;168(2):689-95.
118. Medot-Pirenne M, Heilman MJ, Saxena M, McDermott PE, and Mills CD. Augmentation of an Antitumor CTL Response In Vivo by Inhibition of Suppressor Macrophage Nitric Oxide. *The Journal of Immunology.* 1999;163(11):5877-82.
119. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood.* 2006;109(1):228-34.
120. Kalluri R, and Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer.* 2006;6(5):392-401.
121. Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, and Kuliopulos A. PAR1 Is a Matrix Metalloprotease-1 Receptor that Promotes Invasion and Tumorigenesis of Breast Cancer Cells. *Cell.* 2005;120(3):303-13.
122. Hlatky L, Tsionou C, Hahnfeldt P, and Coleman CN. Mammary Fibroblasts May Influence Breast Tumor Angiogenesis via Hypoxia-induced Vascular Endothelial Growth Factor Up-Regulation and Protein Expression. *Cancer Research.* 1994;54(23):6083-6.
123. Kim J-w, Evans C, Weidemann A, Takeda N, Lee YS, Stockmann C, et al. Loss of fibroblast HIF-1α accelerates tumorigenesis. *Cancer research.* 2012;72(13):3187-95.
124. McGranahan N, and Swanton C. Biological and Therapeutic Impact of Intratumor Heterogeneity in Cancer Evolution. *Cancer Cell.*27(1):15-26.
125. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant Metastasis Occurs Late during the Genetic Evolution of Pancreatic Cancer. *Nature.* 2010;467(7319):1114-7.
126. Cui Y-L, Wang X, and Li X-F. 18F-fluoromisonidazole PET reveals spatial and temporal heterogeneity of hypoxia in mouse models of human non-small-cell lung cancer. *Future Oncology.* 2015;11(20):2841-9.
127. Zornhagen KW, Hansen AE, Oxboel J, Clemmensen AE, El Ali HH, Kristensen AT, et al. Micro Regional Heterogeneity of (64)Cu-ATSM and (18)F-FDG Uptake in Canine Soft Tissue Sarcomas: Relation to Cell Proliferation, Hypoxia and Glycolysis. *PLoS ONE.* 2015;10(10):e0141379.
128. Erapaneedi R, Belousov VV, Schäfers M, and Kiefer F. A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level. *The EMBO Journal.* 2015;35(1):102-13.

129. Movahedi K, Laoui D, Gysemans C, Baeten M, Stangé G, Van den Bossche J, et al. Different Tumor Microenvironments Contain Functionally Distinct Subsets of Macrophages Derived from Ly6C(high) Monocytes. *Cancer Research.* 2010;70(14):5728-39.
130. Laoui D, Van Overmeire E, Di Conza G, Aldeni C, Keirsse J, Morias Y, et al. Tumor Hypoxia Does Not Drive Differentiation of Tumor-Associated Macrophages but Rather Fine-Tunes the M2-like Macrophage Population. *Cancer Research.* 2014;74(1):24-30.
131. Hanahan D, and Weinberg Robert A. Hallmarks of Cancer: The Next Generation. *Cell.*144(5):646-74.
132. Kojima H, Kobayashi A, Sakurai D, Kanno Y, Hase H, Takahashi R, et al. Differentiation stage-specific requirement in HIF-1α-regulated glycolytic pathway during murine B cell development in bone marrow. *Journal of immunology (Baltimore, Md : 1950).* 2010;184(1):154-63.

Figures

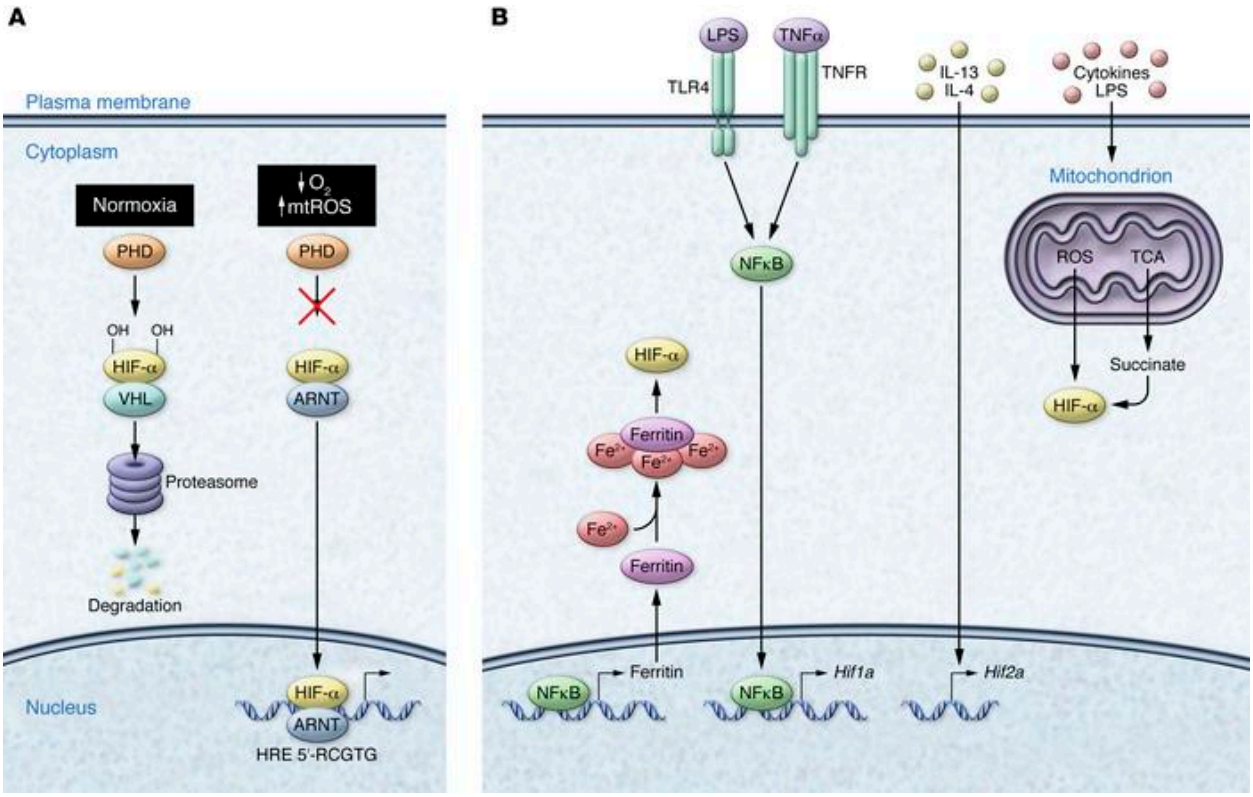

Figure 1.1. Activation of HIFs by hypoxia and inflammation. (**A**) HIF-α subunits are regulated by post-translational hydroxylation of two specific proline residues by PHD enzymes in normoxic conditions. This leads to VHL-mediated proteasomal degradation. As O_2 concentration drops or mitochondrial ROS production increases, HIF-α are stabilized, dimerize with ARNT, translocate to the nucleus and bind to HRE sequences in target genes. (**B**) HIFs are activated by inflammation through cytokine-induced *Hif2α* expression, NF-κB-mediated transcription of *Hif1α*, NF-κB-dependent ferritin iron sequestration, cytokine-mediated mitochondrial ROS production, and TCA metabolite succinate.

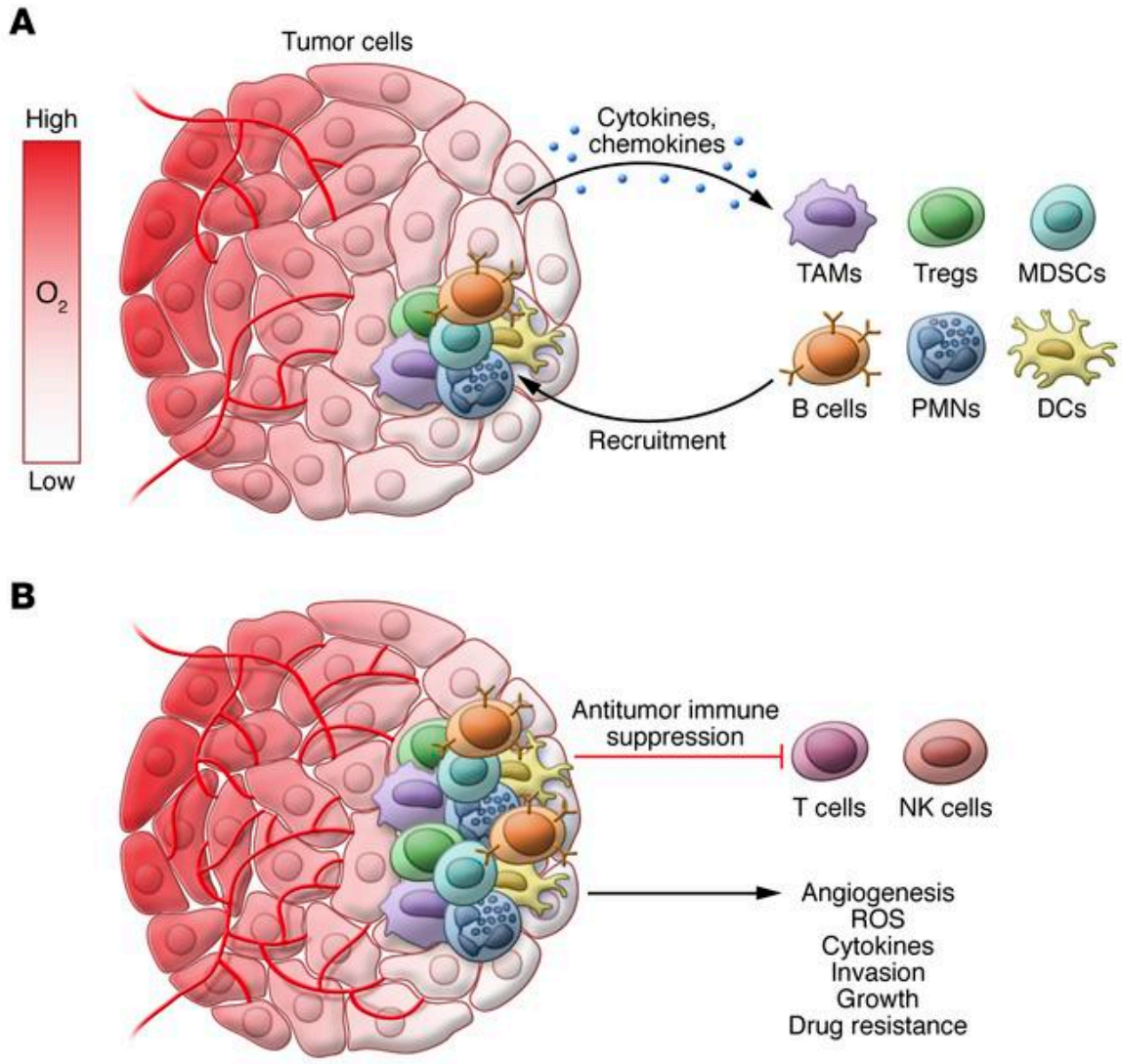

Figure 1.2. Hypoxic activation of tumor-promoting inflammatory responses. (A) As O_2-concentration declines in tumors, hypoxia directed secretion of cytokines and chemokines such as CXCL5, CXCL12, CCL28, and MIF to recruits pro-tumor immune cells including Tregs, TAMs, neutrophils, B cells, and MDSCs. (**B**) Recruited immune cells reside in hypoxic areas of tumors and hypoxia and HIFs regulate inflammatory cell effector functions to promote tumor growth. This includes suppression of anti-tumor immune responses, ROS production, angiogenesis, invasion/metastasis, drug resistance, and direct cytokine expression.

Tables

Table 1.1: Regulation of immune cell effector functions by HIF-1α and HIF-2α

	Immune Cell Recruitment	
	HIF-1α	**HIF-2α**
TAM	Promotes chemoattraction via SDF-1α/CXCL12 expression (83)	Not Known
DC	Not known	Not known
PMN	Not Known	Represses GR-1+ granulocyte recruitment to lung tumors (43)
MDSC	Increased recruitment to HNSCC through MIF secretion (72)	Increased recruitment to HNSCC through MIF secretion (72)
Treg	Regulates CCL28 expression in ovarian cancer (63)	Not known
Th17	Not known	Not known
CD4	Not known	Not known
B cell	Repress B cell infiltration into pancreatic tumors (46)	Not known
	Immune Cell Effector Function	
	HIF-1α	**HIF-2α**
TAM	T-cell suppression (97)	Essential pro-tumor function and inflammatory responses (96)
DC	Not known	Not Known
PMN	Decrease apoptosis (102)	Regulates PMN-mediated inflammatory responses (103)
MDSC	T-cell suppression (99); *Pdl1* expression (101)	Not known
Treg	Regulates *Foxp3* expression (111); essential for immunesuppression	Not Known
Th17	Essential for Th17 differentiation (55)	Not Known
CD4	Represses cytokine secretion (113); regulates CD137 to augment anti-tumor immunity (115)	Not Known
B cell	Essential for B cell maturation (132)	Not Known

Table 1.2 Mouse models

Genotype	Chapter	Description
Vhl$^{F/F}$	2	*Villin*-Cre expressing, *Vhl* WT
Vhl$^{\Delta IE}$	2	*Villin*-Cre expressing, *Vhl* floxed
Hif-1α$^{F/F}$	2	*Villin*-Cre expressing, *Hif-1α* WT
Hif-1α$^{\Delta IE}$	2	*Villin*-Cre expressing, *Hif-1α* floxed
Hif-2α$^{F/F}$	2	*Villin*-Cre expressing, *Hif-2α* WT
Hif-2α$^{\Delta IE}$	2	*Villin*-Cre expressing, *Hif-2α* floxed
Hif-2α^{LSL}	2	*Villin*-Cre expressing, *Hif-2α* overexpression
Hif-2α$^{+/+}$	2	*Villin*-Cre expressing, *Hif-2α* WT
Hif-2α$^{+/+}$/*Apc*$^{Min/+}$	2	*Villin*-Cre expressing, *Hif-2α* WT, ApcMin/+
Hif-2α$^{\Delta IE}$/*Apc*$^{Min/+}$	2	*Villin*-Cre expressing, *Hif-2α* floxed, ApcMin/+
Hif-2α$^{+/+}$/*Apc*$^{Min/+}$	2	*Villin*-Cre expressing, *Hif-2α* WT, ApcMin/+
Hif-2α^{LSL}/*Apc*$^{Min/+}$	2	*Villin*-Cre expressing, *Hif-2α* overexpression, ApcMin/+
LysM^{Cre};*Mcl1*$^{wt/wt}$	3	*LysozymeM*-Cre expressing, *Mcl1* WT
LysM^{Cre};*Mcl1*$^{fl/wt}$	3	*LysozymeM*-Cre expressing, *Mcl1* Het
LysM^{Cre};*Mcl1*$^{fl/fl}$	3	*LysozymeM*-Cre expressing, *Mcl1* floxed
Mrp8^{Cre};*Mcl1*$^{wt/wt}$	3	*Mrp8*-Cre expressing, *Mcl1* WT
Mrp8^{Cre};*Mcl1*$^{fl/fl}$	3	*Mrp8*-Cre expressing, *Mcl1* floxed
Cdx2-CreER^{T2};*Apc*$^{fl/fl}$	3	Tamoxifen inducible Cdx2-CreERT2, *Apc* floxed
vilMAZ	4	*Villin*-promoter MAZ overexpression

Chapter 2[2]

Epithelial Hypoxia-Inducible Factor 2α Facilitates the Progression of Colon Tumors through Recruiting Neutrophils

Abstract

Inflammation is a significant risk factor for colon cancer. Recent work has demonstrated essential roles for several infiltrating immune populations in the metaplastic progression following inflammation. Hypoxia and stabilization of hypoxia-inducible factors (HIFs) are hallmark features of inflammation and solid tumors. Previously, we demonstrated an important role for tumor epithelial HIF-2α in colon tumors, however, the function of epithelial HIF-2α as a critical link in the progression of inflammation to cancer has not been elucidated. In colitis-associated colon cancer models, epithelial HIF-2α was essential in tumor growth. Concurrently, epithelial disruption of HIF-2α significantly decreased neutrophils in the colon tumor microenvironment. Intestinal epithelial HIF-2α overexpressing mice demonstrated that neutrophil recruitment was a direct response to increased epithelial HIF-2α signaling. RNA-seq analysis from HIF-2α overexpressing mice in conjunction with data mining from The Cancer

[2] This chapter represents published manuscript: Triner D, Xue X, Schwartz AJ, Jung I, Colacino JA, and Shah YM. "Epithelial Hypoxia-Inducible Factor 2α Facilitates the Progression of Colon Tumors Through Recruiting Neutrophils." Molecular and Cellular Biology 2017.

Genome Atlas identified the neutrophil chemokine CXCL1 gene was highly upregulated in colon tumor epithelium in a HIF-2α dependent manner. Using selective peptide inhibitors of the CXCL1-CXCR2 signaling axis identified HIF-2α-dependent neutrophil recruitment is an essential mechanism to increase colon carcinogenesis. These studies demonstrate that HIF-2α is a novel regulator of neutrophil recruitment to colon tumors and is essential in shaping the pro-tumorigenic inflammatory microenvironment in colon cancer.

Introduction

Colon cancer remains a significant public health concern and is the second-leading cause of cancer-associated deaths in the United States (1). Patients with chronic inflammation associated with inflammatory bowel disease (IBD), comprising of ulcerative colitis and Crohn's Disease, are at an increased lifetime risk of developing colon cancer; these tumors are termed colitis-associated cancers (CAC) (2). The genetic changes of sporadic colon cancer have been well defined, and a comprehensive genetic analysis of CAC was recently reported (3). In contrast to sporadic colon cancer, CAC are associated with early loss of the TP53 tumor suppressor and less frequent inactivation of adenomatous polyposis coli (APC) (4). Inflammation is an important component in the progression of sporadic cancer and the inflammatory response is essential in the initiation and progression of CAC (5). The precise mechanisms that initiate the pro-tumorigenic response following inflammation remain unknown.

Hypoxia is a characteristic feature of IBD and nearly all solid tumors including those of the colon (6). Hypoxia promotes activation of the hypoxia-inducible factors (HIFs). HIFs consist of a heterodimer of an O_2-labile α-subunit (HIF-1α, HIF-2α, and HIF-3α) and an O_2-stable β-subunit (ARNT) (7). HIFs regulate transcription of target genes that mediate cellular responses to

hypoxic microenvironments. HIFs are also essential factors promoting tumorigenesis and regulate several neoplastic processes including growth, evasion of apoptosis, and chemoresistance (8). Previously, we have shown that overexpression of intestinal epithelial HIF-2α, but not HIF-1α, can increase colon tumor progression in mouse models of sporadic colon tumorigenesis (9, 10). The essential role and mechanisms by which HIF-2α regulates CAC have not been defined.

Inflammation is a critical component of the colon tumor microenvironment and colon tumors are highly infiltrated with cells of both the innate and adaptive immune systems (5). Neutrophils are granulocytic myeloid cells with a critical role in the innate immune response (11) and are highly prevalent in colon tumor microenvironment (12), but the function of neutrophils in the initiation and progression of cancer remains controversial. Previous studies have shown neutrophils can be polarized into anti-tumorigenic (N1) and pro-tumorigenic (N2) (13). N2 neutrophils promote tumorigenesis through suppression of anti-tumor immunity, activation of oncogenic signaling through secretion of neutrophil elastase, and activation of angiogenesis (14-16). On the other hand, anti-tumorigenic N1 neutrophils can suppress tumorigenesis through direct tumor cytotoxicity and activation of anti-tumor immunity (17). Neutrophil recruitment into tumors can be regulated by tumor-derived secretion of a variety of chemokines and cytokines. However, the precise mechanisms mediating recruitment of neutrophils into colon tumors are not well defined.

In the present study, we show that the colon epithelial hypoxic response through activation of HIF-2α is essential in colon tumorigenesis in mouse models of CAC. Mechanistically, intestinal epithelial HIF-2α is a critical mediator of neutrophil recruitment to colon tumors through direct transcriptional regulation of the potent neutrophil chemokine,

CXCL1, in colon tumors. Taken together, these studies provide novel insights into hypoxic inflammatory responses in the progression of colon tumors and suggest rationale for the targeting of HIF-2α in colon tumors.

Methods

Animals

Vhl$^{F/F}$, *Vhl*$^{\Delta IE}$, *Hif-1α*$^{\Delta IE}$, *Hif-1α*$^{F/F}$, *Hif-2α*$^{\Delta IE}$, *Hif-2α*$^{F/F}$, *Hif-2α*^{LSL}, *Hif-2α*$^{+/+}$ mice were previously described (10, 18). For all experiments, male and female mice aged 6- to 8-weeks were used. To evaluate HIF-2α in colon tumorigenesis, *Hif-2α*$^{\Delta IE}$ and *Hif-2α*^{LSL} were crossed to *Apc*$^{Min/+}$. To induce colon tumorigenesis in *Hif-2α*$^{\Delta IE}$/*Apc*$^{Min/+}$, animals were treated with 2% DSS in their drinking water for 5 days then placed back on regular drinking water for 28 days. For AOM/DSS experiments, animals were injected I.P. with azoxymethane (10mg/kg) then cycled on 1.5% DSS in their drinking water for five days followed by regular drinking water for 2-weeks for three cycles. For the CXCR2 pepducin experiment, following the third cycle, *Vhl*$^{\Delta IE}$ mice were treated with CXCR2 pepducin (pal-RTLFKAMGQKHR) or control peptide (pal-TRFLAKMHQGHKR) (Genscript) for 35 consecutive days (s.c. 2.5mg/kg) (19). All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Flow cytometry

Single cell suspensions from fresh normal colon or colon tumor tissue were prepared by finely mincing tissue and incubating with Collagenase Type II (Sigma-Aldrich) (1mg/ml) at 37^{o}

C for 1-hour then passed through a 40um cell strainer. Single-cell suspensions were stained in HBSS in 2% FBS with eFluor780-conjugated anti-CD45 (eBioscience), PE-conjugated anti-Ly6G (BD), APC-conjugated anti-Cd11b (eBioscience), and eFluor450-conjugated anti-F4/80 (eBioscience). Flow cytometry was performed using an LSRFortessa (BD). Flow cytometry data was analyzed using FlowJo software.

Neutrophil isolation and transwell assay

Bone marrow cells were suspended in HBSS buffer supplemented with 20mM HEPES and 0.5% FBS. The isolated bone marrow was disaggregated through 18G needle. To lyse the residual RBCs, 0.2% NaCl was added to the cells for 45 seconds and the reaction was stopped with 1.2% NaCl. The cells were resuspended in HBSS buffer and carefully layered over 62% Percoll. The centrifugation was performed at 2,200 rpm for 30 minutes. The cell pellet was washed twice with PBS and used for antibody staining to confirm purity. The antibodies used were PerCP-Cy5.5-conjugated anti-CD45 (ebioscience), APC-conjugated anti-CD11b (ebioscience), and PE-conjugated anti-Ly6G (BD) and eFluor450-conjugated anti-F4/80 (ebioscience) antibodies. Debris (FSC-A/SSC-A) and doublets (FSC-A/FSC-H) were excluded and CD45+ cells were then sub-gated on CD11b+ and Ly6G+ neutrophils. The numbers indicate the relative percentages of each population. For transwell assay, $1x10^6$ neutrophils were cultured in the top well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% anti-biotic/anti-mycotic and fresh colon explants were plated in the bottom well. Migration was assessed at 2-hours after plating.

Isolation of colon epithelial cells and intraepithelial lymphocytes

Isolation of colon epithelial cells (CECs) and intraepithelial lymphocytes was performed as previously described (10). Briefly, colon tissue was isolated and incubated with EDTA and DTT in Hanks' balanced salt solution (HBSS). To separate CECs and IELs, the tissue was passed through a cell strainer and exposed to a 67%:44% Percoll gradient separation. CECs were collected from the top and IELs were collected at the interface of the Percoll gradient.

Histology

Colon tissue and tumors were excised, fixed, sectioned and stained as previously described (9). Antibodies for immunofluorescence were as follows: BrdU (eBioscience) Ki67 (1:100) (Vector Labs), Ly6G (1:100) (BD), and Alexa Fluor® 488 goat anti-mouse IgG (1:500) (Molecular Probes Inc).

RNA isolation, qPCR analysis and high throughput RNA sequencing (RNA-seq)

RNA was isolated and qPCR analysis was conducted as previously described (9). Primers are listed in Table 2.1. RNA sequencing libraries were prepared using the TruSeq RNA Library Prep Kit v2 (Illumina) following the manufacturer's recommended protocol. Libraries were sequenced using single end 50 cycle reads on a HiSeq 2500 sequencer (Illumina) at the University of Michigan DNA Sequencing Core Facility.

RNA-seq data analysis

Raw sequencing read quality was assessed utilizing FastQC. Reads were aligned to the reference mouse transcriptome (UCSC mm10) using Bowtie v 2.1.0.0 (20) and TopHat v 2.0.9 (21). Default parameters were used for the alignment, with the exception of ""--b2-very-

sensitive", "--no-coverage-search" and "--no-novel-juncs". Mate inner pair distances were estimated by TopHat, and these values were used in the alignment. Expression quantification and differential expression analysis between *Hif-2α*^{LSL}, and *Hif-2α*$^{+/+}$ mice was conducted using CuffDiff v2.1.1 (22) using the parameter settings "--multi-read-correct", "--compatible-hits-norm", and "--upper-quartile –norm" for normalization of expression calculations across samples. For the CuffDiff analysis, we used UCSC mm10.fa as the reference genome and UCSC mm10.gtf as the reference transcriptome. Genes were considered differentially expressed between conditions at a false discovery rate adjusted p-value < 0.05 (23).

Pathway analyses

A directional analysis was conducted on all genes by including p-value of the differential expression test as a measure of effect size and log2 fold difference in expression as a measure of effect direction using iPathways (Advaita). Differentially expressed pathways were identified utilizing PANTHER Classification System (http://pantherdb.org/). KEGG biological pathways and Gene Ontology biological processes were considered differentially expressed at a p-value <0.05.

CXCL1 luciferase reporter activity

The *Cxcl1* promoter was cloned using primers listed in Table 2.1. *Cxcl1* promoter fragments were subsequently cloned into pGL3-basic vector (Promega). Luciferase activity assays were performed as previously described and normalized to β-galactosidase activity (24). HCT116 cells expressing MAZ targeting shRNAs were generated as previously described (10).

Enteroid culture

Enteroids were generated from colon tissue from mice with inducible, colon epithelial specific deletion of *Apc*, activation *Kras*, and loss of *Tp53* (*Cdx2*^{CreER};*Apc*$^{fl/fl}$;*Kras*^{G12V};*Tp53*$^{fl/fl}$). Mice were sacrificed and the colon was cut open longitudinally. All plasticware was pre-coated with 0.1% BSA and all steps were carried out on ice unless specified. The tissue was incubated for 15 minutes at room temperature in 2.5 µg/mL amphotericin B (Fungizone, ThermoFisher) in Dulbecco's Phosphate-Buffered Saline containing 25 µg/mL gentamicin (Gibco) and 50 µg/mL normocin (InvivoGen) (*DPBSgn*). Colon tissue was cut into lengthwise strips (approximately 3 mm x 5 mm). Tissue was incubated in 10 mM DTT for 15 minutes at room temperature, changing to fresh DTT every 5 minutes. Tissue was rinsed in *DPBSgn*, rinsed once with 8 mM EDTA, and then incubated/rotated in 8mM EDTA at 4°C for 75 minutes. EDTA was removed and tissue was washed three times with *DPBSgn*. Tissue was then "snap-shook" 10x to manually separate colon crypts. The crypt-containing supernatant was immediately added to 1.5 mLs of cold FBS in a BSA-coated 50 mL tube and the shake step was repeated twice more. Crypts were spun at 40 x g for 2 minutes at 4°C. The pellet was washed in DPBSgn and spun again at 40 x g for 2 minutes at 4°C. The pellet was resuspended in a solution of 66% matrigel (Corning), 33% KGMG-media (KGMG Bullet Kit, Lonza), and 10 µM of the ROCK inhibitor, Y27632, (Miltenyi) at a concentration of 2 crypts/µL, accounting for 250 µL per well in a 6-well plate. Four diagonal strips of 60 µL of the culture was added to each well in a pre-warmed cell culture plate using a cut tip. After 30 minutes, media containing 10 µM Y27632 was added. Media was changed daily for three days. On the fourth day, cultures were treated with either vehicle or 100 µM of the PHD inhibitor, FG-4592 (Cayman Chemicals), overnight and then lysed directly in Trizol for qPCR analysis.

Data analysis

Error bars represent the standard deviation (25). P values were calculated by independent t test, paired t-test, one-way ANOVA, Dunnett t test, and two-way ANOVA. Immunofluorescence staining and Western blot analysis were quantified with Image J.

Results

HIF-2α is essential in inflammation-induced colon tumorigenesis

One of the most commonly utilized models to study intestinal tumorigenesis is the $Apc^{Min/+}$ model. These mice harbor a germline truncation mutation to the *Apc* gene and spontaneously develop intestinal adenomas (26). However, this model does not completely recapitulate human colon tumorigenesis as the vast majority of these tumors develop in the small intestine with few colon tumors observed. Moreover, few of these tumors progress beyond adenoma and rarely become invasive. Inflammation is an essential component of the colon tumor microenvironment and previous studies have shown that acute colonic inflammation induced by dextran sulfate sodium (DSS) can increase the incidence of colon tumorigenesis in the $Apc^{Min/+}$ mouse (27). To directly determine the functional role for HIF-2α expression in inflammation-induced colon tumorigenesis, mice with intestine-epithelial specific disruption of HIF-2α (*Hif-2α*$^{\Delta IE}$) by villin-cre mediated recombination were crossed to $Apc^{Min/+}$ mice (*Hif-2α*$^{\Delta IE}$/$Apc^{Min/+}$) and compared to littermate controls with intact HIF-2α expression (*Hif-2α*$^{F/F}$/$Apc^{Min/+}$). The *Hif-2α*$^{\Delta IE}$/$Apc^{Min/+}$ mice had significantly reduced colon tumor number and reduced tumor burden (Fig. 2.1A-D). Furthermore, tumors from *Hif-2α*$^{\Delta IE}$/$Apc^{Min/+}$ mice had significantly increased apoptosis as measured by terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining (Fig. 2.1E and F). These results show that colon-epithelial HIF-2α is important in inflammation-driven colon tumorigenesis.

Epithelial expression of HIF-2α can promote inflammatory responses (10) and can modulate the immune environment in tumors. Colon tumors from *Hif-2α*$^{F/F}$/$Apc^{Min/+}$ had a significant increase in the pan-myeloid cell marker CD11b compared to adjacent normal tissue. However, tumors from *Hif-2α*$^{\Delta IE}$/$Apc^{Min/+}$ had a significant reduction in tumor CD11b compared

to those from *Hif-2α$^{F/F}$/Apc$^{Min/+}$*, suggesting tumor epithelial expression of HIF-2α regulates myeloid cell influx into tumors. To determine the precise myeloid cell type absent, qPCR analysis was conducted for monocyte, macrophage, and neutrophil markers. Significantly less expression of neutrophil markers *Ly6g* and myeloperoxidase (*Mpo*) were observed in *Hif-2α$^{\Delta IE}$/Apc$^{Min/+}$* colon tumors compared to *Hif-2α$^{F/F}$/Apc$^{Min/+}$* mice, whereas no changes in expression of the monocyte marker CD68 or macrophage markers *Emr1*, *iNos*, or *Arg1* were observed (Fig. 2.1G). Moreover, the calcium binding protein, S100a8, which is abundantly expressed by neutrophils is significantly reduced in both normal and tumor tissue from *Hif-2α$^{\Delta IE}$/Apc$^{Min/+}$* (Fig. 2.1H) (28). These data suggest epithelial cell expression of HIF-2α is essential for the recruitment of neutrophils in colon tumors.

To assess if HIF-2α is sufficient to drive colon tumorigenesis, mice with overexpression of O_2-stable HIF-2α downstream of a *loxP*-STOP-*loxP* (LSL) cassette knocked-in to the *Rosa26* allele (10) were crossed to *Apc$^{Min/+}$* mice to generate *Hif-2α^{LSL}/Apc$^{Min/+}$* mice. These mice develop significantly more colon tumors compared to age-matched HIF-2α wild-type controls (*Hif-2α$^{+/+}$/Apc$^{Min/+}$*) at 3 months (Fig. 2.2A and B). Moreover, these tumors have significantly higher proportion of proliferating cells as shown by incorporation of Bromodeoxyuridine (BrdU) (Fig. 2.2C and D). Concurrent with increased tumorigenesis, tumors from *Hif-2α^{LSL}/Apc$^{Min/+}$* mice have higher presence of neutrophils compared to *Hif-2α$^{+/+}$/Apc$^{Min/+}$* mice by Ly6G immunofluorescence (Fig. 2.2E).

HIF-2α regulates intratumoral neutrophils in colitis-associated colon cancer

In order to appropriately model the role for HIF-2α in the inflammatory progression of colon cancer, we used the AOM/DSS model of CAC in mice with intestine-epithelial specific

deletion of HIF-2α (*Hif-2α$^{\Delta IE}$*) (29). *Hif-2α$^{\Delta IE}$* and littermate control mice (*Hif-2α$^{F/F}$)* were injected i.p. with AOM (10mg/kg) on day 0 and treated with DSS (1.5%wt/vol) in their drinking water beginning day five for five-days and changed back to regular drinking water for two-weeks for three cycles. Consistent with the inflammation-induced *Apc$^{Min/+}$* model, *Hif-2α$^{\Delta IE}$* mice have significantly reduced colon tumor number compared to littermate controls, suggesting HIF-2α expression is essential for inflammation-induced colon tumorigenesis (Fig. 2.3A). To analyze neutrophil infiltration in tumors, flow cytometry was performed on individual colon tumors. Colon tumors from *Hif-2α$^{\Delta IE}$* had a significant reduction of $CD11b^{+}Ly6G^{+}$ neutrophils from the colon tumor microenvironment compared to *Hif-2α$^{F/F}$* colon tumors (Fig. 2.3B and C). No changes in tumor macrophages (CD11b+/F4/80+) were observed (Fig. 2.3D and E). These data suggest epithelial HIF-2α modulates the colon tumor microenvironment through regulating infiltration of tumor-associated neutrophils.

HIF-1α does not impact colon tumorigenesis or neutrophil recruitment

Our studies suggest that HIF-2α is a major regulator of colitis associated colon tumorigenesis through recruitment of pro-tumorigenic neutrophils. To address overlap in function of HIF-2α and HIF-1α in colon tumorigenesis, we assessed the role of HIF-1α using mice with intestine epithelial disruption of HIF-1α (*Hif-1α$^{\Delta IE}$*). In the AOM/DSS model of colitis associated colon cancer, no difference in tumorigenesis was observed in *Hif-1α$^{\Delta IE}$* compared to WT controls (*Hif-1α$^{F/F}$*) (Fig. 2.4A and B). Concurrently, no changes in expression of neutrophil markers (*Ly6g* and *Cd11b*) were observed in *Hif-1α$^{F/F}$* colon tumor tissue relative to *Hif-1α$^{\Delta IE}$* colon tumor tissue (Fig. 2.4C).

Colon-epithelial HIF-2α regulates neutrophil chemotaxis

To determine if epithelial HIF-2α can regulate neutrophil chemotaxis, we used a transwell assay. Neutrophils were isolated from bone marrow and were shown to be highly pure (over 80%) (Fig. 2.5A). Isolated colon explants used were from mice with epithelial deletion of Von-Hippel Lindau ($Vhl^{\Delta IE}$). Previous work has shown that these mice promote intestinal epithelial stabilization of HIF-2α in normoxic conditions (18). A dramatic increase in neutrophil migration through the transwell towards colon explants from $Vhl^{\Delta IE}$ compared to WT ($Vhl^{F/F}$) colon tissue explants was observed (Fig. 2.5B and C). To more directly assess the effects of HIF-2α, media was conditioned with colon tissue from HIF-2α-overexpressing mice ($Hif\text{-}2\alpha^{LSL}$) and this led to a significant increase in neutrophil transwell chemotaxis compared to colon tissues explant from wild-type littermate mice ($Hif\text{-}2\alpha^{+/+}$) (Fig. 2.5B and C). These data demonstrate that HIF-2α is important in neutrophil recruitment *in vitro*. Flow cytometery analysis of normal colon tissue from mice overexpressing HIF-2α in the intestinal epithelium ($Hif\text{-}2\alpha^{LSL}$) showed a significant increase in intra-colonic neutrophils compared to wild-type littermate mice (Fig. 2.5D and E). Together, these data demonstrate an essential and sufficient role of epithelial HIF-2α in neutrophil recruitment in the colon.

CXCL1 is highly induced by intestinal epithelial HIF-2α.

Neutrophils are recruited to solid tumors through tumor secretion of cytokines and chemokines (30). To determine the precise mechanism for how epithelial HIF-2α recruits neutrophils into intestine, RNA-seq analysis were performed in colon tissues from $Hif\text{-}2\alpha^{LSL}$ and WT ($Hif\text{-}2\alpha^{+/+}$) mice. Pathway analysis identified neutrophil attractive chemokines such as *Cxcl1*, *Cxcl2* and *Cxcl5* and neutrophil markers such as *S100a8* were highly increased in the

colon tissues from *Hif-2α*^{LSL} mice (Fig. 2.6A and B). Expression of several members of the CXC family of chemokines was examined by qPCR from colon tissue of HIF-1α overexpressing mice (*Hif-1α*^{LSL}), and *Hif-2α*^{LSL} mice, and littermate controls. Only *Cxcl1* expression was robustly increased ($p<0.001$) in colon tissue in *Hif-2α*^{LSL} mice compared to WT and *Hif-1α*^{LSL} mice (Fig. 2.6C). These data suggest that activated epithelial HIF-2α in colon tumors may recruit neutrophils through secretion of cytokines and chemokines.

HIF-2α is an essential regulator of CXCL1 expression in colon tumors

Oncomine data analysis indicated that *CXCL1* was highly increased in human colon tumors compared to normal colon tissues (Fig. 2.7A and B). We further confirmed by qPCR that *CXCL1* was significantly increased in a set of colon tumor tissues compared to their adjacent normal colon tissues (Fig. 2.7C). CXCL1 expression is induced in colon tumors but the major regulators of CXCL1 expression in colon tumors are currently unknown. Consistent with patient tumor analysis, CXCL1 expression was highly induced in AOM/DSS-induced colon tumors in *Hif-2α*$^{F/F}$ mice compared to normal adjacent tissue. This increase was significantly attenuated from tumors isolated from *Hif-2α*$^{\Delta IE}$ mice (Fig. 2.7D and E). No difference in *Cxcl1* expression was observed in tumors from *Hif-1α*$^{F/F}$ or *Hif-1α*$^{\Delta IE}$ mice (Fig. 2.7F).

The tumor microenvironment is a complex milieu of tumor epithelial cells, immune cells, and stromal cells. Previous genetic analysis has suggested that in tumor xenograft models, greater than 99% of the *Cxcl1* transcripts are expressed directly by the tumor epithelial cells relative to tumor stromal cells (31). To evaluate if epithelial hypoxia signaling directly regulates *Cxcl1* expression, we generated colon enteroids from mice with colon epithelial specific deletion of *Apc*, activation of the oncogene *Kras*, and loss of *Tp53* tumor suppressor, which are

commonly observed mutations in human colon tumors (Fig. 2.7F). Colon enteroids are an ideal model to mechanistically study CRC because they maintain cell polarization and tight junctions in three-dimensions and the cultures can be generated from primary colon epithelial tissue harboring mutations that are most commonly selected for in human colon tumors (32). To activate hypoxia signaling, these enteroids were treated with the potent PHD inhibitor FG-4592, which stabilizes HIF in normoxic conditions (33). Compared to untreated enteroids, activation of hypoxia significantly induced *Cxcl1* expression, demonstrating that epithelial hypoxia signaling is sufficient to activate *Cxcl1* expression (Fig. 2.7G). Taken together, our data suggest that epithelial HIF-2α is a master regulator of CXCL1 expression in colon tumors.

HIF-2α-regulates CXCL1 promoter through HRE- and MAZ-dependent mechanisms

HIFs activate target gene transcription through binding to hypoxia response elements (HREs), which are defined as 5'-RCGTG-3', in promoter and enhancer regions. Analysis of the CXCL1 proximal promoter identified six canonical HREs clustered at distal and proximal sites (Fig. 2.8A). The proximal promoter region of *Cxcl1* was cloned into the pGL3-luciferase reporter construct. Using co-transfection in HCT116 cells, overexpression of an oxygen-stable HIF-2α was shown to directly activate the *Cxcl1* promoter similar to HIF-2α activation of the well-characterized HREs of the enolase promoter (P2.1) (Fig. 2.8B). To evaluate the dependence of these HREs in HIF-2α-mediated CXCL1 induction, a series of deletion constructs to disrupt the HREs were generated. HIF-2α activation of *Cxcl1* promoter was attenuated when the distal HREs were deleted and completely ameliorated when both the distal and proximal HREs were removed. These data demonstrate that the HREs are essential for HIF-2α-mediated CXCL1 promoter induction (Fig. 2.8C).

It has been suggested that target gene specificity for HIF-1α and HIF-2α is mediated by interactions with other transcription cofactors (34). Previously, our work has shown that HIF-2α inflammatory target gene activation is dependent upon interaction with an essential cofactor, Myc-associated zinc finger (35) (10, 36). MAZ is a cys2his2-type zinc finger transcription factor that is highly upregulated in several human cancers and regulates tumor growth (37). To determine if MAZ is essential for HIF-2α-dependent CXCL1 promoter induction, we used two targeting shRNAs to generate stable knockdowns of MAZ expression in HCT116 cells (MAZ sh1 and MAZ sh2) (Fig. 2.8D). Compared to cells stably expressing scrambled shRNA (Ctrl cells), MAZ sh1 & MAZ sh2 significantly attenuated CXCL1-promoter activation in response to HIF-2α (Fig. 2.8E).

CXCR2 inhibition reduces HIF-2α-driven colon tumorigenesis

CXCL1 induces neutrophil recruitment through binding its cognate receptor, CXCR2, expressed on the cell surface of neutrophils. To determine if CXCL1 signaling through CXCR2 is the major mechanism by which epithelial HIF-2α mediates neutrophil recruitment, we use a well-characterized CXCR2 blocking peptide mimetic, CXCR2 pepducin. CXCR2 is a G-protein coupled receptor and pepducins block CXCR2 signaling and decrease neutrophil influx into sites of inflammation and tumors (38, 39). Bone marrow derived neutrophils were isolated and treated with the CXCR2 pepducin (Pep) or control pepducin (Veh). Blocking CXCR2 completely attenuated HIF-2α-induced neutrophil migration (Fig. 2.9A and B).

We next assessed the functional role for neutrophil recruitment in HIF-2α-driven colon tumorigenesis. To investigate this axis, we used the AOM/DSS model of CAC in $Vhl^{\Delta IE}$ mice. Previous work has shown these mice have a higher propensity to develop colon tumors in a HIF-

2α-dependent manner (9). Unlike the *Hif-2α*^{LSL} mice, *Vhl*$^{\Delta IE}$ mice can survive 3 cycles of DSS. To confirm that epithelial deletion of *Vhl* increases *Cxcl1* expression, we isolated purified colon epithelial cells (CECs) and intraepithelial lymphocytes (40) in *Vhl*$^{\Delta IE}$ and *Vhl*$^{F/F}$ mice. Cxcl1 expression is significantly increased in CECs of *Vhl*$^{\Delta IE}$ mice compared to *Vhl*$^{F/F}$ mice but not in intraepithelial lymphocytes (IELs) (Fig. 2.9C). Moreover, CXCL1 expression is induced in *Vhl*$^{\Delta IE}$ mice in a HIF-2α-dependent manner using mice with double disruption of VHL and HIF-2α (*Vhl*$^{\Delta IE}$/ *Hif-2α*$^{\Delta IE}$) (Fig. 2.9D). To address the role of CXCR2-mediated neutrophil recruitment to colon tumors, *Vhl*$^{\Delta IE}$ were randomized to treatment with Ctrl-pepducin (Veh) or CXCR2-pepducin (Pep) once daily for days 65-100 by subcutaneous injection following the third cycle of DSS, (Fig. 2.9E). Compared to littermate control *Vhl*$^{F/F}$ mice, *Vhl*$^{\Delta IE}$ mice treated with Ctrl-pepducin developed significantly more colon tumors and had higher tumor burden and increased neutrophil influx (Fig. 2.9F-H). The *Vhl*$^{\Delta IE}$ mice treated with CXCR2-pepducin significantly reduced HIF-2α-driven colon tumorigenesis and neutrophil infiltration. In addition, tumors from *Vhl*$^{\Delta IE}$ mice had a significant increase in tumor cell proliferation measured by Ki67 immunofluorescence staining, which was attenuated in *Vhl*$^{\Delta IE}$ mice treated with CXCR2-pepducin (Fig. 2.9I and J). Taken together, our studies suggest that HIF-2α-mediated neutrophil recruitment through CXCL1-CXCR2 axis is essential for its role in colon tumorigenesis.

Discussion

Inflammation and hypoxia are intimately linked and hypoxia has been previously shown to regulate the inflammatory microenvironment of many tumor types. Hypoxia increases ovarian cancer tumor growth through secretion of CCL28, which facilitates recruitment of immune suppressive T regulatory (Treg) cells to promote tumor growth (41). In pancreatic ductal

adenocarcinoma, HIF-1α is a tumor suppressor through blockade of pro-tumorigenic B cell recruitment to tumors (42). Interestingly, our data shows that epithelial expression of HIF-2α can modulate the inflammatory milieu of colon tumors through regulating the recruitment of intratumoral neutrophils. Hypoxic regulation of cytokines and chemokine secretion from tumor cells can modulate neutrophil recruitment to promote hepatocellular carcinoma (43). Additionally, neutrophils tend to be localized to hypoxic zones within uterine tumors (25). Mechanistically, we have discovered a novel HIF-2α target gene, CXCL1. CXCL1 is a member of the C-X-C family of chemokines and is a potent neutrophil chemoattractant to sites of inflammation or tumors through binding its cognate receptor, CXCR2 (44). Consistent with our work showing that epithelial CXCL1 is induced by HIF-2α, previous studies using xenograft models demonstrate the vast majority (>99%) of *Cxcl1* transcripts in colon tumors are derived from tumor epithelial cells (31). Our studies clearly demonstrate that the epithelial HIF-2α can regulate *Cxcl1* induction in colon tumors.

The functional role for neutrophils in the progression of tumors is not clear, as both anti-tumorigenic (N1) and pro-tumorigenic (N2) neutrophils have been described (13). Neutrophils expressing the hepatocyte growth factor (HGF) receptor, c-MET, have been shown to be largely anti-tumorigenic in colon tumors (35). However, large-scale meta-analysis studies have shown that neutrophils correlate highly with adverse outcomes across more than 25-different tumor types (45). Neutrophils are critical mediators of metastasis in murine models of breast cancer (40). It has also been suggested that neutrophils are essential in the inflammatory progression of colon tumors as depletion of neutrophils with anti-Ly6G antibody significantly reduced colon tumors (46). Moreover, a high neutrophil to lymphocyte ratio portends poor prognosis for colon cancer patients (47). In our study, we show that HIF-2α-driven colon tumorigenesis is dependent

upon neutrophil influx into colon tumors through the neutrophil CXCL1 receptor, CXCR2. A critical role for CXCR2 in the initiation and progression of colon cancer and pancreatic ductal adenocarcinoma has been described (48, 49). Inhibition of neutrophil influx via CXCR2 decreased HIF-2α-driven colon tumorigenesis, progression, and proliferation. These studies demonstrate mechanistically how hypoxic inflammatory responses can modulate the colon tumor immune microenvironment to promote tumor growth. More work is needed to determine the precise mechanisms by which neutrophils promote colon tumorigenesis.

The studies reported herein suggest that that epithelial HIF-2α, but not HIF-1α, selectively modulates neutrophil recruitment into tumors without affecting other myeloid cell populations. Neutrophils are critical to setup an oxygen gradient in the intestine (50). These oxygen gradients promote tissue repair in a HIF-1α-dependent manner. HIF-1α is highly active in intestinal inflammation and genetic deletion of intestinal epithelial HIF-1α exacerbates colitis (51). HIF-1α is an essential regulator of expression of intestinal barrier protective genes such as intestinal trefoil factor (*Itf*), *CD73*, and multidrug resistance gene 1 (*Mdr-1*) (51, 52). The bidirectional signaling of hypoxia and neutrophil may be a feed forward mechanism mediated by HIF-2α critical to establish an oxygen gradient during acute inflammation for HIF-1α-dependent injury repair (50). However, our data suggest in chronic inflammation this mechanism exacerbates tumorigenesis.

Previously, we have shown that activation of HIF-2α can promote colon tumor cell growth in a cell-autonomous manner. Epithelial expression of HIF-2α is a potent activator of inflammatory responses and increases the progression of intestinal inflammation (10). Additionally, HIF-2α is a transcriptional regulator of pro-inflammatory cyclooxygenase 2 (COX2) and microsomal prostaglandin e synthase (mPGES) to increase tumor inflammation and

treatment of HIF-2α-overexpressing mice with the anti-inflammatory, nimesulide, can reduce colon tumorigenesis (24). Interestingly, HIF-1α activation has no effect on colon tumorigenesis (53). Similarly, we find that HIF-1α has no effect on the expression of *Cxcl1*. Previous studies have highlighted the dichotomous role for HIF-1α and HIF-2α in several cancer models. For example, in renal cell carcinoma, HIF-2α is essential for tumor cell growth whereas HIF-1α decreases cell growth (54). In pancreatic cancer, genetic mouse models demonstrate that HIF-2α is essential for tumorigenesis whereas HIF-1α decreases tumorigenesis through repression of infiltrating pro-tumorigenic B cells (55, 56). However, in lung cancer it has been shown that HIF-2α exerts a tumor suppressive effect (57). These studies demonstrate the careful need to evaluate the tumor specific roles for HIF-1α and HIF-2α for therapeutic targeting. HIF-2α-specific inhibitors have been developed which target a novel ligand-binding pocket that is located within the PAS-B domain of HIF-2α but not in HIF-1α (58). These novel tools may provide an exciting therapeutic avenue to decrease tumor cell proliferation as well as decrease tumor-promoting inflammatory responses in colon cancer.

Funding information: This work was supported by NIH grants (CA148828 and DK095201 to Y.M.S.), the University of Michigan Gastrointestinal Peptide Center (Y.M.S.), a pilot grant from the University of Michigan GI Spore (CA130810 to Y.M.S.), the Crohn's Colitis Foundation of America (grant number 276556 to X.X.), a Research Grant from University of Michigan Comprehensive Cancer Center P30CA046592 (J.A.C.), funding from Ravitz Foundation (J.A.C.), and the Research Scholar Award from American Gastroenterological Association (to X.X.). D.T. was supported by the T32 training grant (T32 DK 094775) "Training in Basic and Translational Digestive Sciences."

References

1. Siegel RL, Miller KD, and Jemal A. Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians.* 2016;66(1):7-30.
2. Kim ER, and Chang DK. Colorectal cancer in inflammatory bowel disease: The risk, pathogenesis, prevention and diagnosis. *World Journal of Gastroenterology : WJG.* 2014;20(29):9872-81.
3. Fearon ER. Molecular Genetics of Colorectal Cancer. *Annual Review of Pathology: Mechanisms of Disease.* 2011;6(1):479-507.
4. Robles AI, Traverso G, Zhang M, Roberts NJ, Khan MA, Joseph C, et al. Whole-Exome Sequencing Analyses of Inflammatory Bowel Disease−Associated Colorectal Cancers. *Gastroenterology.* 2016;150(4):931-43.
5. Terzić J, Grivennikov S, Karin E, and Karin M. Inflammation and Colon Cancer. *Gastroenterology.* 2010;138(6):2101-14.e5.
6. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, et al. The Expression and Distribution of the Hypoxia-Inducible Factors HIF-1α and HIF-2α in Normal Human Tissues, Cancers, and Tumor-Associated Macrophages. *The American Journal of Pathology.* 2000;157(2):411-21.
7. Semenza GL. Hypoxia-Inducible Factors in Physiology and Medicine. *Cell.* 2012;148(3):399-408.
8. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends in pharmacological sciences.* 2012;33(4):207-14.
9. Xue X, Taylor M, Anderson E, Hao C, Qu A, Greenson JK, et al. Hypoxia-inducible factor-2α activation promotes colorectal cancer progression by dysregulating iron homeostasis. *Cancer Research.* 2012;72(9):2285-93.
10. Xue X, Ramakrishnan S, Anderson E, Taylor M, Zimmermann EM, Spence JR, et al. Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice. *Gastroenterology.* 2013;145(4):831-41.
11. Fridlender ZG, and Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis.* 2012.
12. Rao H-L, Chen J-W, Li M, Xiao Y-B, Fu J, Zeng Y-X, et al. Increased Intratumoral Neutrophil in Colorectal Carcinomas Correlates Closely with Malignant Phenotype and Predicts Patients' Adverse Prognosis. *PLoS ONE.* 2012;7(1):e30806.
13. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of Tumor-Associated Neutrophil (TAN) Phenotype by TGF-β: "N1" versus "N2" TAN. *Cancer cell.* 2009;16(3):183-94.
14. Houghton AM, Rzymkiewicz DM, Ji H, Gregory AD, Egea EE, Metz HE, et al. Neutrophil Elastase-Mediated Degradation of IRS-1 Accelerates Lung Tumor Growth. *Nature medicine.* 2010;16(2):219-23.
15. Nozawa H, Chiu C, and Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America.* 2006;103(33):12493-8.
16. Mishalian I, Bayuh R, Eruslanov E, Michaeli J, Levy L, Zolotarov L, et al. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17—A new mechanism of impaired antitumor immunity. *International Journal of Cancer.* 2014;135(5):1178-86.

17. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *The Journal of Clinical Investigation.*124(12):5466-80.
18. Shah YM, Ito S, Morimura K, Chen C, Yim S-h, Haase VH, et al. Hypoxia-Inducible Factor Augments Experimental Colitis Through a MIF-Dependent Inflammatory Signaling Cascade. *Gastroenterology.* 2008;134(7):2036-48.e3.
19. Jamieson T, Clarke M, Steele CW, Samuel MS, Neumann J, Jung A, et al. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. *The Journal of Clinical Investigation.*122(9):3127-44.
20. Langmead B, Trapnell C, Pop M, and Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology.* 2009;10(3):1-10.
21. Trapnell C, Pachter L, and Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics.* 2009;25(9):1105-11.
22. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, and Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotech.* 2013;31(1):46-53.
23. Benjamini Y, and Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological).* 1995;57(1):289-300.
24. Xue X, and Shah YM. Hypoxia-inducible factor-2α is essential in activating the COX2/mPGES-1/PGE(2) signaling axis in colon cancer. *Carcinogenesis.* 2013;34(1):163-9.
25. Blaisdell A, Crequer A, Columbus D, Daikoku T, Mittal K, Dey Sudhansu K, et al. Neutrophils Oppose Uterine Epithelial Carcinogenesis via Debridement of Hypoxic Tumor Cells. *Cancer Cell.* 2015;28(6):785-99.
26. Taketo MM, and Edelmann W. Mouse Models of Colon Cancer. *Gastroenterology.* 2009;136(3):780-98.
27. Tanaka T, Kohno H, Suzuki R, Hata K, Sugie S, Niho N, et al. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in ApcMin/+ mice: Inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. *International Journal of Cancer.* 2006;118(1):25-34.
28. Pruenster M, Kurz ARM, Chung K-J, Cao-Ehlker X, Bieber S, Nussbaum CF, et al. Extracellular MRP8/14 is a regulator of β2 integrin-dependent neutrophil slow rolling and adhesion. *Nature Communications.* 2015;6:6915.
29. Taylor M, Qu A, Anderson ER, Matsubara T, Martin A, Gonzalez FJ, et al. Hypoxia-Inducible Factor-2α Mediates the Adaptive Increase of Intestinal Ferroportin During Iron Deficiency in Mice. *Gastroenterology.* 2011;140(7):2044-55.
30. Powell DR, and Huttenlocher A. Neutrophils in the Tumor Microenvironment. *Trends in Immunology.*37(1):41-52.
31. Isella C, Terrasi A, Bellomo SE, Petti C, Galatola G, Muratore A, et al. Stromal contribution to the colorectal cancer transcriptome. *Nat Genet.* 2015;47(4):312-9.
32. Dame MK, Jiang Y, Appelman HD, Copley KD, McClintock SD, Aslam MN, et al. Human colonic crypts in culture: segregation of immunochemical markers in normal versus adenoma-derived. *Laboratory investigation; a journal of technical methods and pathology.* 2014;94(2):222-34.

33. Ramakrishnan SK, Anderson ER, Martin A, Centofanti B, and Shah YM. Maternal intestinal HIF-2α is necessary for sensing iron demands of lactation in mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2015;112(28):E3738-E47.
34. Hu C-J, Sataur A, Wang L, Chen H, and Simon MC. The N-Terminal Transactivation Domain Confers Target Gene Specificity of Hypoxia-inducible Factors HIF-1α and HIF-2α. *Molecular Biology of the Cell.* 2007;18(11):4528-42.
35. Finisguerra V, Di Conza G, Di Matteo M, Serneels J, Costa S, Thompson AAR, et al. MET is required for the recruitment of anti-tumoural neutrophils. *Nature.* 2015;522(7556):349-53.
36. Xie L, Xue X, Taylor M, Ramakrishnan SK, Nagaoka K, Hao C, et al. Hypoxia-Inducible Factor/MAZ-Dependent Induction of Caveolin-1 Regulates Colon Permeability through Suppression of Occludin, Leading to Hypoxia-Induced Inflammation. *Molecular and Cellular Biology.* 2014;34(16):3013-23.
37. Smits M, Wurdinger T, van het Hof B, Drexhage JAR, Geerts D, Wesseling P, et al. Myc-associated zinc finger protein (MAZ) is regulated by miR-125b and mediates VEGF-induced angiogenesis in glioblastoma. *The FASEB Journal.* 2012;26(6):2639-47.
38. Kaneider NC, Agarwal A, Leger AJ, and Kuliopulos A. Reversing systemic inflammatory response syndrome with chemokine receptor pepducins. *Nat Med.* 2005;11(6):661-5.
39. Steele CW, Karim SA, Foth M, Rishi L, Leach JDG, Porter RJ, et al. CXCR2 inhibition suppresses acute and chronic pancreatic inflammation. *The Journal of Pathology.* 2015;237(1):85-97.
40. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau C-S, et al. IL17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature.* 2015;522(7556):345-8.
41. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang L-P, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells. *Nature.* 2011;475(7355):226-30.
42. Lee KE, Spata M, Bayne LJ, Buza EL, Durham AC, Allman D, et al. Hif1a Deletion Reveals Pro-Neoplastic Function of B Cells in Pancreatic Neoplasia. *Cancer Discovery.* 2016;6(3):256-69.
43. Zhou S-L, Zhou Z-J, Hu Z-Q, Huang X-W, Wang Z, Chen E-B, et al. Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib. *Gastroenterology.*150(7):1646-58.e17.
44. Zlotnik A, and Yoshie O. Chemokines: A New Classification System and Their Role in Immunity. *Immunity.* 2000;12(2):121-7.
45. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med.* 2015;21(8):938-45.
46. Shang K, Bai Y-P, Wang C, Wang Z, Gu H-Y, Du X, et al. Crucial Involvement of Tumor-Associated Neutrophils in the Regulation of Chronic Colitis-Associated Carcinogenesis in Mice. *PLoS ONE.* 2012;7(12):e51848.
47. Pine JK, Morris E, Hutchins GG, West NP, Jayne DG, Quirke P, et al. Systemic neutrophil-to-lymphocyte ratio in colorectal cancer: the relationship to patient survival,

tumour biology and local lymphocytic response to tumour. *Br J Cancer.* 2015;113(2):204-11.
48. Steele Colin W, Karim Saadia A, Leach Joshua DG, Bailey P, Upstill-Goddard R, Rishi L, et al. CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma. *Cancer Cell.*29(6):832-45.
49. Katoh H, Wang D, Daikoku T, Sun H, Dey SK, and DuBois RN. CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis. *Cancer cell.* 2013;24(5):631-44.
50. Campbell Eric L, Bruyninckx Walter J, Kelly Caleb J, Glover Louise E, McNamee Eóin N, Bowers Brittelle E, et al. Transmigrating Neutrophils Shape the Mucosal Microenvironment through Localized Oxygen Depletion to Influence Resolution of Inflammation. *Immunity.* 2014;40(1):66-77.
51. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, and Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *Journal of Clinical Investigation.* 2004;114(8):1098-106.
52. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, et al. Hypoxia-Inducible Factor 1–Dependent Induction of Intestinal Trefoil Factor Protects Barrier Function during Hypoxia. *The Journal of Experimental Medicine.* 2001;193(9):1027-34.
53. Xue X, Ramakrishnan SK, and Shah YM. Activation of HIF-1α does not increase intestinal tumorigenesis. *American Journal of Physiology - Gastrointestinal and Liver Physiology.* 2014;307(2):G187-G95.
54. Raval RR, Lau KW, Tran MGB, Sowter HM, Mandriota SJ, Li J-L, et al. Contrasting Properties of Hypoxia-Inducible Factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-Associated Renal Cell Carcinoma. *Molecular and Cellular Biology.* 2005;25(13):5675-86.
55. Criscimanna A, Duan L-J, Rhodes JA, Fendrich V, Wickline E, Hartman DJ, et al. PanIN-specific regulation of Wnt signaling by HIF2α during early pancreatic tumorigenesis. *Cancer research.* 2013;73(15):4781-90.
56. Lee K, Spata M, Bayne LJ, Buza EL, Durham AC, Allman D, et al. Hif1α deletion reveals pro-neoplastic function of B cells in pancreatic neoplasia. *Cancer discovery.* 2016;6(3):256-69.
57. Mazumdar J, Hickey MM, Pant DK, Durham AC, Sweet-Cordero A, Vachani A, et al. HIF-2α deletion promotes Kras-driven lung tumor development. *Proceedings of the National Academy of Sciences.* 2010;107(32):14182-7.
58. Scheuermann TH, Li Q, Ma H-W, Key J, Zhang L, Chen R, et al. Allosteric Inhibition of Hypoxia Inducible Factor-2 with Small Molecules. *Nature chemical biology.* 2013;9(4):271-6.

Table 2.1 Primer list

Cloning Primers	
pGL3-mCxcl1-luc1685 F	5'-ACGTGGTACCAGCTAATCTTAGGA
pGL3-mCxcl1-luc345 F	5'-ACGTGGTACCCACTGTAGTACACC
pGL3-mCxcl1-luc285 F	5'-ACGTGGTACCTGACCCACCTCG
pGL3-mCxcl1-R	5'-ACGTCTCGAGGTGGAGCTCTAG
qPCR Primers	
mCxcl1 F	5'-TCTCCGTTACTTGGGGACAC
mCxcl1 R	5'-CCACACTCAAGAATGGTCGC
hCxcl1 F	5'-AACAGCCACCAGTGAGCTTC
hCxcl1 R	5'-GAAAGCTTGCCTCAATCCTG
CD11b F	5'-ATGGACGCTGATGGCAATACC
CD11b R	5'-TCCCCATTCACGTCTCCCA
CD11c F	5'-CTGGATAGCCTTTCTTCTGCTG
CD11c R	5'-GCACACTGTGTCCGAACTCA
Emr1 F	5'-CCCCAGTGTCCTTACAGAGTG
Emr1 R	5'-GTGCCCAGAGTGGATGTCT
CD68 F	5'-CTTCCCACAGGCAGCACAG
CD68 R	5'-AATGATGAGAGGCAGCAAGAGG
iNOS F	5'-ACCCTAAGAGTCACCAAAATGGC
iNOS R	5'-TTGATCCTCACATACTGTGGACG
Ly6G F	5'-TGGACTCTCACAGAAGCAAAG
Ly6G R	5'-GCAGAGGTCTTCCTTCCAACA
Mpo F	5'-AGTTGTGCTGAGCTGTATGGA
Mpo R	5'-CGGCTGCTTGAAGTAAAACAGG
S100a8 F	5'-CCAATTCTCTGAACAAGTTTTCG
S100a8 R	5'-TCACCATGCCCTCTACAAGA

Figures

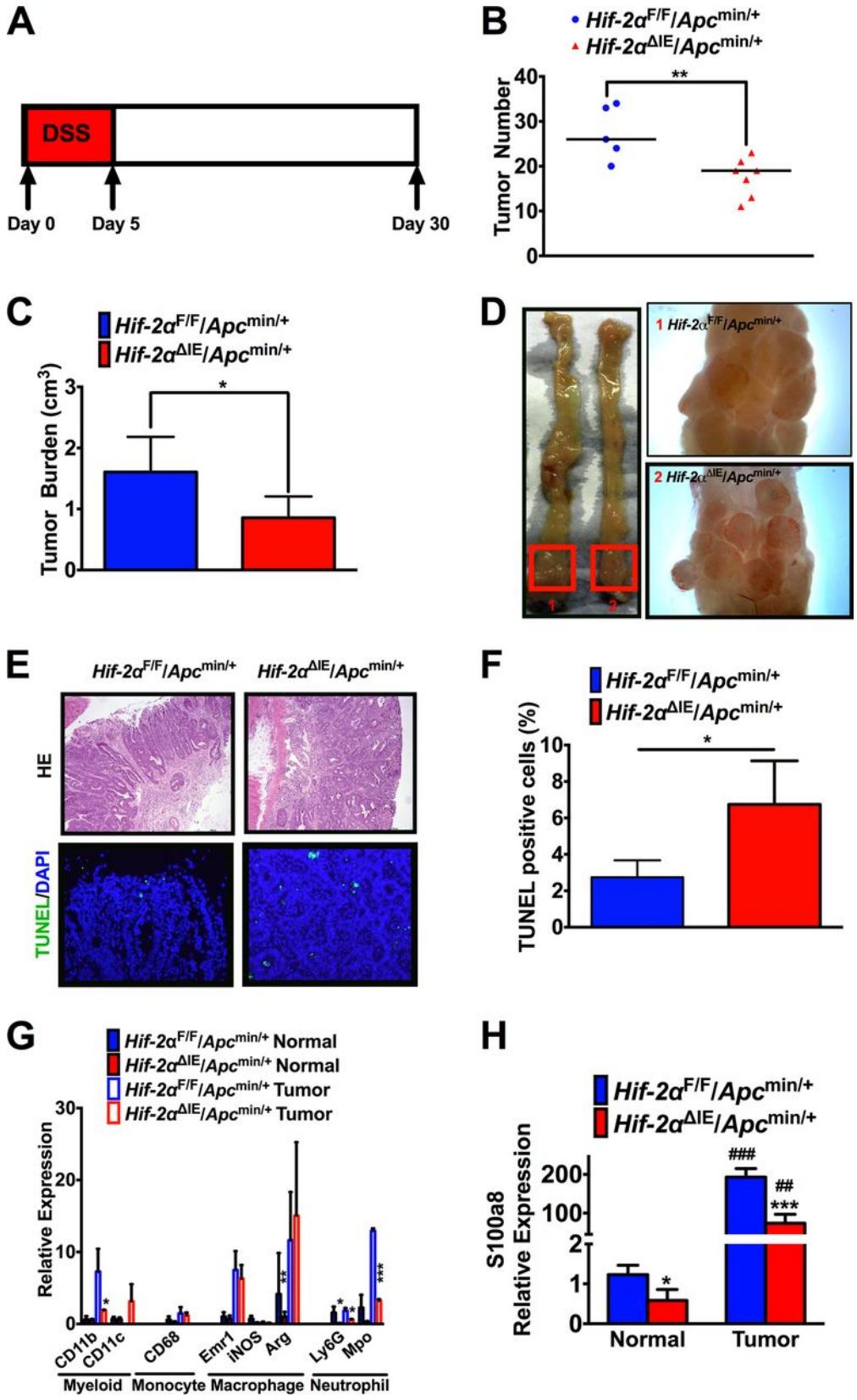
A
DSS
Day 0
Day 5
Day 30
B
Hif-2α^F/F/Apc^min/+
Hif-2α^ΔIE/Apc^min/+
**
Tumor Number
C
Tumor Burden (cm^3)
*
D
1 Hif-2α^F/F/Apc^min/+
2 Hif-2α^ΔIE/Apc^min/+
E
HE
TUNEL/DAPI
F
TUNEL positive cells (%)
*
G
Normal
Tumor
Relative Expression
CD11b
CD11c
CD68
Emr1
iNOS
Arg
Ly6G
Mpo
Myeloid
Monocyte
Macrophage
Neutrophil
H
S100a8
Relative Expression
###
##
***
Normal
Tumor

Figure 2.1. HIF-2α is essential for inflammation-induced colon tumorigenesis.
(A) *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ (n=5) and *Hif-2α*$^{\Delta IE}$/*Apc*$^{Min/+}$ (n=7) were treated for 5-days with DSS and analyzed 28-days following final day of DSS administration. (B) Tumor number, (C) tumor burden and (D) Gross image in the colon from *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ and *Hif-2α*$^{\Delta IE}$/*Apc*$^{Min/+}$ mice 25 days following DSS administration. (E) Representative images of H&E analysis and TUNEL staining, (F) quantification of TUNEL positive cells in tumor tissue from *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ and *Hif-2α*$^{\Delta IE}$/*Apc*$^{Min/+}$ mice. (G) qPCR expression analysis of myeloid cell markers and (H) S100a8 in tumors and/or adjacent normal tissue from *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ and *Hif-2α*$^{\Delta IE}$/*Apc*$^{Min/+}$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$mice, ## $p<0.01$, ### $p<0.001$ compared to normal tissue. Statistical analysis was performed by student's t-test or two-way ANOVA followed by Sidak's multiple comparisons test.

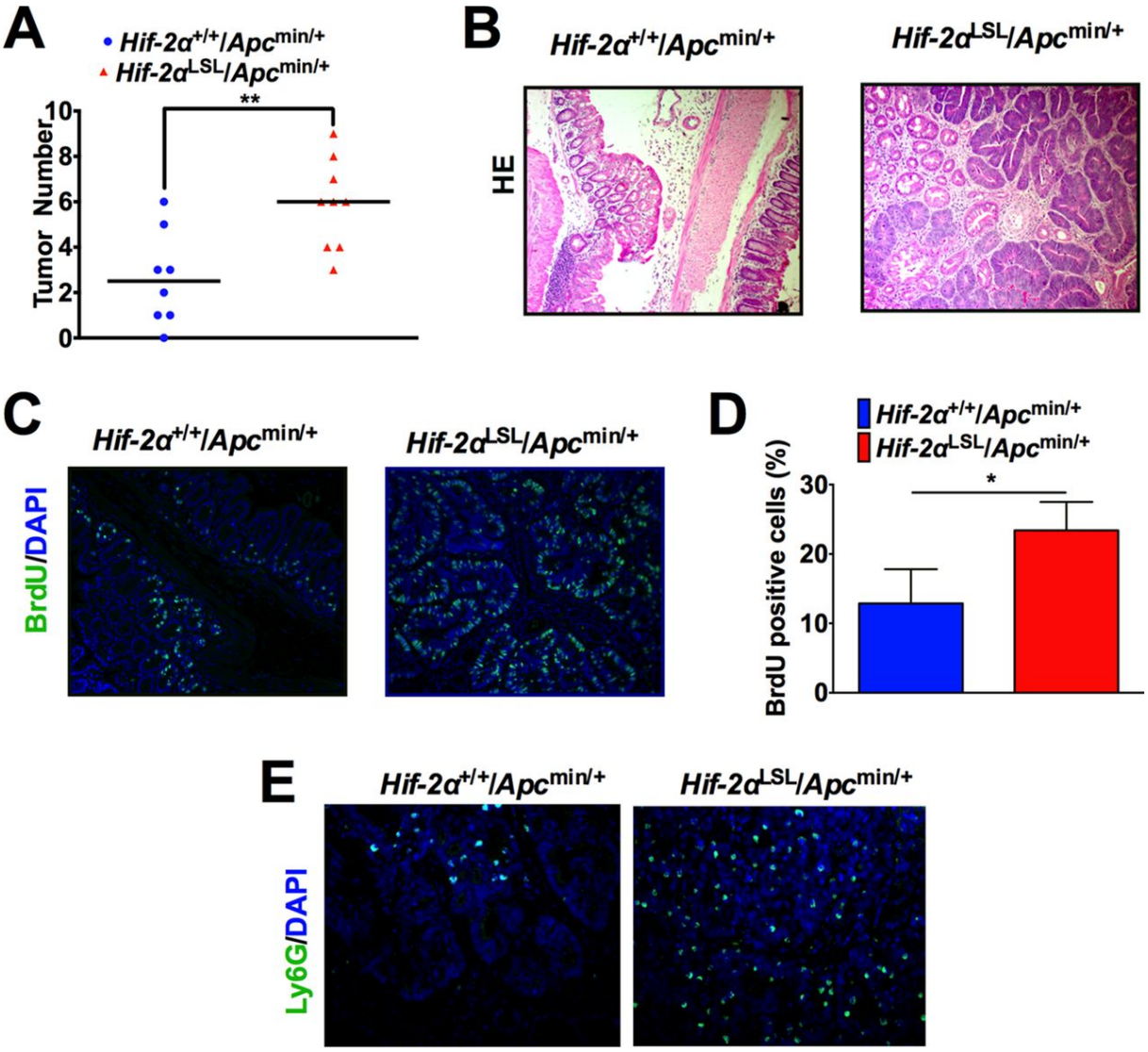

Figure 2.2. HIF-2α increases inflammation-induced colon tumorigenesis.
(A) Tumor number from colon tissue of *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ (n=8) and *Hif-2α*^{LSL}/*Apc*$^{Min/+}$ mice (n=9). (B) Representative images of H&E analysis. (C) Representative images of BRDU incorporation in tumors from *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ and *Hif-2α*^{LSL}/*Apc*$^{Min/+}$ mice. (D) Quanitficaiton from (C). * $p<0.05$. (E) Representative images of Ly6G immunofluorescence in tumors from *Hif-2α*$^{+/+}$/*Apc*$^{Min/+}$ and *Hif-2α*^{LSL}/*Apc*$^{Min/+}$ mice. Statistical analysis was performed by Student's t-test.

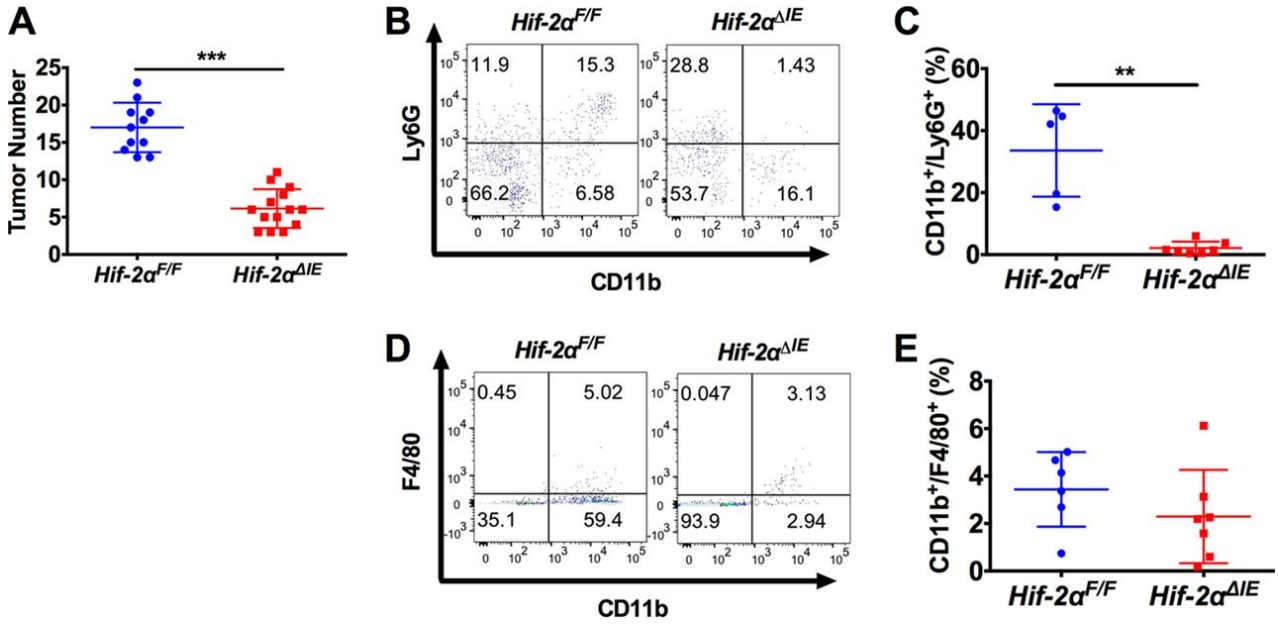

Figure 2.3. Disruption of intestinal epithelial HIF-2α decreases colon tumors and intratumoral neutrophils in a colitis-associated colon cancer model (CAC).
(A) Tumor number from colons of *Hif-2α*$^{F/F}$ (n=11) and *Hif-2α*$^{\Delta IE}$ (n=14) following AOM/DSS induced CAC. (B) Flow cytometry analysis of CD11b/Ly6G double positive cells or (D) CD11b/F4/80 double positive cells gated from CD45+ cells in tumors from *Hif-2α*$^{F/F}$ and *Hif-2α*$^{\Delta IE}$ mice. Tumors were collected from individual mice. (C and E) Quantification from flow cytometry data in tumors ** $p<0.01$ and *** $p<0.001$ compared to *Hif-2α*$^{+/+}$ mice. Statistical analysis was performed by Student's t-test.

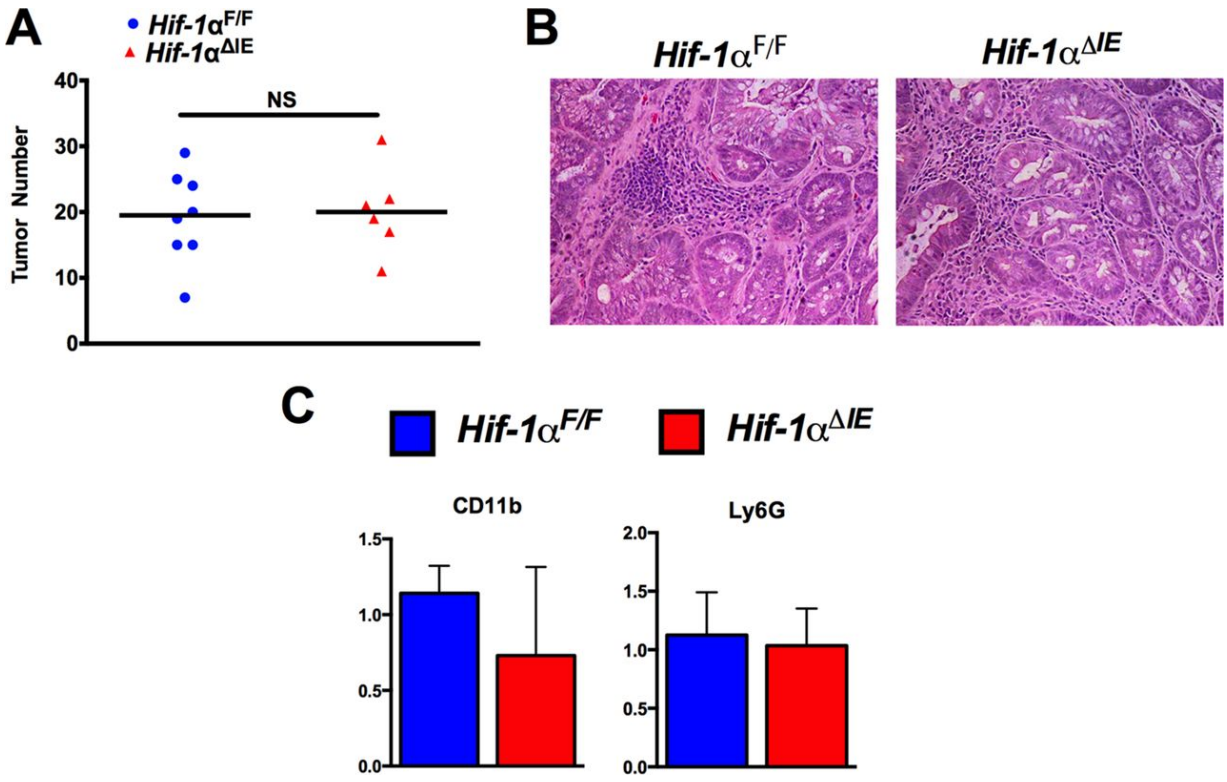

Figure 2.4. HIF-1α does not impact colon tumorigenesis or neutrophil recruitment. (A) Tumor number from colons of *Hif-1α*$^{F/F}$ (n=8) and *Hif-1α*$^{\Delta IE}$ (n=6) following AOM/DSS induced CAC. (B) Representative images of H&E staining from *Hif-1α*$^{F/F}$ and *Hif-1α*$^{\Delta IE}$ tumor tissue. (C) qPCR of *Cd11b* and *Ly6g* expression in tumor tissue from (A). Statistical analysis was performed by Student's t-test.

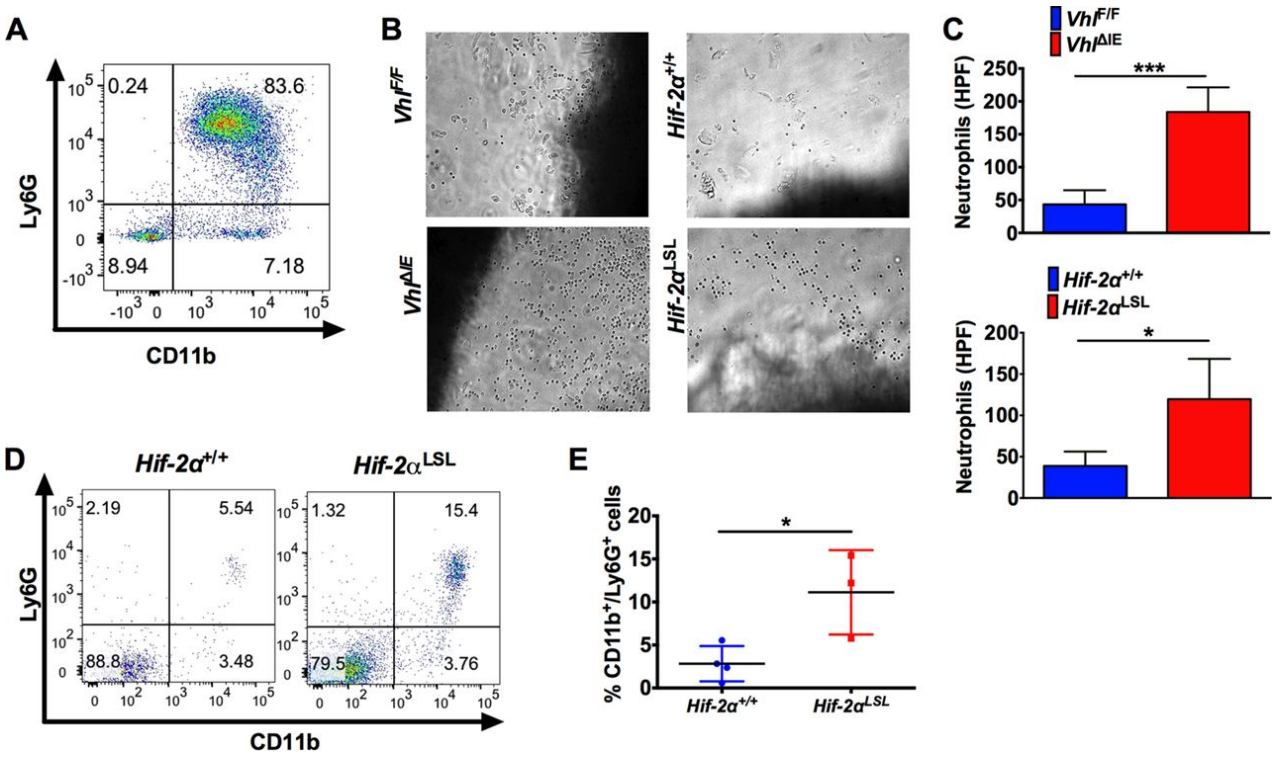

Figure 2.5. Intestinal HIF-2α activation promotes recruitment of neutrophils to the colon. (A) Flow cytometric analysis of CD11b/Ly6G staining of bone marrow isolated neutrophils. (B) Representative images of neutrophil or (C) quantification of cell numbers migrated into the bottom well of a transwell towards $Vhl^{F/F}$, $Vhl^{\Delta IE}$, $Hif\text{-}2\alpha^{+/+}$, $Hif\text{-}2\alpha^{LSL}$ colon tissue explants. (D) Flow cytometry analysis and (E) quantification of CD11b/Ly6G double positive cells gated from CD45+ cells in colon tissue from $Hif\text{-}2\alpha^{LSL}$ mice compared to $Hif\text{-}2\alpha^{+/+}$ mice. * $p<0.05$ and *** $p<0.001$ compared to $Vhl^{F/F}$ or $Hif\text{-}2\alpha^{+/+}$ mice. Statistical analysis was performed by Student's t-test.

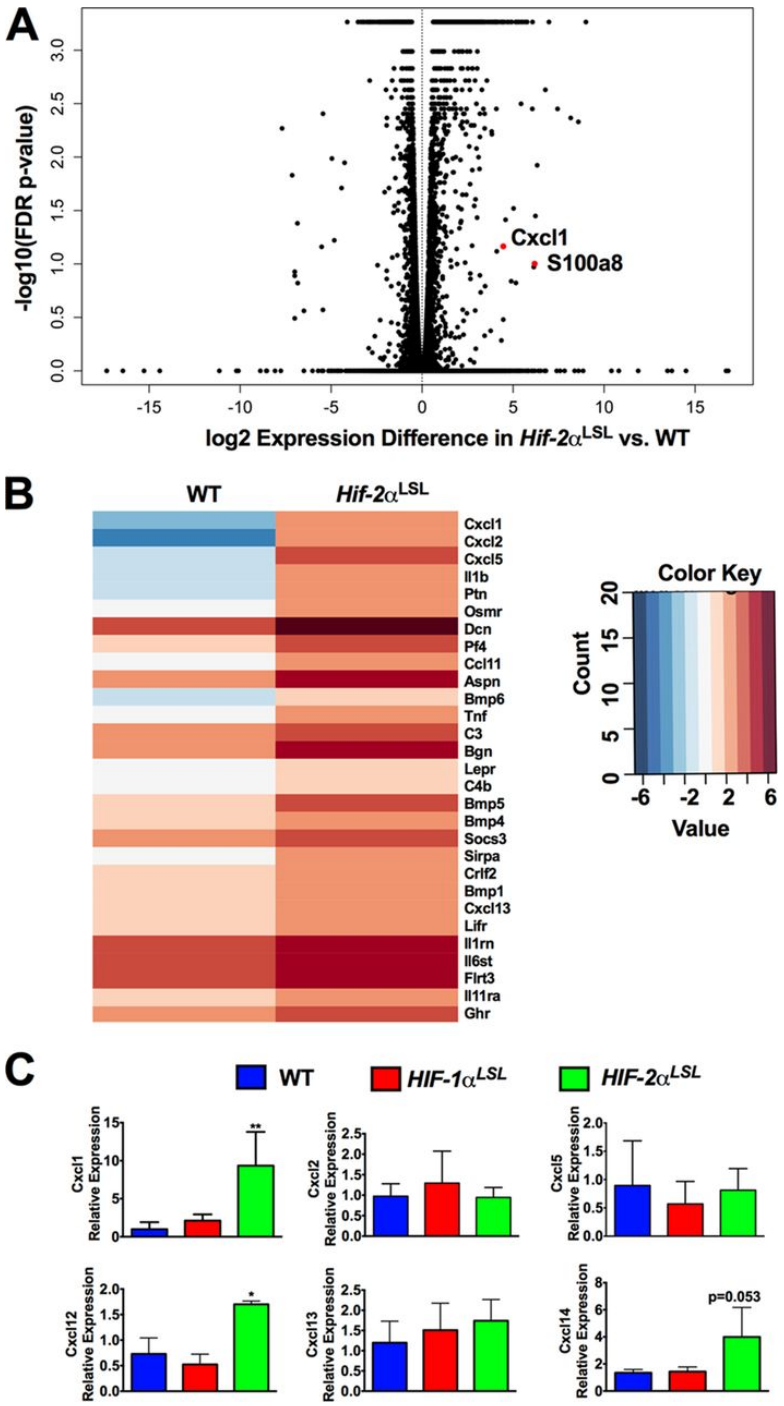

Figure 2.6. Activation of intestinal epithelial HIF-2α increases CXCL1 expression. Volcano plot of RNA-Seq analysis in colon tissues from *Hif-2α*$^{+/+}$ (n=6) and *Hif-2α*^{LSL} mice (n=6). (B) Heatmap of genes related to cytokine activity enriched by PANTHER gene ontology analysis from *Hif-2α*$^{+/+}$ and *Hif-2α*^{LSL} colon tissues. (C) qPCR analysis of CXC-family of chemokines in colon tissue from WT, *Hif-1α*^{LSL}, and *Hif-2α*^{LSL} mice. * $p<0.05$ and ** $p<0.001$ compared to normal colon tissues. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test.

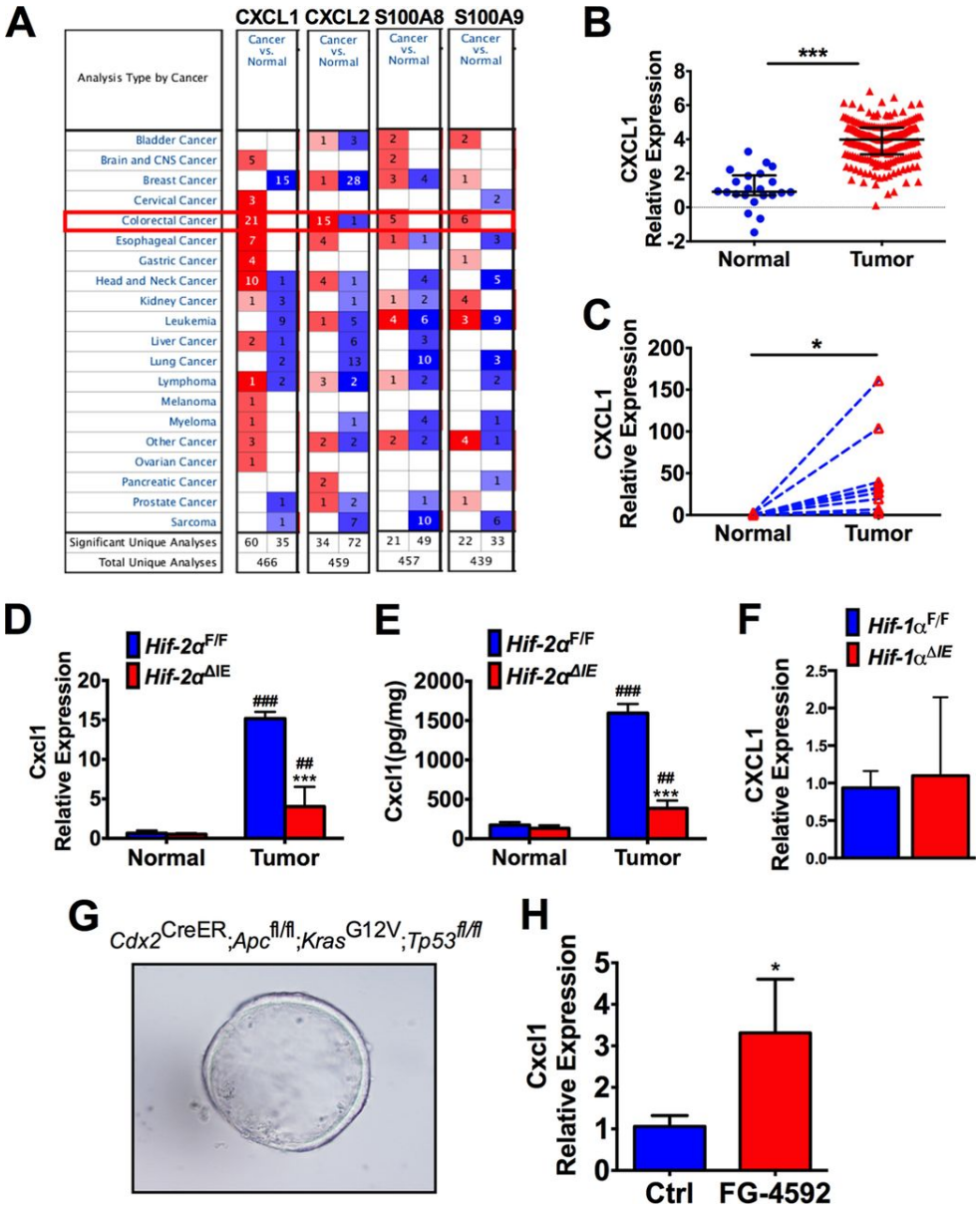

Figure 2.7. Epithelial HIF-2α is essential for CXCL1 expression in colon tumors
Oncomine database analysis of CXCL1, CXCL2 and neutrophil markers in several independent microarrays analysis from colon cancer and normal tissue. CXCL1 gene expression in (B) The Cancer Genome Atlas (TCGA) dataset and (C) a set of 8 pairs of colon tumor and adjacent normal tissue collected at University of Michigan (Umich). (D) qPCR analysis of *Cxcl1* mRNA or (E) ELISA analysis of CXCL1 protein in CAC tumor tissue and adjacent normal tissue from *Hif-2α*$^{F/F}$ and *Hif-2α*$^{\Delta IE}$ mice. (F) qPCR analysis of *Cxcl1* expression in tumors from *Hif-1α*$^{F/F}$ and *Hif-1α*$^{\Delta IE}$ mice. (G) Representative image of colon enteroids following deletion of *Apc* and *TP53* and activation of Kras. (H) qPCR of *Cxcl1* expression in colon enteroids treated with FG-4592 or vehicle control (Ctrl). * p<0.05. *p<0.05, ** p<0.01 and *** p<0.001 compared to WT, *Hif-2α*$^{F/F}$ or Ctrl. ## p<0.01, ### p<0.001 compared to normal tissue. Expression was normalized to β-Actin. Statistical analysis was performed by Student's t-test, paired t-test or two-way ANOVA followed by Sidak's multiple comparisons test.

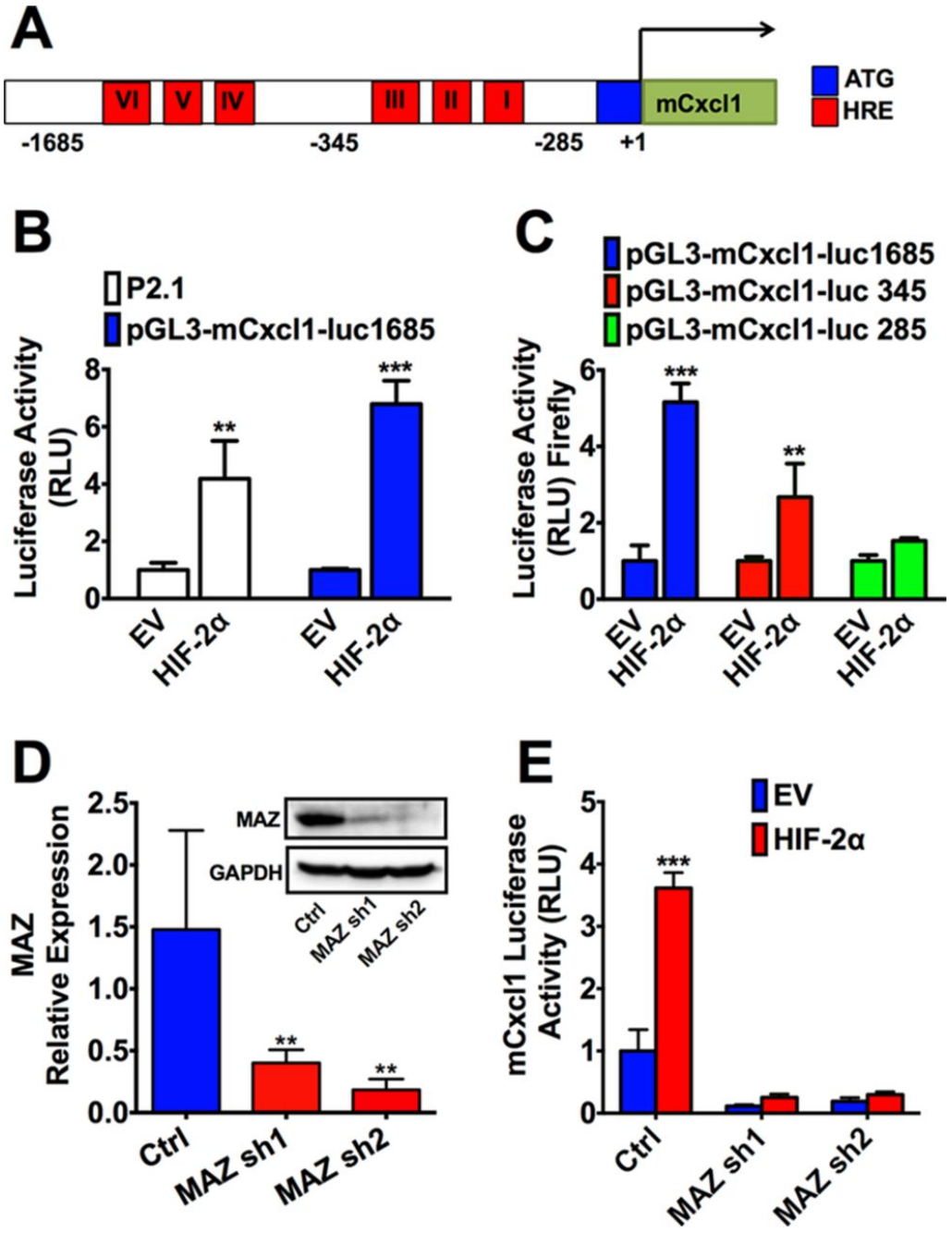

Figure 2.8. HIF-2α and MAZ are essential for CXCL1 activation.
Analysis of the *Cxcl1* proximal promoter shows there are six HREs present (I-VI) in proximal and distal areas. (B) *Cxcl1* or the enolase (P2.1) promoter luciferase activity assays in HCT116 cells expressing HIF-2α. (C) *Cxcl1* promoter luciferase activity assays with deletion constructs in HCT116 cells expressing HIF-2α. (D) qPCR and western blot analysis of MAZ knockdown efficiency in HCT116 cells expressing MAZ targeting shRNAs (MAZ sh1 & MAZ sh2). (E) CXCL1-promoter luciferase activity assay in HCT116 cells expressing MAZ targeting shRNAs. *p<0.05, ** p<0.01 and *** p<0.001 compared to EV or Ctrl. Statistical analysis was performed by two-way ANOVA followed by Sidak's multiple comparisons test.

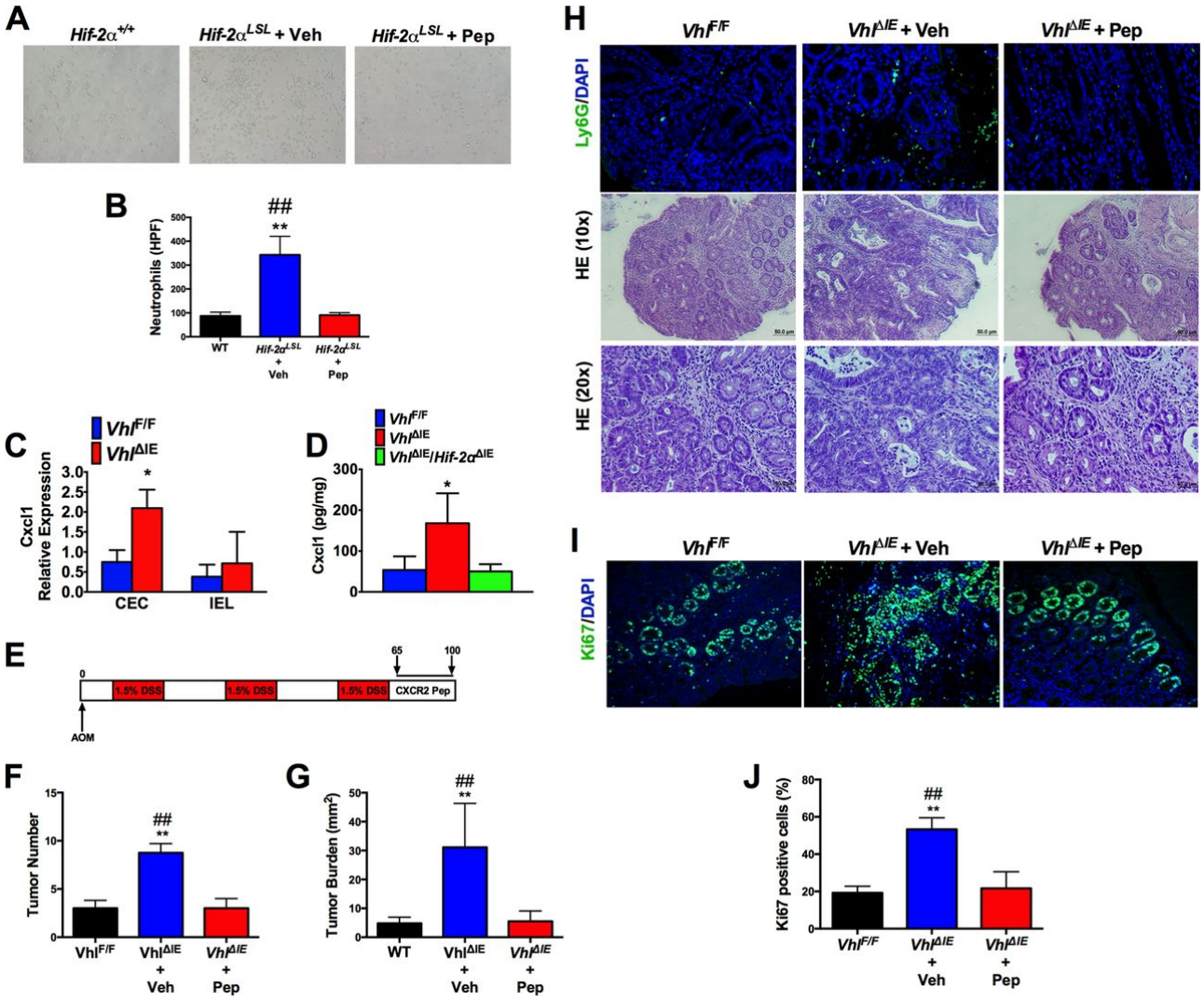

Figure 2.9. HIF-2α-mediated neutrophil recruitment is essential for colon tumorigenesis. (A) Representative images of neutrophils and (B) quantification from Ctrl pepducin (Veh) or CXCR2 pepducin (PEP) treated migrated into the bottom well of a transwell towards *Hif-2α*$^{+/+}$ and *Hif-2α*^{LSL} colon tissue explants. (C) qPCR analysis of *Cxcl1* expression in colon epithelial cells (CEC) compared to intraepithelial lymphocytes (IEL) of *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice. (D) ELISA analysis of CXCL1 protein in colon tissue of *Vhl*$^{F/F}$ (n=6), *Vhl*$^{\Delta IE}$ (n=3), *Vhl*$^{\Delta IE}$*/Hif-2α*$^{\Delta IE}$ (n=4) mice. (E) Schematic diagram for AOM/DSS-induced CAC and CXCR2 pepducin treatment protocol. (F) Tumor counting (G) tumor burden analysis, (H) representative images of H&E staining and Ly6G staining and (I) Ki67 staining and (J) quantification of Ki67 staining in colon tissue from *Vhl*$^{F/F}$ (n=4), *Vhl*$^{\Delta IE}$ + Veh (n=4), and *Vhl*$^{\Delta IE}$ + PEP (n=3) groups. ** p<0.01 compared to *Vhl*$^{F/F}$. ## p<0.01 compared to *Vhl*$^{\Delta IE}$ + CXCR2 pepducin. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test.

Chapter 3

Neutrophils Restrict Tumor-Associated Microbiota to Dampen Colon Tumor Growth and Progression

Abstract

Neutrophils are among the most prevalent immune cells in the microenvironment of colon tumors. Neutrophils are largely believed to favor colon tumor growth and are predictive biomarkers for colon cancer patients. Clinical trials targeting neutrophil trafficking in cancer are initiated. However, very little is known about neutrophil function in the early steps of colon tumorigenesis. To clearly understand the role of neutrophils in colon cancer we utilized mice with constitutive genetic depletion of neutrophils. In an inflammation-induced and sporadic colon tumor models, depletion of neutrophils increased progression and invasion of colon tumors. Mechanistically, neutrophil depletion correlated with increased tumor-associated bacteria, high intratumoral B-cells, increased proliferation, heightened DNA damage, and heightened inflammatory response through IL-17 and NF-*k*B. Antibiotic treatment or B-cell depletion in neutrophil deficient animals dramatically reversed tumor progression. Our findings indicate a critical role for neutrophils in the repression of colon tumor growth and progression.

Introduction

It has become widely appreciated that in addition to genetic hits such as *APC* loss, *KRAS* activation, and *TP53* loss, the tumor immune response plays a critical role in the neoplastic progression of colon cancer (1). Chronic inflammation of the colon, in the form of inflammatory bowel disease (IBD), is a significant risk factor for the development of colon cancer. The colon tumor immune microenvironment consists of cells in the innate and adaptive response with variable roles in tumor growth (1). Anti-tumor immune cells, such as T-cell and natural killer cells dampen tumorigenesis and tumor progression and pro-tumorigenic immune cells such as T-regulatory cell and tumor associated macrophages foster tumor growth and immune evasion (2). As tumors progress, mechanisms such as decreased tumor O_2 tension alter immune cell differentiation and effector functions to tip the balance in favor of the pro-tumor immune response and enhance tumor growth and progression (2). Better understanding of the tumor immune response may provide novel therapeutic avenues for treatment of disease.

Polymorphonuclear neutrophils (PMNs) are myeloid cells of the innate immune system known for their role in the acute host response to infection. PMN effector functions consist of bacterial phagocytosis, generation and release of cytotoxic granule proteins, and production of superoxide radicals (3). Evidence has emerged that PMNs are among the most prevalent immune cells type in several human cancers, including colon cancer, pancreatic cancer, and lung cancer (4). The functional role for PMNs in the progression of cancer has largely been suggested to be pro-tumorigenic through suppression of antitumor immunity and direct activation of tumor cell growth (5, 6). Recent meta-analysis demonstrates PMNs have the highest correlation with adverse outcomes across all cancers (7). Despite these observations, the primary contribution of PMNs to the initiation of colon tumorigenesis is not clear. Therefore, a more thorough

investigation of PMNs in cancer is necessary to identify their potential for therapeutic targeting in colon cancer.

The majority of studies have relied upon antibody mediated PMN depletion and PMN trafficking receptor inhibitors which may have off-target effects and may not completely eradicate intra-tumoral PMNs in colon cancer. In the current study, a murine model of genetic PMN depletion was assessed in mouse models of colitis and colon cancer. Genetic depletion of PMNs robustly enhanced tumor progression and invasion in a colitis-associated tumor model as well as a sporadic colon tumor model. Interestingly, genetic PMN depletion promoted a dramatic expansion of tumor-associated bacteria that was critical for the increased tumor progression. This also led to accumulation of intra-tumoral B-cells which played an important role in increased tumor growth and progression. Therapeutic targeting of granulocytic myeloid cells and PMNs is currently being evaluated for clinical utility in several cancer types (4). Our data delineates an important role for neutrophils in blunting colon tumor progression and invasion and provides caution against anti-PMN therapy in colon cancer, particularly in the context of preceding inflammation and in earliest stages of neoplastic disease.

Methods

Animals

For all experiments, male and female mice aged 6 to 8-weeks were used. All mice are C57BL/6 background. $LysM^{Cre};Mcl1^{fl/fl}$ mice were previously described (8). All animals used are C57BL/6 background. To delineate the role of neutrophils in colon cancer, the AOM/DSS model was utilized. For AOM/DSS experiments, animals were injected I.P. with 10mg/kg azoxythmethane then cycled on and off DSS in their drinking water for seven days for three

cycles interspersed with two weeks of regular drinking water beginning five-days following AOM injection. For acute DSS experiments, animals were treated with 2.5% DSS in drinking water for seven days then changed back to regular drinking water for two days. *Cdx2*-CreER^{T2}*;Apc$^{fl/fl}$* mice have been previously described (9). *Mrp8-Cre* expressing mice were previously described and crossed to *Mcl-1$^{fl/fl}$* mice (10). Bone marrow transplantation was performed by isolation of single cell suspension of bone marrow cells from femur and tibia of 6- to 8-week old mice *LysM^{Cre};Mcl1$^{fl/fl}$*, *Mrp8^{Cre};Mcl1$^{fl/f}$*, and *LysM^{Cre};Mcl1$^{wt/wt}$* mice. 1x10^6 bone marrow cells were injected into the tail vein of recipient mice lethally irradiated with two split doses of 6gy radiation four-hours apart. Mice were supplemented with Neomycin (1g/ml) in their drinking water for two-weeks. Four-weeks post transplantation, recipient mice were injected I.P. with tamoxifen (100mg/kg) in corn oil and sacrificed 14-days after administration. For antibiotics treatment, AOM/DSS was induced in *LysM^{Cre};Mcl1$^{wt/wt}$* and *LysM^{Cre};Mcl1$^{fl/fl}$* mice with 1.5% DSS. 14-days after administration of AOM, mice were placed on antibiotics drinking water (neomycin 1mg/ml, ampicillin 1mg/ml, and streptomycin 0.5mg/ml). Every other day, mice were orally gavaged with antibiotics solution (neomycin 2.5mg/ml, ampicillin 2.5mg/ml, streptomycin 1.25mg/ml, and metronidazole 1.25mg/ml) and sacrificed 72-days after AOM administration. For B-cell inhibition experiments, colon tumorigenesis was initiated in *LysM^{Cre};Mcl1$^{wt/wt}$* and *LysM^{Cre};Mcl1$^{fl/fl}$* mice using AOM and 1.5% DSS. One-week following the third cycle of DSS, *LysM^{Cre};Mcl1$^{fl/fl}$* mice were randomized into Ctrl and anti-B220 treatment groups (BioXcell). Mice were treated every fourth day with 400ug I.P. anti-B220 diluted in sterile 1x PBS pH 7.0.

Flow cytometry

Fresh normal and colon tumor tissue were finely minced followed by incubation with Collagenase Type II (Sigma-Aldrich) (1mg/ml) 1-hour in cold HBSS. Digested tissues were then passed through a 40um cell strainer. Peripheral blood was prepared by lysing one drop of tail blood for twenty minutes with RBC lysis buffer. White blood cells were then pelleted and passed through a 40um cell strainer. Single-cell suspensions were stained with eFluor780- anti-CD45 (eBioscience), PE-anti-Ly6G (BD), APC-anti-Cd11b (eBioscience), FITC-anti-B220 (eBioscience) and eFluor450 anti-F4/80 (eBioscience). Flow cytometry was performed using an LSRFortessa (BD). Flow cytometry data was analyzed using FlowJo software (BD Biosciences).

Histology and immunofluorescence

Colon tissue was excised, cut longitudinally, swiss-rolled, and fixed in 10% formalin overnight. Tissues were then embedded in paraffin and cut in 5μm sections and stained with hematoxylin and eosin and scored by a blinded gastroenterology pathologist. Tumor invasion scoring was done as previously described (11). Immunofluorescence was performed following antigen retrieval of 5um paraffin slides. Antibodies for immunofluorescence were as follows: Ki67 (1:100, Vector Labs), p-H2AX (1:200, Cell Signaling Technologies), cCasp3 (1:800, Cell Signaling Technologies) and Alexa Fluor® 488 goat anti-rabbit IgG (1:500, Molecular Probes Inc). B220 staining was performed following antigen retrieval of 5um paraffin slides using anti-B220 FITC antibody (eBioscience). Bacteria labeling was performed with Cy3 labeled EUB338 probe (5'-Cy3 GCTGCCTCCCGTAGGAGT). Briefly, paraffin tissue sections were deparaffinized in xylenes (2x for 5 minutes) followed by 100% ethanol (2x for 5 minutes) then rinsed in D.I. water. Slides were incubated with 100ul of 5ng/ul EUB338 for two hours at 46^{O}C in hybridization buffer (35% formamide, 20mM Tris-HCl pH7.2, 0.9M Nacl, 0.01% SDS) then

washed for 40 minutes at 48^{O}C and mounted with ProLong^{TM} Gold reagent with Dapi (Invitrogen). cCasp3 and p-H2AX were quantified using ImageJ software as number of positive cells per high powered field (HPF). EUB338 was quantified as percent of EUB338 positive area per Dapi positive area.

RNA isolation, qPCR analysis

RNA was isolated using TRIzol reagent (Ambion). RNA was reverse transcribed to cDNA using MMLV reverse transcriptase (Fisher Scientific). qPCR analysis was performed using Radiant Green qPCR mix (Alkali Scientific Inc.).

Protein isolation and Western blotting

Tissues were lysed in radioimmunoprecipitation assay buffer (RIPA) (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP-40, 0.1% SDS). Proteins were separated and using SDS-PAGE and transferred to nitrocellulose membrane. Antibodies used are as follows: phosphor-P65 (1:1000, Cell Signaling Technologies), total-P65 (1:1000 Cell Signaling Technologies), phospho-STAT3 (1:1000, Cell Signaling Technologies), total-STAT3 (1:1000 Cell Signaling Technologies), GAPDH (1:1000, Santa Cruz Biotechnology), PCNA (1:1000, Cells Signaling Technologies).

IgA ELISA

Fecal IgA was measured using Mouse IgA ELISA Quantitation Kit according to manufacturer's protocol (Bethyl Laboratories, Inc).

High throughput RNA sequencing (RNA-seq) and RNA-Seq data analysis

TruSeq RNA library prep kit v2 (Illumina) was used to prepare RNA sequencing libraries. Libraries were sequenced using single-end 50-cycle reads on a Illumina HiSeq 2500 sequencer. The Flux high-performance computer cluster at the University of Michigan was used for computational analysis. RNA-seq read quality was assessed utilizing FastQC. Reads were aligned to a splice junction aware build of the mouse genome (mm10) using STAR(12) with the options "outFilterMultimapNmax 10" and "sjdbScore 2". Differential expression testing between WT and *Mcl-1*$^{+/-}$, *Mcl-1*$^{-/-}$ colon samples was conducted with CuffDiff v 2.1.1 with the parameter settings "-compatible-hits-norm," and "–frag-bias-correct". UCSC mm10.fa was used and the GENCODE mouse M12 primary assembly annotation GTF as the reference genome and reference transcriptome, respectively. Genes were considered differentially expressed at a false-discovery rate-adjusted (FDR) *P* value of <0.05.

16S rRNA gene sequencing and bacterial community analysis

Feces were collected at 65 and 75-days after AOM/DSS induced colon tumorigenesis in *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice. At day 75, tumors were collected and snap frozen. Bacterial sequencing was performed at the Microbial Systems Molecular Biology Lab. The Dual-indexing sequencing strategy was used to amplify the V4 region of the 16s rRNA gene from each sample. PCR was performed under the following conditions 95^{o}C, 2min; [95^{0}C, 20 secs; 55^{0}C, 15 secs] – 30 cycles; 72^{0}C, 10 min; 4^{0}C using Accuprime High Fidelity Taq (ThermoFisher). Samples were normalized using the SequalPrep Normalization plate kit (ThermoFisher). Sequencing was done on the Illmina MiSeq platform, using a MiSeq Regent Kit

V2 500 cycles, according to the manufacturer's instructions (13). Bacterial Community analysis was done base on Mothur wiki.

Statistical analysis

P-values were calculated by students t-test or one-way ANOVA. Error bars represent standard error of the mean.

Results

Neutrophil deficiency enhances the acute inflammatory response in colitis

PMN infiltration in IBD is a marker of disease severity and progression (14). In acute DSS-induced colitis in mice, neutrophils robustly infiltrated inflamed mucosal tissues as early as 4-days after initiation of colitis, were maintained through active inflammation at 7-days, and were quickly lost during resolution (Fig. 1A & B). Macrophage influx is milder and less robust (Fig. S1A & B). To dissect the role for PMNs in the inflammatory progression of colitis, we utilized mice with a genetic neutrophil deficiency. Mcl-1, an anti-apoptotic member of the Bcl-2 family, is a PMN-specific survival molecule when disrupted in myeloid cells using *LysM*-cre (*LysM*^{Cre};*Mcl1*$^{fl/fl}$) (Fig. 1C) (8). Mice with myeloid deficiency of *Mcl-1* had greater than 50% reduction of circulating PMNs in heterozygous floxed animals (*LysM*^{Cre};*Mcl1*$^{fl/wt}$) and greater than 90% reduction in PMNs in homozygous floxed animals (*LysM*^{Cre};*Mcl1*$^{fl/fl}$) (Fig. 1D & E). This was accompanied by no significant change in the number of circulating monocytes as previously reported (Fig. S2A) (15). Furthermore, no changes in basal histological colon architecture and cell proliferation were observed (Fig. S2B& C).

To evaluate the function of PMN depletion of the acute response in the DSS-injury model, *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice were treated with 2.5% DSS in their drinking water for 7-days then changed back to regular drinking water for 2-days. Compared to *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ mice, *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice failed to recover body weight by 9-days and had significant colon length shortening (Fig. 1F & G). Histologic analysis shows significant destruction of colonic architecture in *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ colon tissue compared to *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and higher histopathologic inflammation score than *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ (Fig. 1H & I). Interestingly, PMN-deficiency did not change expression of *Tnfa* and *Il22*, but correlated with increased expression of *Il17* and *Il6* (Fig. 1J).

Neutrophils inhibit colitis-associated colon tumorigenesis.

Next, the functional role for genetic PMN depletion in inflammation-induced cancer of the colon was assessed. PMNs are among the most prevalent immune cell type in the colon tumor microenvironment in the AOM/DSS model (Fig. S3A & B). The function for PMNs in the progression of inflammation-induced colon cancer has largely been suggested to be pro-tumorigenic (16). However, genetic PMN ablation early in colon tumorigenesis has not been assessed. To address this, *LysM*^{Cre}*;Mcl1*$^{wt/wt}$, *LysM*^{Cre}*;Mcl1*$^{fl/wt}$, and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice were I.P. injected with AOM (10mg/kg) and cycled on and off 2% DSS in their drinking water and sacrificed at day 87 after AOM administration (Fig. 2A). Weight loss is a surrogate for morbidity in AOM/DSS-induced colitis-associated colon tumorigenesis. By the end of the third cycle of DSS, *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice failed to recover body weight by day 87 (Fig. 2B). Compared to *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ animals, *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice developed significantly larger tumors (Fig. 2C & D). No change in the total tumor number was observed between *LysM*^{Cre}*;Mcl1*$^{wt/wt}$,

LysM^{Cre}*;Mcl1*$^{fl/wt}$, and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice (Fig. 2E). However, the tumor burden and average tumor size were significantly increased in *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice (Fig. 2F & G). To confirm depletion of neutrophils was maintained in colon tumors, flow cytometric analysis of primary tumor tissue was performed. Whereas 30-40% of the white blood cells (WBCs) present in tumors from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ mice were double positive for CD11b and Ly6g, less than 10% of the WBCs were double positive in *LysM*^{Cre}*;Mcl1*$^{fl/wt}$ mice, and virtually no double positive cells were detected in the *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice (Fig. 2H and I). No change in the percentage of tumor-associated macrophages was observed (Fig. S4A & B). These data suggest that PMNs play a critical role in inhibiting colitis-associated colon tumorigenesis by restricting tumor size and growth.

Neutrophil depletion increases colon tumor invasion and proliferation.

In the AOM/DSS model, highly invasive disease is rare and in many cases requires additional genetic hits (11). Further histologic characterization of colon tumors showed that no tumors in *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ mice developed into invasive adenocarcinomas (Fig. 3A). Strikingly, 25% of tumors from *LysM*^{Cre}*;Mcl1*$^{fl/wt}$ mice were invasive adenocarcinomas and this was potentiated in the *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice as greater than 60% of colon tumors in *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice were invasive (Fig. 3B). 16% of tumors from *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ had invaded through muscularis propria and many tumors could be detected in perirectal fat (Fig. 3C & D). In addition to increased tumor invasion, PMN depletion dramatically increased colon tumor proliferation as measured by Ki67 incorporation and proliferating cell nuclear antigen (PCNA) expression (Fig. 3E & F). No significant difference in tumor cell apoptosis was observed (Fig.

S5A & B). These data delineate an important function for intra-tumoral PMNs in restricting tumor invasion and proliferation.

Neutrophil depletion enhances tumor progression in genetic colon cancer models.

The data thus far shows that PMNs restrict tumor progression in inflammation-driven colon tumors. However, the vast majority of colon tumors are not preceded by chronic inflammation and develop due to mutations in the Adenomatous polyposis coli (*APC*) gene (17). In mice, truncation of a single *Apc* allele is sufficient to cause spontaneous intestinal tumorigenesis, albeit the vast majority of these tumors are localized to the small intestine (18). Mice with a colon-specific disruption of *Apc* using the colon-specific homeobox 2 (*Cdx2*) Cre (*Cdx2*-CreER^{T2}; $Apc^{fl/fl}$) develop colon tumors after administration of tamoxifen (9). To understand the role of PMN depletion in sporadic tumorigenesis, *Cdx2*-CreER^{T2}; $Apc^{fl/fl}$ mice were transplanted with bone marrow from either $LysM^{Cre};Mcl1^{wt/wt}$ or PMN-deficient $LysM^{Cre};Mcl1^{fl/fl}$ mice (Fig. 4A). Chimeras transplanted with $LysM^{Cre};Mcl1^{fl/fl}$ bone marrow had depleted circulating PMNs (Fig. 4B and C). *Cdx2*-CreER^{T2};$Apc^{fl/fl}$ mice were administered a single injection of tamoxifen (100mg/kg, I.P.) and were sacrificed and analyzed for early neoplastic changes 14-days after tamoxifen injection. In $LysM^{Cre};Mcl1^{wt/wt}$ chimeras, only low-grade adenomas were observed. In $LysM^{Cre};Mcl1^{fl/fl}$ chimeras, 3 out of 7 mice developed submucosal invasive adenocarcinoma (AdenoCa-T1) (Fig. 4D & E). This suggests that PMNs restrict early tumor progression in colon cancer independent of acute exacerbations of colitis in the AOM/DSS model.

LysM-Cre is expressed in multiple myeloid cell types including monocytes, mature macrophages, and granulocytes (19). Therefore, off-target effects cannot be excluded. We next

assessed the dependence of PMN-specific *Mcl-1*-deletion for the enhanced tumor progression. To specifically delete *Mcl-1* in PMNs, *Mcl-1* floxed animals were crossed to the PMN-specific *Mrp8-Cre* transgenic mice (10). *Cdx2*-CreER^{T2};*Apc*$^{fl/fl}$ animal chimeras transplanted with *Mrp8*^{Cre};*Mcl1*$^{-/-}$ bone marrow chimeras had significant reduction of circulating PMNs (Fig. 4 F-H). *Mrp8*^{Cre};*Mcl1*$^{-/-}$ mice had no significant difference in circulating monocytic cells (Fig. S6A & B). Strikingly, at fourteen days after tamoxifen administration, *Mrp8*^{Cre};*Mcl1*$^{-/-}$ bone marrow recapitulated the increased invasiveness observed in *LysM;Mcl-1*$^{-/-}$ mice with areas of invasion into and through the muscularis propria (Fig. 4I & J). These data indicate that PMN depletion early in sporadic colon tumorigenesis dramatically increases progression and invasion.

Neutrophils restrict colon tumor-associated bacteria expansion

To identify mechanisms by which PMN-depletion enhanced colon tumor progression, RNA-seq analysis was performed on tumor tissue from *LysM*^{Cre};*Mcl1*$^{wt/wt}$, *LysM*^{Cre};*Mcl1*$^{fl/wt}$, and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. Several genes with known roles in anti-microbial defense were significantly reduced (*Cfd*, *Lyz2*, *Defa27*, *Mcpt1*) (Fig. 5A). Additionally, several B-cell associated genes (*Ighd, Ighg1, Fcmr*) were highly upregulated (Fig. 5A). Neutrophils are well described for their anti-microbial and phagocytic functions. Neutrophil deficient *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice have previously been shown to have higher attachment and decreased eradication of the pathogenic bacteria *Citrobacter rodentium* in the colon (15). To assess for tumor-associated bacteria, we performed fluorescence *in situ* hybridization (FISH) using a universal bacterial probe (EUB338). Relative to AOM/DSS tumors from *LysM*^{Cre};*Mcl1*$^{wt/wt}$ mice, PMN-deficient tumors have increase in tumor-associated bacteria (Fig. 5B & C). Bacteria can induce intra-tumoral genetic instability through promoting DNA double-stranded breaks (20). PMN-deficient

tumors had significantly more phosphorylated histone p-H2AX (Fig. 5D & E). IL-17 was identified as a highly-expressed cytokine in RNA-seq analysis in *Mcl-1*$^{-/-}$ colon tumors (Fig. 5A). Colon tumor-associated microbes drive tumor progression through activation of IL-17 expression (21). Moreover, epithelial attachment of pathogenic microbes can directly induce an IL-17 response (22). Our data has shown in acute DSS-colitis, *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice had an increased colon IL-17 response. *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ colon tumors also had significantly increased *Il17* expression compared to *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ (Fig. S7A). High activation of bacterial-activated inflammatory pathways such as NF-κB and STAT3 were also observed (Fig. S7B).

We next assessed alterations in microbiota by bacterial sequencing of fecal and primary tumor samples from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice after AOM/DSS induced colon tumorigenesis.. No significant changes in tumor-associated and fecal bacterial diversity were detected (Fig. S7C). However, several bacteria species were significantly altered in tumors and feces of *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice (Fig. 5F). Interestingly, Akkermansia, a mucinolytic bacteria previously shown to be associated with human colon tumors was significantly increased in tumors from *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice (23)REF. Furthermore, Proteobacteria was significantly decreased in tumors from *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice, which has previously been suggested to be decreased in human colon tumors (24). Collectively, these data suggest that PMNs play an important role in restricting bacterial dysbiosis in colon tumors.

Colon microbiota are essential for increased tumor growth in PMN-deficient mice

PMN-deficiency greatly increased intra-tumoral bacteria and bacteria-dependent responses (Fig. 5). The colon microbiota is strongly associated with initiation and progression of colon tumors (25). Several bacterial species have been proposed to have a direct causal role in

colon cancer. *Fusobacterium nucleatum* can directly potentiate colon cancer growth and enhances ß-Catenin signaling (26). To evaluate the contribution of bacteria to tumor growth, $LysM^{Cre};Mcl1^{wt/wt}$ and $LysM^{Cre};Mcl1^{fl/fl}$ were induced by AOM/DSS and beginning on day 14 after AOM treatment $LysM^{Cre};Mcl1^{wt/wt}$ and $LysM^{Cre};Mcl1^{fl/fl}$ mice were randomized into Ctrl and antibiotics treatment groups (Fig. 6A). $LysM^{Cre};Mcl1^{fl/fl}$ bone marrow chimeras had significantly reduced PMNs one-day prior to AOM administration (Fig. 6B). Whereas, $LysM^{Cre};Mcl1^{fl/fl}$ mice developed dramatically larger colon tumors than $LysM^{Cre};Mcl1^{wt/wt}$ mice, antibiotic treatment decreased tumor size and decreased tumor burden with no change in total tumor number in $LysM^{Cre};Mcl1^{fl/fl}$ mice (Fig. 6C-F). Antibiotics treatment reduced PMN-deficient tumor-associated genetic instability (Fig. 6G). Furthermore, antibiotics dramatically reduced tumor invasion in $LysM^{Cre};Mcl1^{fl/fl}$ mice (Fig. 6H). Collectively, these data define an important contribution of colon microbiota to enhanced tumor growth and progression observed in PMN-deficient animals.

Neutrophils restrict expansion of colon tumor-associated B cells

Antibiotics treatment partially but not completely reversed the PMN-deficient increase in colon tumor progression, suggesting other mechanisms promote growth and invasion. In addition to anti-microbial genes, RNA-seq data showed many genes associated with B-cells were higher in $LysM^{Cre};Mcl1^{fl/fl}$ colon tumors. Immunofluorescence and flow cytometric analysis of tumors showed robust infiltration of B-cells into primary tumors from PMN-depleted mice compared to $LysM^{Cre};Mcl1^{wt/wt}$ mice (Fig. S8A & B). No changes in circulating B-cells or fecal IgA were observed in untreated $LysM^{Cre};Mcl1^{fl/fl}$ mice (Fig. S8C-E). In addition to heightened B-cell infiltration into the primary tumor, B-cells were also directly associated with highly invasive

areas in colitis-associated and sporadic tumors from *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice (Fig. 7A and Fig S8F). The close association of B-cells with invasive tumors suggested a potential role for these cells in the neoplastic progression of PMN-depleted colon tumors. To define the functional role of B-cells in *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice, we decided to target B-cells using anti-B220 antibody-mediated B-cell inhibition (27). To evaluate the ability of anti-B220 therapy to decrease B-cells from colonic tissue during inflammation, mice were treated with 2.5% DSS and were randomized to Ctrl or anti-B220 antibody (400ug I.P.) treatment in acute DSS-colitis (Fig. S9A). Circulating B-cells and intracolonic B-cells were significantly decreased after three doses anti-B220 antibody (Fig. S9B & C). This treatment did not have a significant effect on the acute inflammatory response in colitis (Fig. S9D-F).

To assess B-cells in the progression of PMN-deficient tumors, colon tumorigenesis was induced in *LysM*^{Cre};*Mcl1*$^{wt/wt}$ and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice using AOM/DSS. Immediately following the third cycle of DSS, *LysM*^{Cre};*Mcl1*$^{fl/fl}$ animals were randomized into Ctrl and anti-B220 treated groups and treated beginning every fourth day from Day 50 through Day 75 (Fig. 7B). 75-days post induction of colon tumorigenesis, anti-B220 treatment reduced colon B-cells (Fig. 7C). No change in tumor number was detected between any groups (Fig. S10A). However, tumor burden and tumor size were dramatically increased in *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice relative to *LysM*^{Cre};*Mcl1*$^{wt/wt}$ control animals (Fig. 7D & E). B-cell inhibition from *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice improved the observed increase in tumor burden and tumor size (Fig. 7D & E). Furthermore, B-cell depletion led to a reduction in the percentage of invasive adenocarcinomas and increased percentage of non-invasive adenomas (Fig. 7F & S9B).

Discussion

The functional role for neutrophils in neoplastic progression has been controversial, however, the vast majority of work suggests a pro-tumorigenic role for PMNs in tumorigenesis. PMN and granulocytic-cell targeted therapeutics have been proposed for the treatment of several tumor types (4). Peptide-mimetics targeting the G-protein coupled receptor CXCR2 which mediates PMN and myeloid-derived suppressor cells (MDSCs) infiltration into tumors is efficacious in dampening colon tumorigenesis in both colitis-associated as well as sporadic colon tumor models (28). Peptide Fc-fusion proteins (peptibodies) targeting the S100 family of protein expressed on MDSCs and PMNs have also shown to dampen tumor growth in preclinical models (29). Clinical trials in breast cancer targeting PMNs have been initiated (30). However, PMN infiltration into human colon tumors was associated with both positive and adverse clinical outcomes (31, 32). Experiments using mouse models further raise questions about the function of PMNs in the initiation and progression of colon tumors as conflicting data on the role of PMNs have been shown. Our work clearly demonstrates depletion of neutrophils early in colon tumorigenesis profoundly enhances tumor growth, proliferation, and invasion.

Discrepancies between neutrophil function in our study relative to others could perhaps be explained by differences in models. For example, anti-Ly6G antibody depletion of PMNs is commonly used in tumor studies (33). Interestingly, flow cytometric analysis suggests $Cd11b^{-}/Ly6G^{+}$ exist, which could perhaps suggest non-PMN targets of this treatment may affect the robust reduction in colitis-associated tumors observed in this model (34). Although CXCR2 is not completely PMN selective, deletion of the chemokine receptor CXCR2 results in reduced $Cd11b^{+}/Ly6g^{+}$ infiltration into colon tumors and significant reduction in colon tumorigenesis (34). Strategies to inhibit CXCR2 may be effective in inhibiting a subset of neutrophils.

Intratumoral PMN heterogeneity has been proposed to partly explain the differential effects of PMN depletion on tumorigenesis (35-38). Moreover, a landmark study showed that PMN plasticity can be driven towards protumor "N2" PMNs or antitumor "N1" PMNs in a TGF-ß-dependent manner. Approaches to target all tumor-associated neutrophils as described in the present work potentiate disease progression. Moreover, this is the first study to our knowledge to use a complete neutrophil deficient mouse in the study of colon cancer. Other models of genetic neutrophil depletion have been used in the study of other cancers. The *Csf3r* knockout mouse fail to develop PMNs and enhance tumor growth in a murine uterine tumor model (39).

The colon microbiota and microbial dysbiosis drive colon tumorigenesis (25). This idea is highlighted with studies from germfree mouse models which show decreased colon tumorigenesis in the absence of microbiota (40). Furthermore, *E. coli* species were directly linked to tumor invasion (41). Key factors regulating the expansion, diversity, and evolution of tumor-associated microbiota in colon cancer are not well understood. Our studies have delineated a novel mechanism for intra-tumoral PMNs restricting microbiota-dependent tumor progression. Deletion of PMNs profoundly increased tumor-associated bacteria and many known microbiota-dependent responses, including genetic instability, IL-17 response, and NF-κB activation, all of which with known critical roles in neoplastic progression. Additionally, PMN depletion impacted the microbiota associated with colitis-associated colon tumors in mice. Importantly, antibiotics treatment reduced PMN-deficient tumor progression and invasion. Future studies aimed at determining the precise bacteria associated with PMN-deficient tumors are currently in progress.

The precise function for PMNs in the progression of IBD is not well known. Chronic granulomatous disease (CGD), a primary deficiency in the NADPH oxidase complex resulting in

inability of neutrophils to generate reactive oxygen species to kill pathogens, can predispose patients to development of IBD-like disease that resembles Crohn's disease (42). This suggests a role for PMN anti-microbial functions in restricting spontaneous colitis. Recently, it has been suggested that in addition to the pathogen killing function, decreased PMN-mediated O_2-consumption through decreased ROS production in Chronic Granulomatous Disease reduces hypoxia-inducible transcription factor-1α (HIF-1α) activity, an essential regulator of colon epithelial barrier integrity (43). In addition to the ROS-mediated mucosal protection, PMN-secreted IL-22 has also been shown to have an important role in activating expression of epithelial-derived antimicrobial peptides, although no changes in *Il22* mRNA abundance was observed in our studies (44). Several studies suggesting a prominent role for PMNs in promoting inflammatory responses have been reported (14). The data in our studies suggest that neutrophil depletion in $LysM^{Cre};Mcl1^{fl/fl}$ mice recapitulates aspects of the enhanced inflammatory response in colitis seen in Chronic Granulomatous Disease patients. Future experiments more specifically addressing mechanisms of increased colitis in $LysM^{Cre};Mcl1^{fl/fl}$ mice and the contribution to microbiota are currently under investigation

One of the more surprising finings of our study is the enhanced tumor invasion in response to PMN depletion. This response was not due solely to enhanced inflammation in colitis, as genetic colon tumor models also displayed increased local invasiveness following PMN depletion. PMNs have been generally characterized as critical drivers of tumor cell invasion and metastasis. PMNs are purported to increase tumor invasion be secreting enzymes such as matrix metalloproteinase 9 (MMP9) (45). In hepatocellular carcinoma, PMNs were shown to directly initiate an epithelial-mesenchymal transition in tumor cells (46). PMNs were recently shown to suppress Natural Killer (NK) cell-mediated breast cancer cell killing and

promote tumor cell extravasation (47). Also in breast cancer, PMNs induced by IL-17 producing T-cells were found to increase tumor metastasis through repression of CD8 T-cells (48). However, PMNs have also been shown to limit metastatic seeding of tumors in the lung through generation of H_2O_2 (49). Our findings demonstrate in colon cancer that depleting neutrophils early in tumors leads to cell invasion through all layers of the colon mucosae. Thus, targeting of PMNs therapeutically in human colon cancer may exacerbate disease.

PMN-deficient tumors were highly infiltrated with $B220^+$ B-cells. Analogous to PMNs, B-cell function in tumors is not well understood and could perhaps have both pro- and anti-tumor functions (50-53). Our data indicate that B-cells expand in PMN-deficient tumors and are highly associated with invasive areas. Moreover, inhibition of B-cells decreased tumor size and decreased tumor invasion in PMN-deficient mice. Previous reports suggested that B-cells in colon lymphoid follicles were an early site of tumor cell invasion in rat and mouse models of colon cancer (54). It has also been suggested that human colon cancers are highly associated with B-cells and lymphoid follicles (55). B-cells associated with invasive areas and promoted tumor growth and invasion in our model. These data suggest further study into the regulation, recruitment, and functional role of B-cells in colon cancer are warranted.

It is possible that the effects observed in our studies are due to complete absence of PMNs prior to induction of tumorigenesis. The role for PMNs in the initiation of colon cancer are controversial. Our data suggest a mechanism by which PMNs restrict tumor-associated bacteria and B-cells, which are essential for colon tumor growth and progression (Fig. 7G). Clinical studies suggest PMNs are a positive prognostic indicator in early stage colon cancer (31). Studies in mice have proposed that PMNs in early stage tumors retain antitumor functions but a phenotypic switch to a more immunosuppressive state occurs throughout tumor progression

(56). It is possible that similar mechanisms exist in colon cancer whereby the earliest tumor infiltrating PMNs serve to inhibit expansion of colon microbiota and B-cells to limit tumorigenesis and progression. Whereas, PMNs in established tumors evolve a more pro-tumorigenic phenotype (Fig. 7H).

References

1. Terzić J, Grivennikov S, Karin E, and Karin M. Inflammation and Colon Cancer. *Gastroenterology.* 2010;138(6):2101-14.e5.
2. Triner D, and Shah YM. Hypoxia-inducible factors: a central link between inflammation and cancer. *The Journal of Clinical Investigation.* 2016;126(10):3689-98.
3. Nicolás-Ávila JÁ, Adrover JM, and Hidalgo A. Neutrophils in Homeostasis, Immunity, and Cancer. *Immunity.*46(1):15-28.
4. Coffelt SB, Wellenstein MD, and de Visser KE. Neutrophils in cancer: neutral no more. *Nature Reviews Cancer.* 2016;16:431.
5. Houghton AM, Rzymkiewicz DM, Ji H, Gregory AD, Egea EE, Metz HE, et al. Neutrophil elastase–mediated degradation of IRS-1 accelerates lung tumor growth. *Nature Medicine.* 2010;16:219.
6. Wang T-t, Zhao Y-l, Peng L-s, Chen N, Chen W, Lv Y-p, et al. Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. *Gut.* 2017.
7. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nature Medicine.* 2015;21:938.
8. Dzhagalov I, St. John A, and He Y-W. The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood.* 2007;109(4):1620-6.
9. Feng Y, Sentani K, Wiese A, Sands E, Green M, Bommer GT, et al. Sox9 Induction, Ectopic Paneth Cells, and Mitotic Spindle Axis Defects in Mouse Colon Adenomatous Epithelium Arising From Conditional Biallelic Apc Inactivation. *The American Journal of Pathology.* 2013;183(2):493-503.
10. Németh T, Futosi K, Sitaru C, Ruland J, and Mócsai A. Neutrophil-specific deletion of the CARD9 gene expression regulator suppresses autoantibody-induced inflammation in vivo. *Nature Communications.* 2016;7:11004.
11. Dubé PE, Yan F, Punit S, Girish N, McElroy SJ, Washington MK, et al. Epidermal growth factor receptor inhibits colitis-associated cancer in mice. *The Journal of Clinical Investigation.* 2012;122(8):2780-92.
12. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013;29(1):15-21.
13. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, and Schloss PD. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology.* 2013;79(17):5112-20.
14. Wéra O, Lancellotti P, and Oury C. The Dual Role of Neutrophils in Inflammatory Bowel Diseases. *Journal of Clinical Medicine.* 2016;5(12):118.
15. Kamada N, Sakamoto K, Seo S-U, Zeng MY, Kim Y-G, Cascalho M, et al. Humoral Immunity in the Gut Selectively Targets Phenotypically Virulent Attaching-and-Effacing Bacteria for Intraluminal Elimination. *Cell host & microbe.* 2015;17(5):617-27.
16. Shang K, Bai Y-P, Wang C, Wang Z, Gu H-Y, Du X, et al. Crucial Involvement of Tumor-Associated Neutrophils in the Regulation of Chronic Colitis-Associated Carcinogenesis in Mice. *PLOS ONE.* 2012;7(12):e51848.

17. Fearon ER. Molecular Genetics of Colorectal Cancer. *Annual Review of Pathology: Mechanisms of Disease.* 2011;6(1):479-507.
18. Xue X, Taylor M, Anderson E, Hao C, Qu A, Greenson JK, et al. Hypoxia-Inducible Factor-2α Activation Promotes Colorectal Cancer Progression by Dysregulating Iron Homeostasis. *Cancer Research.* 2012;72(9):2285.
19. Abram CL, Roberge GL, Hu Y, and Lowell CA. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *Journal of immunological methods.* 2014;408:89-100.
20. Nougayrède J-P, Homburg S, Taieb F, Boury M, Brzuszkiewicz E, Gottschalk G, et al. Escherichia coli Induces DNA Double-Strand Breaks in Eukaryotic Cells. *Science.* 2006;313(5788):848-51.
21. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature.* 2012;491(7423):254-8.
22. Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, et al. Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell.*163(2):367-80.
23. Sun J, and Kato I. Gut microbiota, inflammation and colorectal cancer. *Genes & Diseases.* 2016;3(2):130-43.
24. Gao Z, Guo B, Gao R, Zhu Q, and Qin H. Microbiota disbiosis is associated with colorectal cancer. *Frontiers in Microbiology.* 2015;6:20.
25. Brennan CA, and Garrett WS. Gut Microbiota, Inflammation, and Colorectal Cancer. *Annual Review of Microbiology.* 2016;70(1):395-411.
26. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor immune microenvironment. *Cell host & microbe.* 2013;14(2):207-15.
27. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, et al. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T regulatory cells. *Cancer research.* 2011;71(10):3505-15.
28. Jamieson T, Clarke M, Steele CW, Samuel MS, Neumann J, Jung A, et al. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. *The Journal of Clinical Investigation.* 2012;122(9):3127-44.
29. Qin H, Lerman B, Sakamaki I, Wei G, Cha S, Rao SS, et al. Generation of a novel therapeutic peptide that depletes MDSC in tumor-bearing mice. *Nature medicine.* 2014;20(6):676-81.
30. Ocana A, Nieto-Jiménez C, Pandiella A, and Templeton AJ. Neutrophils in cancer: prognostic role and therapeutic strategies. *Molecular Cancer.* 2017;16(1):137.
31. Wikberg ML, Ling A, Li X, Öberg Å, Edin S, and Palmqvist R. Neutrophil infiltration is a favorable prognostic factor in early stages of colon cancer. *Human Pathology.* 2017;68:193-202.
32. Haram A, Boland MR, Kelly ME, Bolger JC, Waldron RM, and Kerin MJ. The prognostic value of neutrophil-to-lymphocyte ratio in colorectal cancer: A systematic review. *Journal of Surgical Oncology.* 2017;115(4):470-9.
33. Wang Y, Wang K, Han GC, Wang RX, Xiao H, Hou CM, et al. Neutrophil infiltration favors colitis-associated tumorigenesis by activating the interleukin-1 (IL-1)/IL-6 axis. *Mucosal Immunology.* 2014;7:1106.

34. Katoh H, Wang D, Daikoku T, Sun H, Dey SK, and DuBois RN. CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis. *Cancer cell.* 2013;24(5):631-44.
35. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: “N1” versus “N2” TAN. *Cancer Cell.*16(3):183-94.
36. Sagiv Jitka Y, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic Diversity and Plasticity in Circulating Neutrophil Subpopulations in Cancer. *Cell Reports.*10(4):562-73.
37. Ostanin DV, Kurmaeva E, Furr K, Bao R, Hoffman J, Berney S, et al. Acquisition of Antigen-Presenting Functions by Neutrophils Isolated from Mice with Chronic Colitis. *The Journal of Immunology.* 2012;188(3):1491.
38. Mishalian I, Bayuh R, Levy L, Zolotarov L, Michaeli J, and Fridlender ZG. Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression. *Cancer Immunology, Immunotherapy.* 2013;62(11):1745-56.
39. Blaisdell A, Crequer A, Columbus D, Daikoku T, Mittal K, Dey SK, et al. Neutrophils Oppose Uterine Epithelial Carcinogenesis via Debridement of Hypoxic Tumor Cells. *Cancer cell.* 2015;28(6):785-99.
40. Li Y, Kundu P, Seow SW, de Matos CT, Aronsson L, Chin KC, et al. Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in APC Min/+ mice. *Carcinogenesis.* 2012;33(6):1231-8.
41. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, et al. Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota. *Science.* 2012;338(6103):120.
42. Schappi M, Smith V, Goldblatt D, Lindley K, and Milla P. Colitis in chronic granulomatous disease. *Archives of Disease in Childhood.* 2001;84(2):147-51.
43. Campbell EL, Bruyninckx WJ, Kelly CJ, Glover LE, McNamee EN, Bowers BE, et al. Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. *Immunity.* 2014;40(1):66-77.
44. Zindl CL, Lai J-F, Lee YK, Maynard CL, Harbour SN, Ouyang W, et al. IL-22–producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis. *Proceedings of the National Academy of Sciences.* 2013;110(31):12768-73.
45. Kessenbrock K, Plaks V, and Werb Z. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell.* 2010;141(1):52-67.
46. Zhou S-L, Zhou Z-J, Hu Z-Q, Li X, Huang X-W, Wang Z, et al. CXCR2/CXCL5 axis contributes to epithelial–mesenchymal transition of HCC cells through activating PI3K/Akt/GSK-3β/Snail signaling. *Cancer Letters.* 2015;358(2):124-35.
47. Spiegel A, Brooks MW, Houshyar S, Reinhardt F, Ardolino M, Fessler E, et al. Neutrophils suppress intraluminal NK-mediated tumor cell clearance and enhance extravasation of disseminated carcinoma cells. *Cancer discovery.* 2016;6(6):630-49.
48. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau C-S, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature.* 2015;522:345.
49. Granot Z, Henke E, Comen E, King T, Norton L, and Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer cell.* 2011;20(3):300-14.

50. Yuen GJ, Demissie E, and Pillai S. B lymphocytes and cancer: a love-hate relationship. *Trends in cancer.* 2016;2(12):747-57.
51. Shalapour S, Lin X-J, Bastian IN, Brain J, Burt AD, Aksenov AA, et al. Inflammation-induced IgA+ cells dismantle anti-liver cancer immunity. *Nature.* 2017;551:340.
52. Lee K, Spata M, Bayne LJ, Buza EL, Durham AC, Allman D, et al. Hif1α deletion reveals pro-neoplastic function of B cells in pancreatic neoplasia. *Cancer discovery.* 2016;6(3):256-69.
53. Liu R, Lu Z, Gu J, Liu J, Huang E, Liu X, et al. MicroRNAs 15A and 16–1 Activate Signaling Pathways That Mediate Chemotaxis of Immune Regulatory B cells to Colorectal Tumors. *Gastroenterology.* 2017.
54. Nauss KM, Locniskar M, Pavlina T, and Newberne PM. Morphology and Distribution of 1,2-Dimethylhydrazine Dihydrochloride-Induced Colon Tumors and Their Relationship to Gut-Associated Lymphoid Tissue in the Rat2. *JNCI: Journal of the National Cancer Institute.* 1984;73(4):915-24.
55. Bronen RA, Glick SN, and Teplick SK. Diffuse lymphoid follicles of the colon associated with colonic carcinoma. *American Journal of Roentgenology.* 1984;142(1):105-9.
56. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *The Journal of Clinical Investigation.* 2014;124(12):5466-80.

Figures

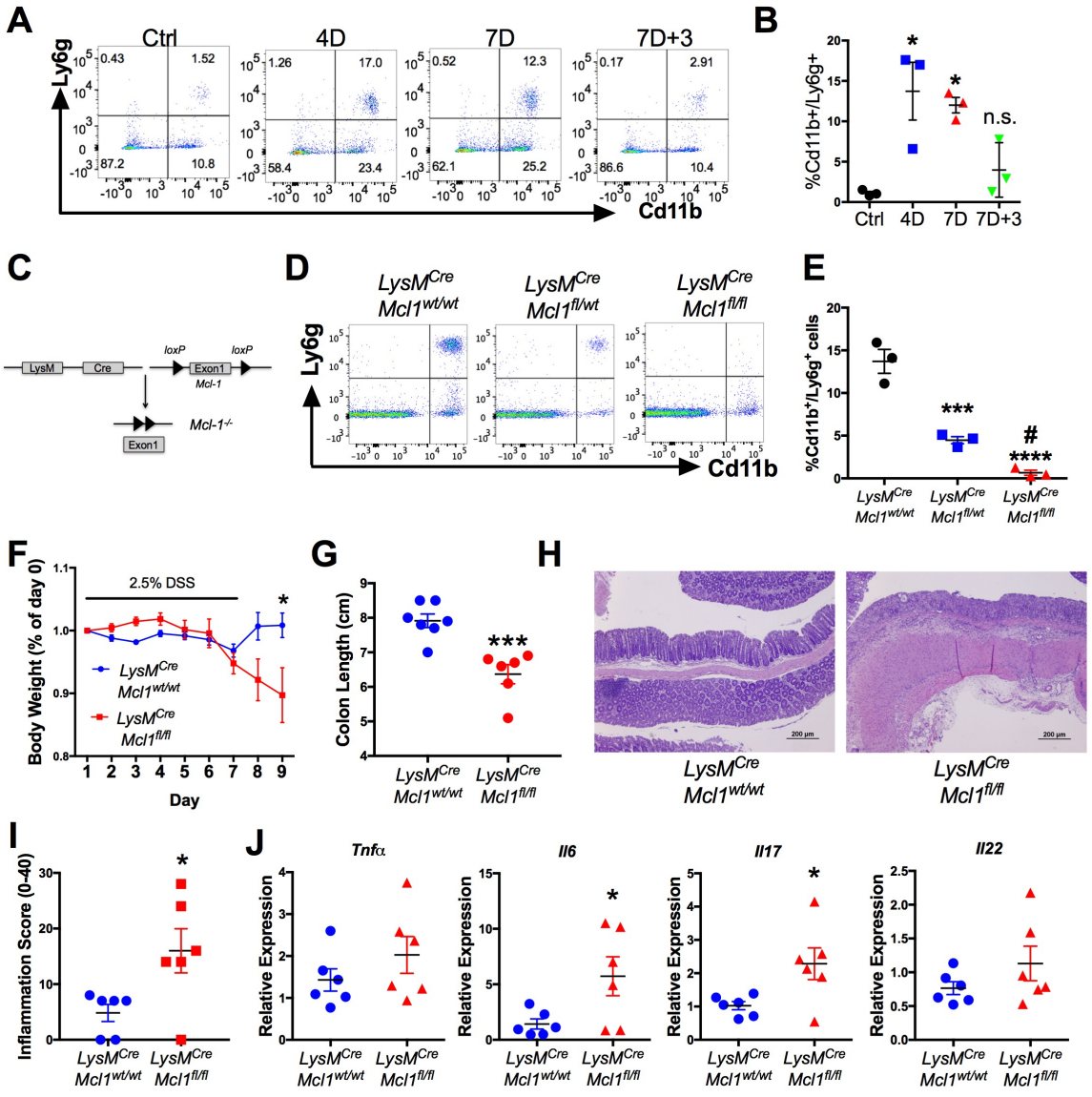

Figure 3.1. Neutrophil depletion exacerbates acute colitis. (A) Representative flow cytometry dot plots (D=days of DSS treatment) and **(B)** quantification of flow cytometric analysis of peripheral blood neutrophils using Ly6g and Cd11b in WT and *Mcl-1*$^{-/-}$ mice treated with 2.5% DSS. **(C)** Schematic of *Mcl-1* deletion in myeloid cells using *LysozymeM (LysM)*-cre (*LysM*^{Cre};*Mcl1*$^{fl/fl}$) **(D)** Representative dot plots and **(E)** quantification of flow cytometric analysis of peripheral blood neutrophils using Ly6g and Cd11b staining gated on total lymphocytes in *LysM*^{Cre};*Mcl1*$^{wt/wt}$, *LysM*^{Cre};*Mcl1*$^{fl/wt}$, and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. ***p<0.001 and ****p<0.0001 relative to *LysM*^{Cre};*Mcl1*$^{wt/wt}$, #p<0.05 compared to *LysM*^{Cre};*Mcl1*$^{fl/wt}$. **(F)** Body weight, **(G)** colon length, **(H)** representative hematoxylin & eosin staining, and **(I)** histopathologic inflammation score of *LysM*^{Cre};*Mcl1*$^{wt/wt}$ and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice treated with 2.5% DSS. ***p<0.001, *p<0.05. **(J)** qPCR analysis of indicated genes *p<0.05 relative to *LysM*^{Cre};*Mcl1*$^{wt/wt}$. Statistical analysis was performed with student's t-test or one-way ANOVA followed by Tukey's multiple comparisons test.

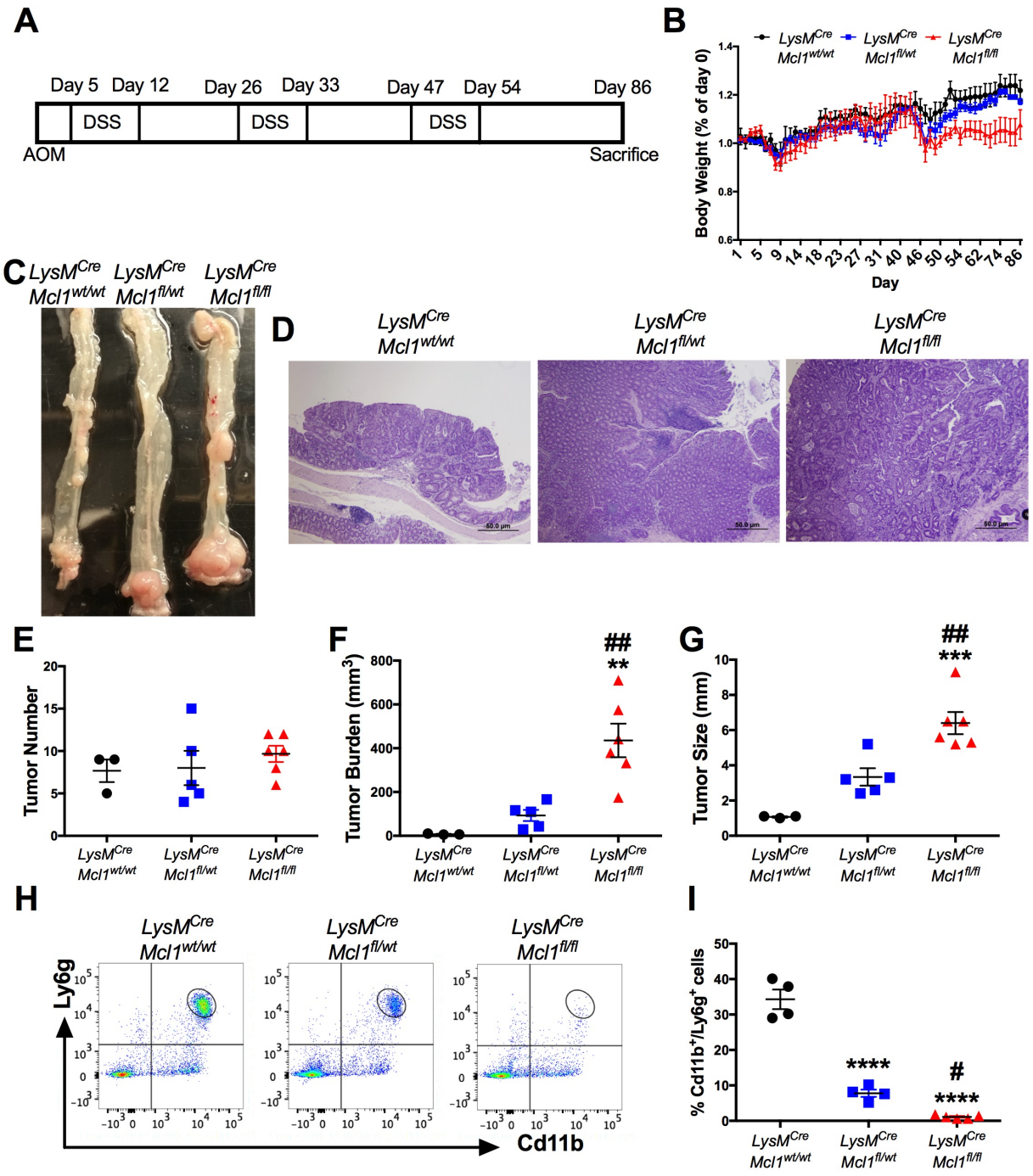

Figure 3.2. Neutrophils restrict colitis-associated colon tumor progression. (A) Schematic of AOM/DSS-induced colon tumorigenesis. Mice were I.P. injected with AOM on Day 0 then cycled on and off 2% DSS in their drinking water for three cycles with two-weeks between then sacked at day 87. **(B)** Body weight, **(C)** representative images of whole mount specimens, **(D)** hematoxylin and eosin (H&E) staining of indicated mice after AOM/DSS. Body weight shown as a percentage of Day 0. of AOM/DSS treated colon tissue from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$, *LysM*^{Cre}*;Mcl1*$^{fl/wt}$, and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice. **(E)** Tumor number, **(F)** tumor burden, and **(G)**

average tumor size of colon tumors. *p<0.05, **p<0.01, ***p<0.001 relative to *LysM*^{Cre};*Mcl1*$^{wt/wt}$, ##p<0.01 relative to *LysM*^{Cre};*Mcl1*$^{fl/wt}$**. (H)** Flow cytometric analysis and **(I)** quantification of Cd11b and Ly6g staining from primary colon tumors. ***p<0.001 relative to *LysM*^{Cre};*Mcl1*$^{wt/wt}$. ##p<0.01 relative to *LysM*^{Cre};*Mcl1*$^{fl/wt}$. Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple comparison's test.

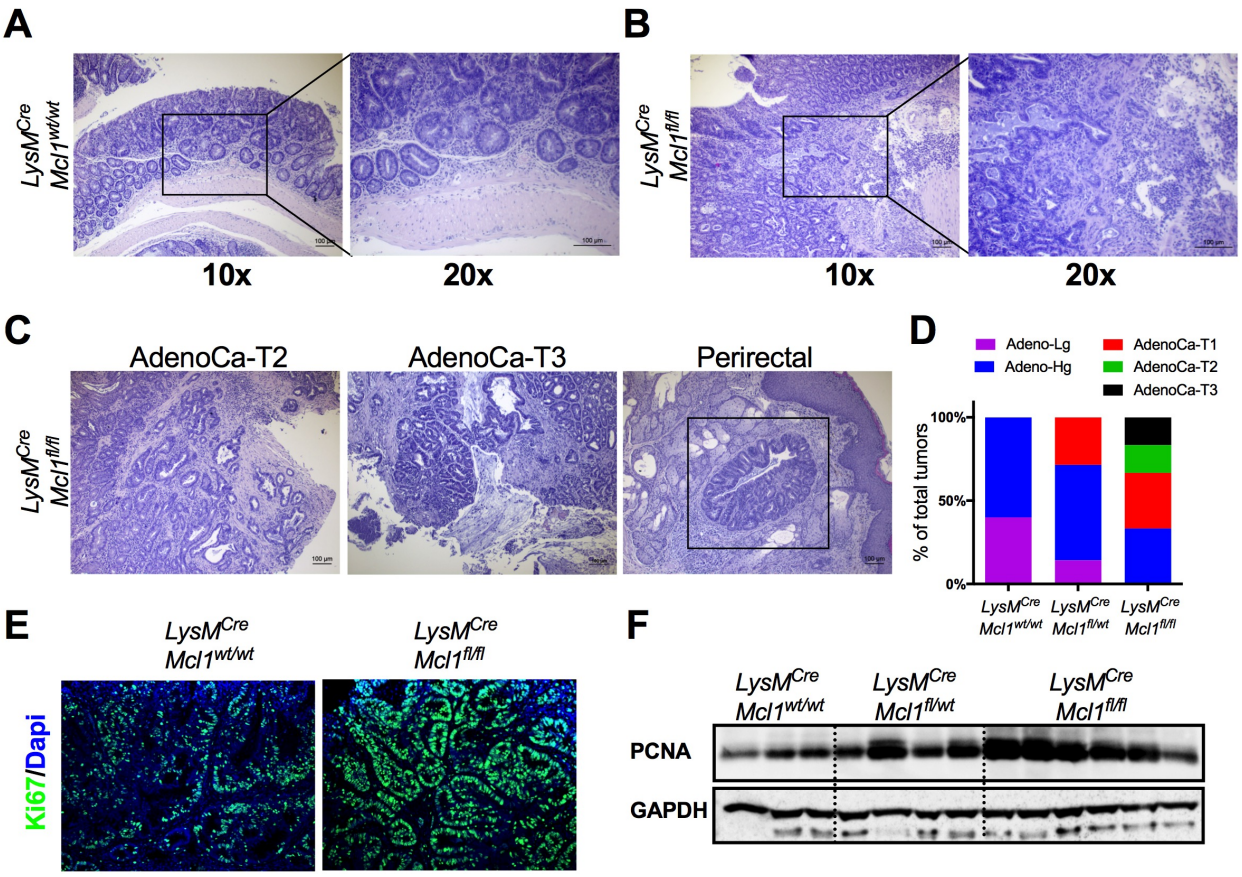

Figure 3.3. Neutrophils limit tumor progression and invasion. (A) Representative H&E of staining of adenoma from *LysM*^{Cre};*Mcl1*$^{wt/wt}$ mice and **(B)** invasive adenocarcinoma from *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice at 10x and 20x magnification. **(C)** Representative image of H&E of invasive AdenoCa-T2, AdenoCa-T3, and colon tumor cells in perirectal fat in *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. **(D)** Quantification of percentage of low-grade adenomas (Adeno-lg), high-grade adenomas (Adeno-Hg), submucosal invasive adenocarcinomas (AdenoCa-T1), muscularis invasive adenocarcinoms (AdenoCa-T2), and invasive through muscularis (AdenoCa-T3) in *LysM*^{Cre};*Mcl1*$^{wt/wt}$, *LysM*^{Cre};*Mcl1*$^{fl/wt}$, and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. **(E)** Representative image of Ki67 staining of colon tumors in *LysM*^{Cre};*Mcl1*$^{wt/wt}$ and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. **(F)** Western blot analysis of proliferating cell nuclear antigen (PCNA) of colon tumors from *LysM*^{Cre};*Mcl1*$^{wt/wt}$, *LysM*^{Cre};*Mcl1*$^{fl/wt}$, and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice.

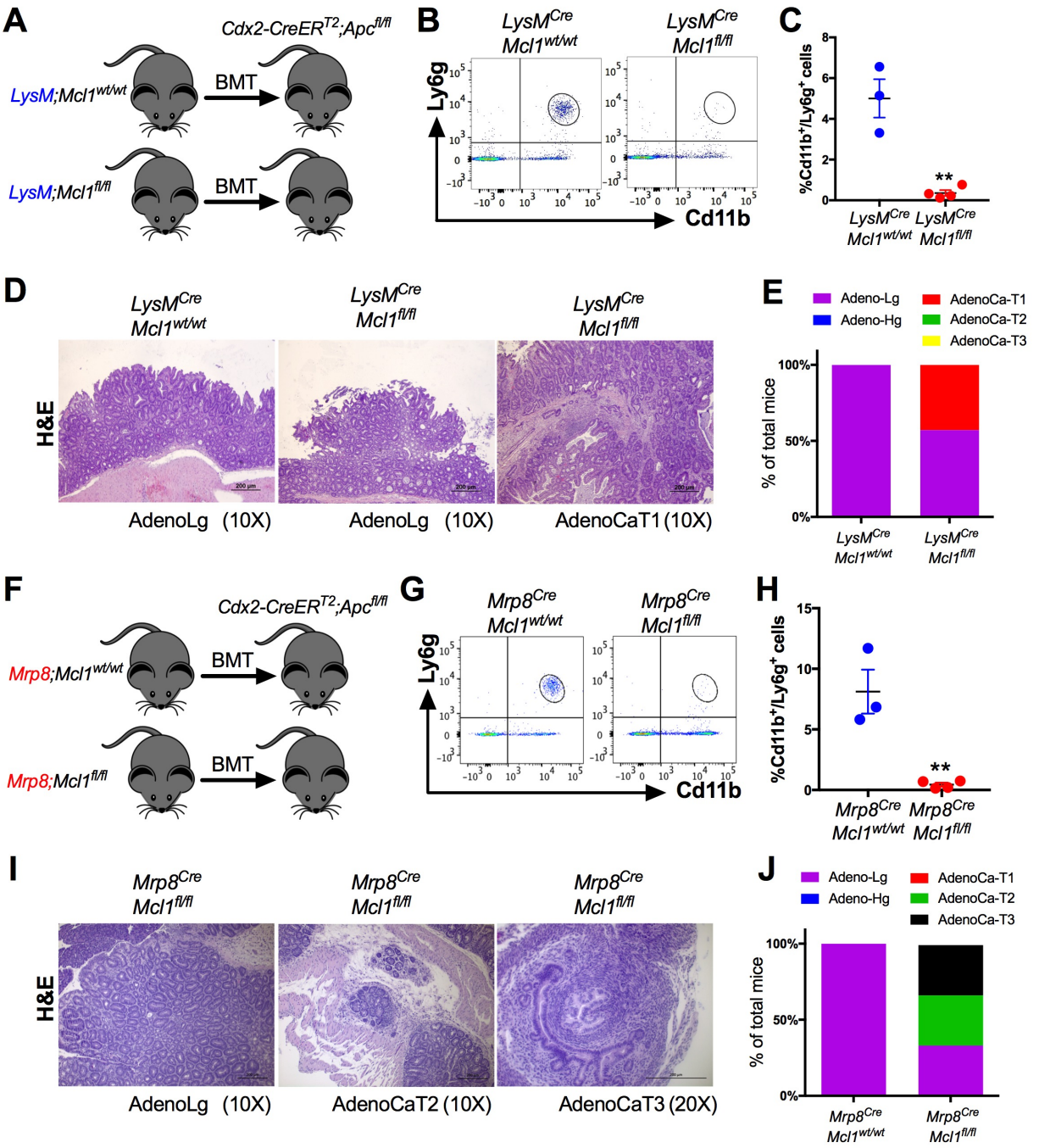

Figure 3.4. Neutrophils restrict progression of sporadic colon tumorigenesis. (A) Schematic of bone marrow transplantation from *LysM^{Cre};Mcl1$^{wt/wt}$* (n=6) and *LysM^{Cre};Mcl1$^{fl/fl}$* (n=7) mouse donors to *Cdx2*-CreER^{T2}*;Apc$^{fl/fl}$* mice. **(B)** Flow cytometric do plot analysis and **(C)** quantification of Cd11b and Ly6g staining of peripheral blood. **p<0.01 relative to WT. **(D)** Representative H&E images of low grade adenomas (AdenoLg) in *LysM^{Cre};Mcl1$^{wt/wt}$* and *LysM^{Cre};Mcl1$^{fl/fl}$* and invasive adenocarcinoma T1 (AdenoCaT1) in *LysM^{Cre};Mcl1$^{fl/fl}$* mice. **(E)** Quantification of colon tumor stage in indicated mice. **(F)** Schematic of bone marrow transplantation from *Mrp8^{Cre};Mcl1$^{wt/wt}$* (n=3) and *Mrp8^{Cre};Mcl1$^{fl/fl}$* (n=3) mouse donors to *Cdx2*-CreER^{T2}*;Apc$^{fl/fl}$* mice. **(G)** Flow cytometric do plot analysis and **(H)** quantification of Cd11b and

Ly6g staining of peripheral blood. **p<0.01 relative to $Mrp8^{Cre};Mcl1^{wt/wt}$. **(I)** Representative H&E images of low grade adenomas (AdenoLg) in $Mrp8^{Cre};Mcl1^{wt/wt}$ and $Mrp8^{Cre};Mcl1^{fl/fl}$ and invasive adenocarcinoma T2 (AdenoCa-T2) and adenocarcinoma T3 (AdenCa-T3). **(J)** Quantification of colon cancer stage in indicated mice. Statistical analysis was performed with student's t-test.

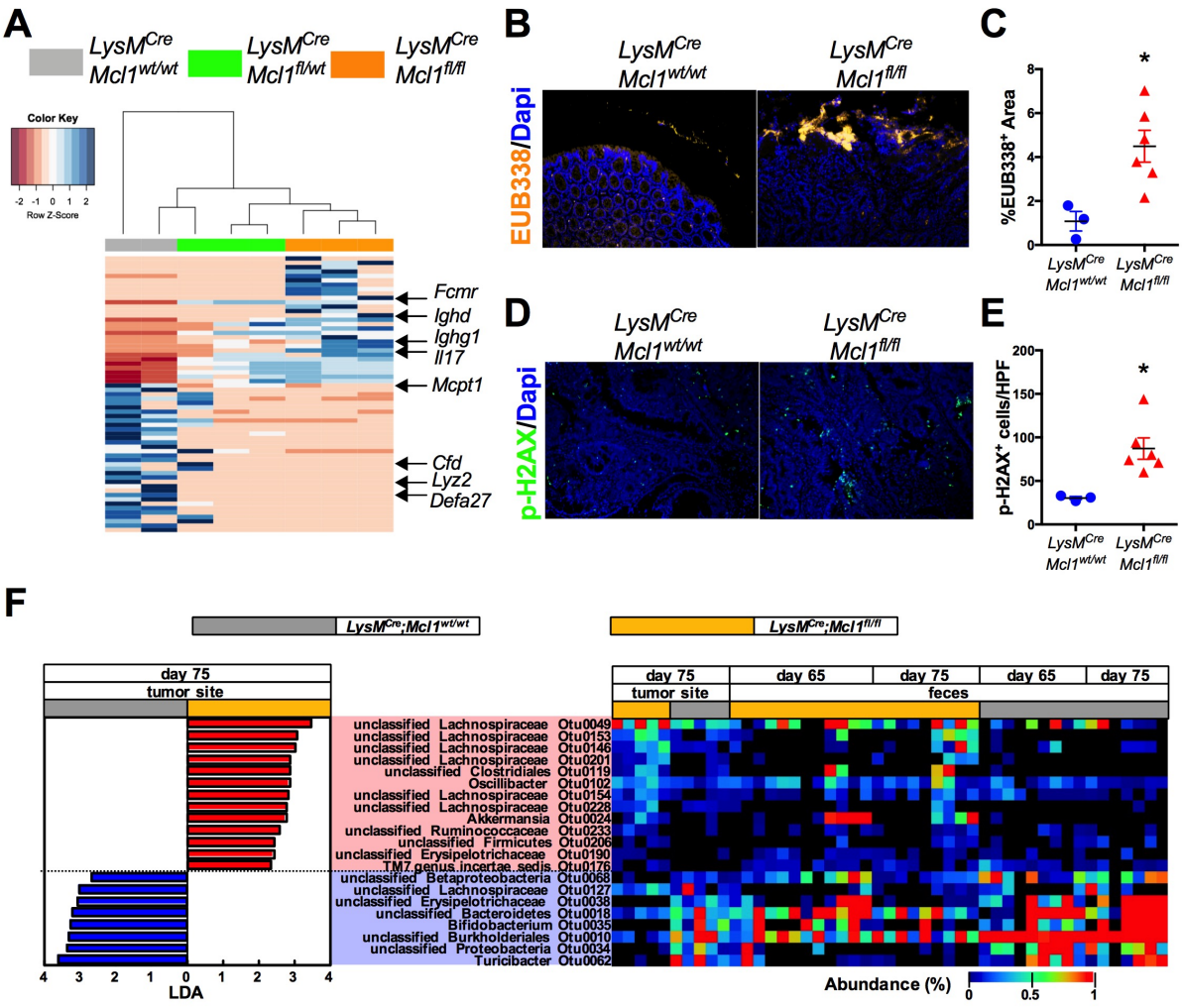

Figure 3.5. Neutrophils limit tumor-associated bacteria. (A) Heat map of RNA-seq analysis of colon tumors from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$, *LysM*^{Cre}*;Mcl1*$^{fl/wt}$, and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice. **(B)** Representative images fluorescence *in situ* hybridization (FISH) using universal bacteria EUB338 probe labeled with Cy3 colon tumors from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice. **(C)** Quantification of EUB338 positive area per high powered field (HPF). *p<0.05. **(D)** Representative images and **(E)** quantification of immunofluorescence staining of p-H2AX in *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ colon tumors. *p<0.05. **(F)** Heat map of significantly altered tumor-associated (Day 75) and fecal bacteria (Day 65 & Day 75) in *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice relative to *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ mice.

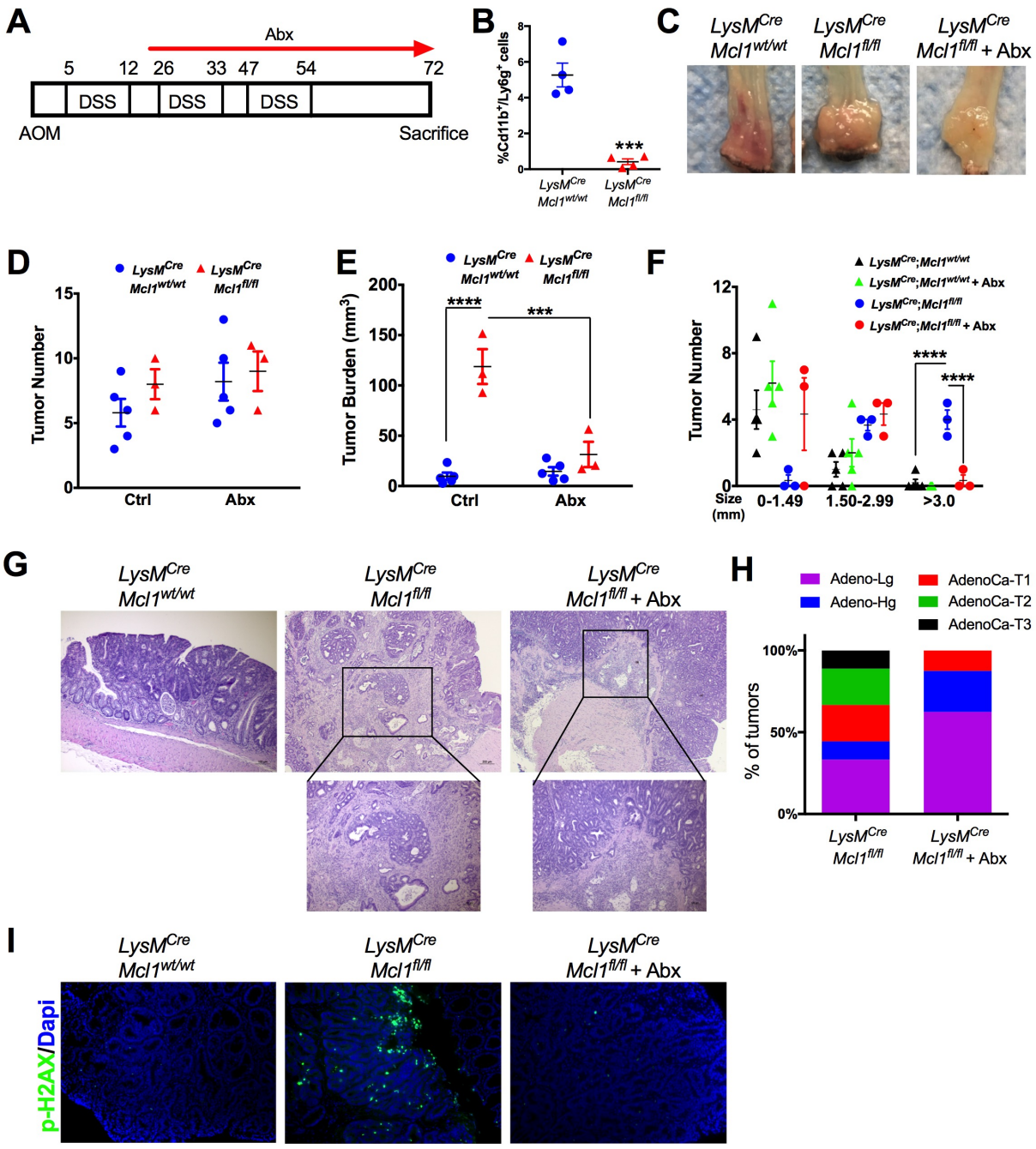

Figure 3.6. Microbiota promotes tumor progression in PMN-deficient mice. (A) Schematic of AOM/DSS-induced colon tumorigenesis. **(B)** Quantification of circulating Cd11b+/Ly6g+ cells in indicated mice. ***p<0.001 **(C)** Representative grow whole mount specimens, **(D)** tumor number, **(E)** tumor burden and **(F)** tumor size of colon tumors in *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ treated with antibiotics or control. ****p<0.0001, ***p<0.001. **(G)** Representative H&E analysis and **(H)** quantification of colon tumor progression from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$, *LysM*^{Cre}*;Mcl1*$^{fl/fl}$, and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice treated with antibiotics. **(I)** Representative p-H2AX immunofluorescence staining. Statistical analysis was performed with student's t-test and one-way ANOVA followed by Tukey's multiple comparisons test.

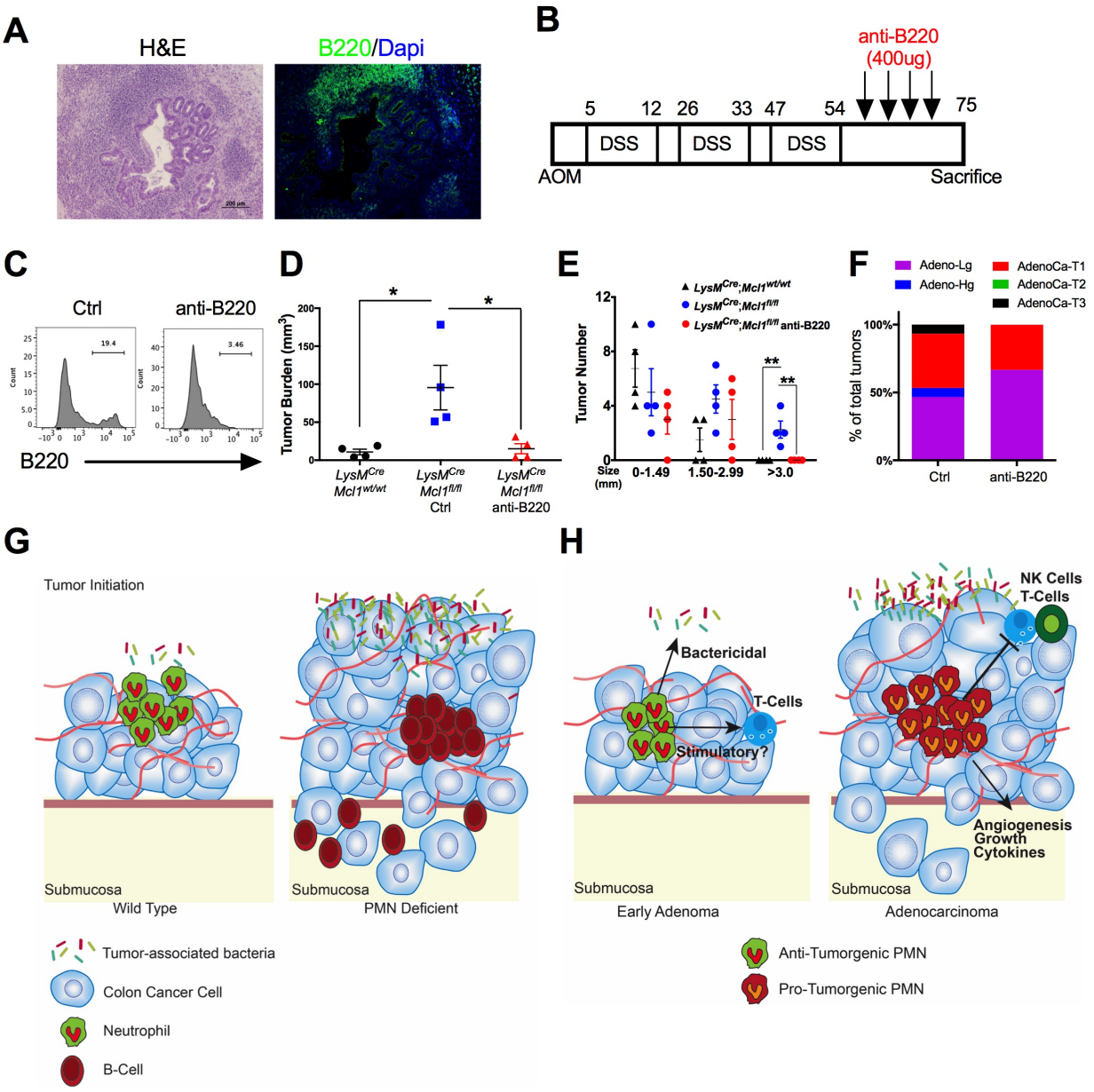

Figure 3.7. B-cells are important in PMN-deficient colitis-associated colon tumor model. **(A)** Representative H&E and B220 staining from *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ tumor tissue of invasive adenocarcinoma. **(B)** Schematic of AOM/DSS-induced colon tumorigenesis. Anti-B220 treatment was initiated at day 54 with 400ug/mouse treatement every fourth day. **(C)** Representative flow cytometric analysis of B220 staining in colon tissue from Ctrl and anti-B220 treated *Mcl-1*$^{-/-}$ mice. **(D)** Tumor burden, **(E)** tumor size, and **(F)** quantification of colon cancer stage in indicated mice. with anti-B220 antibody or control. *p<0.05 relative to *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ plus antibiotics. ** ##p<0.001 relative to *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ + anti-B220, and **p<0.01 relative to all groups >3.0mm. Statistical analysis performed by either student's t-test or one-way ANOVA followed by Tukey's multiple comparison's test. **(G)** Neutrophils restrict colon tumor progression and invasion by restricting outgrowth of tumor-associated microbiota and limiting infiltration of B-cells. **(H)** We propose a model in which

PMNs in the earliest stage colon tumors inhibit tumorigenesis through repression of tumor-associated bacteria as well as other potential mechanisms such as stimulation of T-cells. In later stage colon tumors, PMNs acquire pro-tumorigenic function and inhibit anti-tumor immunity, directly increase tumor growth, secrete inflammatory cytokines, and promote angiogenesis.

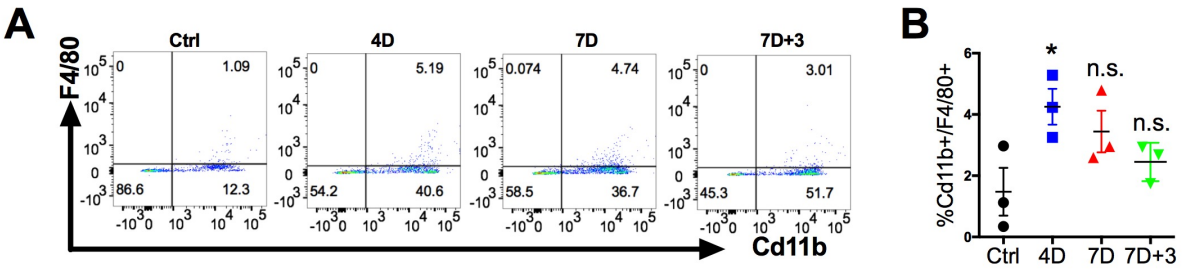

Figure S3.1. Macrophage infiltration in DSS colitis. (A) Representative flow cytometry dot plots and **(B)** quantification of F4/80+/Cd11b+ cells in DSS colitis time course. *p<0.05.

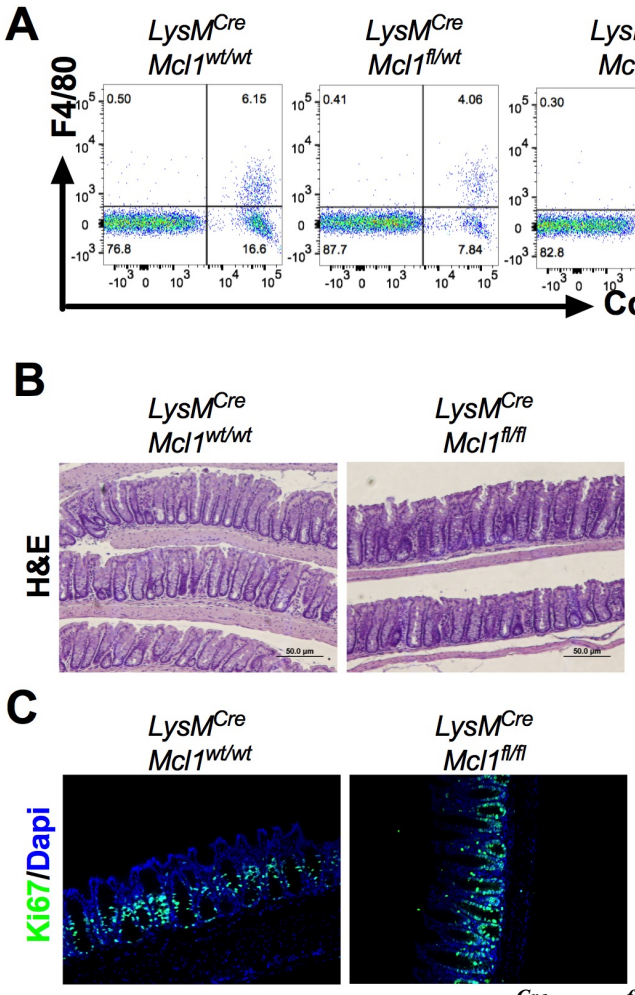

Figure S3.2. Characterization of *LysM^{Cre};Mcl1$^{fl/fl}$* mice. (A) Representative flow cytometry dot plots of F4/80+/Cd11b+ cells in peripheral blood. **(B)** Representative H&E staining and **(C)** Ki67 immunofluorescence from *LysM^{Cre};Mcl1$^{wt/wt}$* and *LysM^{Cre};Mcl1$^{fl/fl}$* mouse colon tissue.

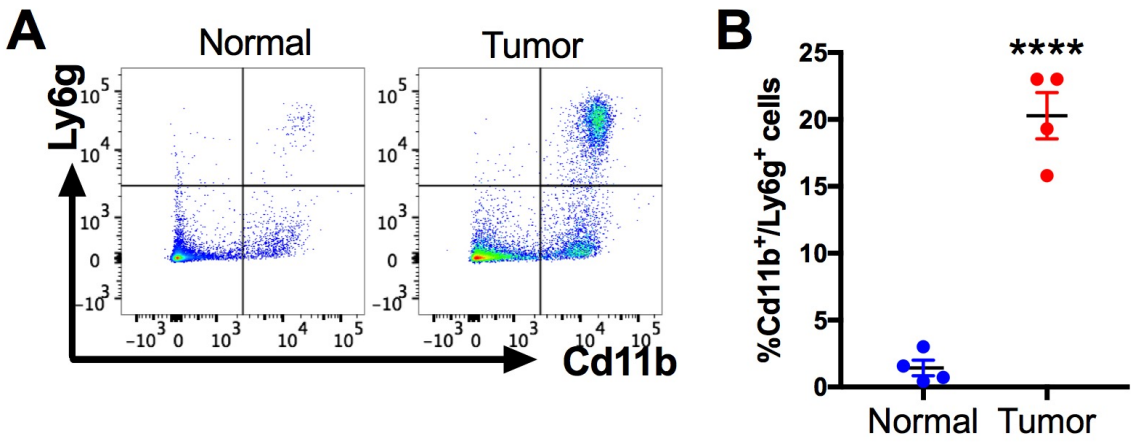

Figure S3.3. Neutrophils are highly infiltrated in AOM/DSS colon tumors. (A) Representative flow cytometry dot plots and **(B)** quantification of Ly6g+/Cd11b+ cells in AOM/DSS colon tumors from WT mice. ****p<0.001. Statistical analysis performed by student's t test.

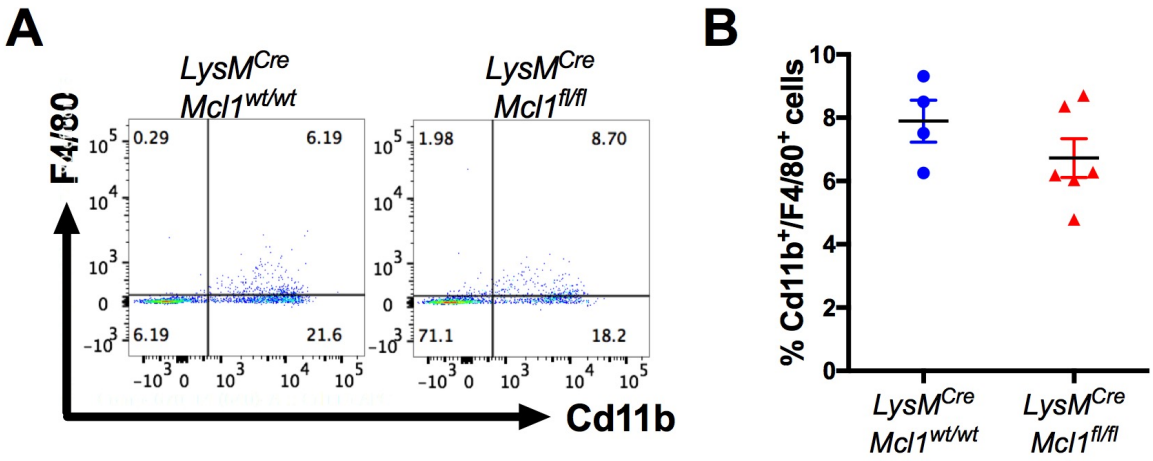

Figure S3.4. Tumor infiltration of macrophages is unaltered in *Mcl-1*$^{-/-}$ mice. (A) Representative flow cytometry dot plots and **(B)** quantification of F4/80+/Cd11b+ cells in colon tumors from *LysM*^{Cre};*Mcl1*$^{wt/wt}$ and *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. Statistical analysis performed by student's t test.

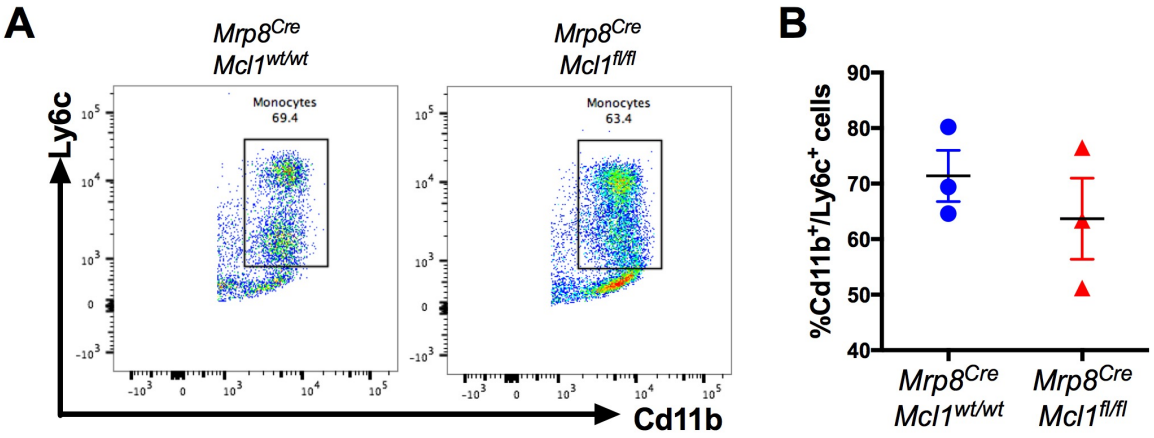

Figure S3.5. PMN depletion does not alter colon tumor apoptosis. (A) Representative immunofluorescence cleaved caspase 3 (cCasp3) and dapi from *LysM^{Cre};Mcl1$^{wt/wt}$* and *LysM^{Cre};Mcl1$^{fl/fl}$* mice colon tumors. **(B)** Quantification of cCasp3 positive cells per high powered field (HPF). Statistical analysis performed by student's t test.

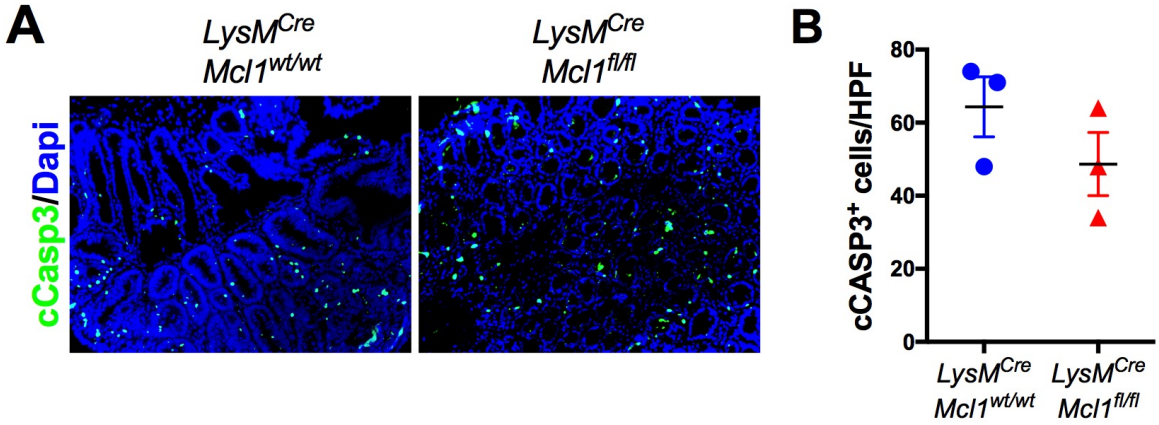

Figure S3.6. Monocytes are unchanged in *Mrp8-Cre/Mcl-1$^{-/-}$* mice. (A) Representative flow cytometry dot plots and **(B)** quantification of Ly6c+/Cd11b+ cells as a percentage of total myeloid cells from *Mrp8^{Cre};Mcl1$^{wt/wt}$* and *Mrp8^{Cre};Mcl1$^{fl/fl}$* mice. Statistical analysis performed by student's t test.

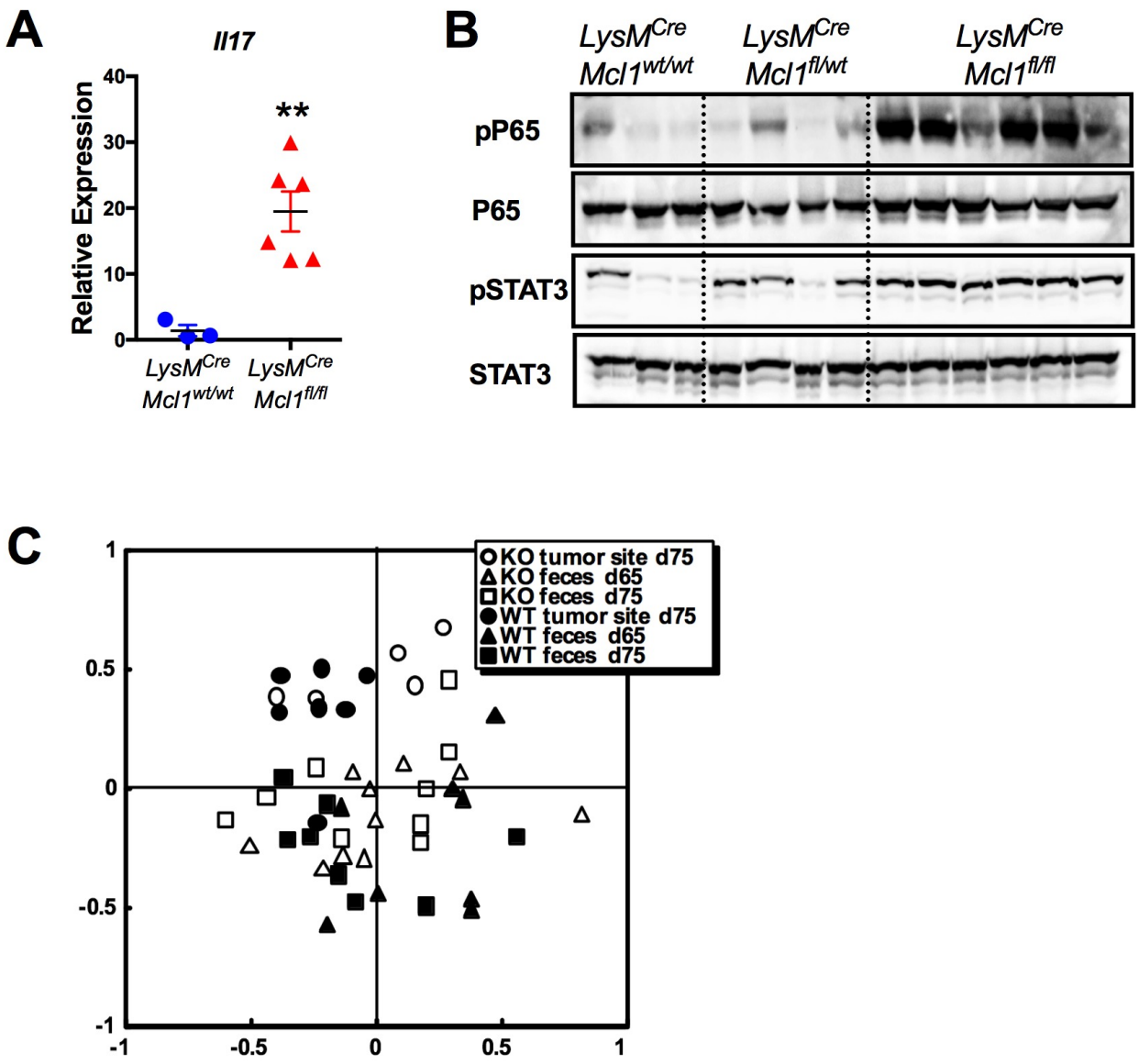

Figure S3.7. PMN depletion increases microbiota-dependent inflammatory responses. (A) Quantitative PCR analysis of *Il17* expression in $LysM^{Cre};Mcl1^{wt/wt}$ and $LysM^{Cre};Mcl1^{fl/fl}$ colon tumors. **p<0.01. **(B)** Western blot analysis of colon tumors from WT, $Mcl\text{-}1^{+/-}$, and $Mcl\text{-}1^{-/-}$ mice. Statistical analysis was performed with student's t-test. **(C)** Principal component analysis of bacterial composition in $LysM^{Cre};Mcl1^{wt/wt}$ and $LysM^{Cre};Mcl1^{fl/fl}$ tumor and fecal samples.

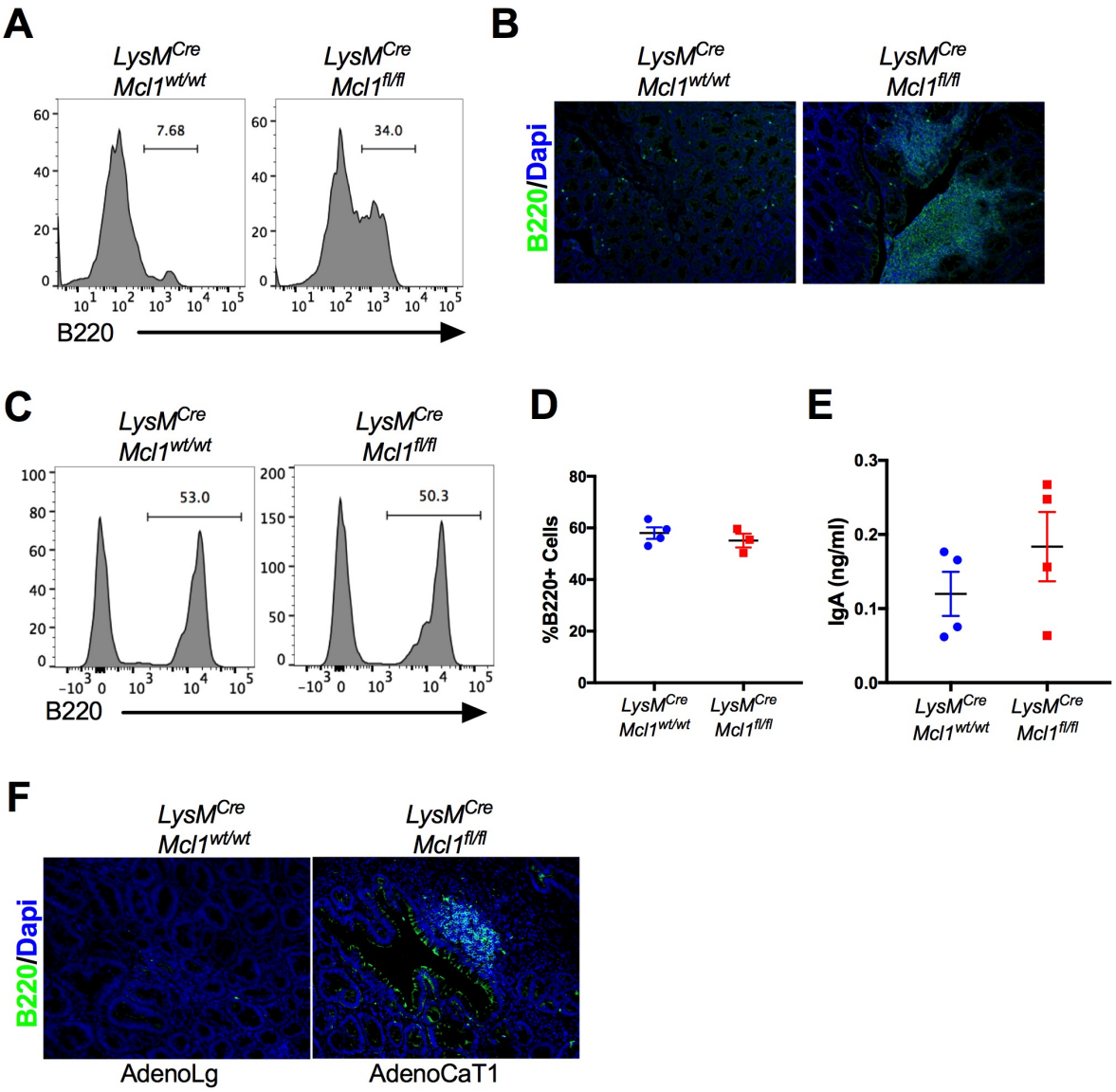

Figure S3.8. Increased B-cells in PMN-deficient colon tumors. (A) Flow cytometric analysis and **(B)** representative images of B220 staining of colon tumor tissue from *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice. **(C)** Flow cytometric analysis of B220 staining and **(D)** quantification of peripheral blood B-cells. **(E)** IgA ELISA analysis from feces of *LysM*^{Cre}*;Mcl1*$^{wt/wt}$ and *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mice. **(F)** Representative immunofluorescence of B220 staining from WT tumor tissue and invasive adenocarcinoma tissue from *LysM*^{Cre}*;Mcl1*$^{fl/fl}$ mouse. Statistical analysis performed by student's t test.

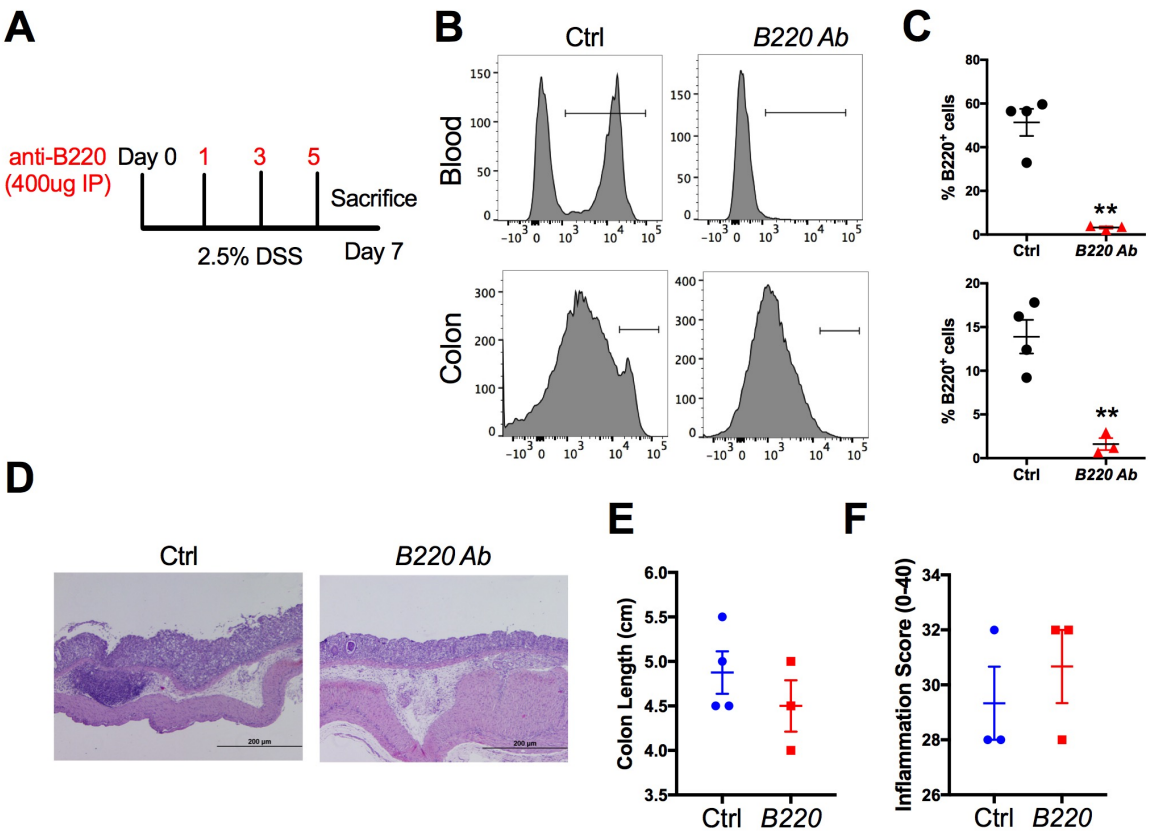

Figure S3.9. B-cell depletion does not exacerbate DSS colitis. (A) Schematic of 2.5% DSS treatment and anti-B220 injections (400ug/mouse I.P.) on days 1, 3, and 5. **(B)** Representative flow cytometric analysis of B220 staining in peripheral blood and colon tissue and **(C)** quantification B-cells of Ctrl and anti-B220 treated animals. **(D)** Representative H&E histologic analysis **(E)** colon length and **(F)** total inflammation score of treated mice. Statistical analysis performed by student's t test.

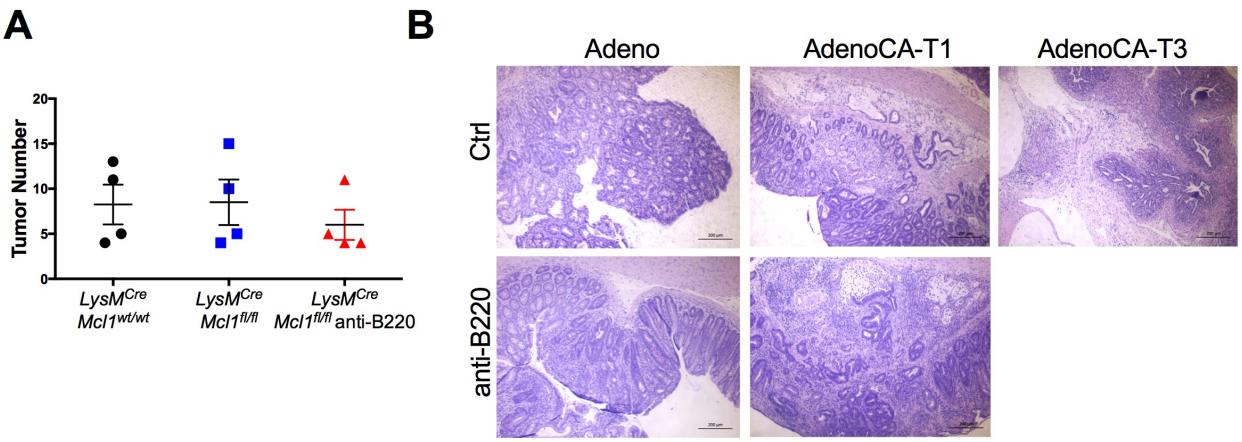

Figure S10. B-cell depletion reduces PMN-deficient tumor progression. (A) Tumor number and **(B)** representative histologic analysis indicated tumor stage from Ctrl and anti-B220 treated *LysM*^{Cre};*Mcl1*$^{fl/fl}$ mice. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test.

Chapter 4

Myc-Associated Zinc Finger Protein Regulates the Pro-Inflammatory Response in Colitis and Colon Cancer via STAT3 Signaling

Abstract

Myc-associated zinc finger (MAZ) is a transcription factor highly upregulated in chronic inflammatory disease and several human cancers. In the current study, we found that MAZ protein is highly expressed in human ulcerative colitis and colon cancer. However, the precise role for MAZ in the progression of colitis and colon cancer is not well defined. To determine the function of MAZ, a novel mouse model of intestine epithelial specific MAZ overexpression was generated. Expression of MAZ in intestinal epithelial cells was sufficient to enhance inflammatory injury in two complementary models of colitis. Moreover, MAZ expression increased tumorigenesis in an *in vivo* model of inflammation-induced colon cancer and was important for growth of human colon cancer cell lines *in vitro* and *in vivo*. Mechanistically, MAZ is critical in the regulation of oncogenic STAT3 signaling. MAZ expressing mice have enhanced STAT3 activation in the acute response to colitis, Moreover, MAZ was essential for cytokine and bacteria-induced STAT3 signaling in colon cancer cells. These data indicate an important functional role for MAZ in the inflammatory progression of colon cancer through regulation of STAT3 signaling and suggest MAZ is a potential therapeutic target to dampen STAT3 signaling in colon cancer.

Introduction

Colon cancer remains a significant public health concern and is the third-leading cause of cancer-related deaths in the United States (1). The tumor-associated inflammatory response is well-characterized as a key contributor to neoplastic progression, in part through activation of oncogenic signaling pathways in tumor cells, such as STAT3 and NF-*k*B (2). Myc-associated zinc finger (MAZ) is an inflammation-induced cys_2his_2-type zinc finger transcription factor that is highly active in inflammatory foci and previously has been suggested to be a critical driver of inflammation in animal models (3). Moreover, MAZ is highly expressed in cancer of the pancreas, liver, breast, and prostate (4-7). MAZ has three splice-variants (MAZ1, MAZ2, and MAZ3). MAZ splice-variants 1 and 3 are activated by inflammation, whereas MAZ2 is anti-inflammatory (8, 9). Our previous work has identified an essential role for MAZ in hypoxia-driven inflammatory responses in both colitis and colon cancer (10). MAZ is a direct protein interactor of the hypoxia-inducible transcription factor (HIF-2α) and is important for HIF-2α transactivation of inflammatory target genes, such as tumor necrosis factor α (*Tnfa*) (11, 12). However, HIF-2α-independent functions for MAZ in the progression of colitis and colon cancer are relatively unknown.

Signal transducer and activator of transcription 3 (STAT3) is an inflammation responsive transcription factor with an important role in the progression of several cancers (13). STAT3 signaling is activated by inflammatory cytokines such as IL-6, IL-10, and IL-22 and plays a key role in the development of Th17 cells (14). In intestinal epithelial cells, STAT3 signaling is an essential mediator of epithelial repair processes in the resolution of colitis downstream of IL-22 (15). Polymorphisms associated with STAT3 and STAT3 signaling pathway have been identified in both ulcerative colitis and Crohn's disease, suggesting an important role in the progression of

human inflammatory bowel disease (IBD) (16). In addition to the prominent role for STAT3 in colitis, STAT3 is an essential transcription factor for the progression of colitis associated colon cancer (CAC). Genetic disruption of STAT3 in intestinal epithelial cells decreases susceptibility to inflammation-induced colon cancer and chronically active STAT3 increases susceptibility to colon tumorigenesis (17). Deletion of the STAT3 activating cytokine IL-6 also reduced CAC (18). STAT3 activation downstream of microbial sensing pathways is also an important mechanism linking inflammation and colon cancer (19). Under normal circumstances, mechanisms exist to rapidly dampen STAT3 activation (20). In tumors STAT3 is chronically activated, suggesting tumor-specific factors maintain high STAT3 and drive neoplastic progression. Discovering factors essential in sustaining high STAT3 activation may provide novel druggable targets for the treatment of colon cancer.

In the current study, it was demonstrated that MAZ was restricted to epithelial cells and was highly expressed in both inflammatory disease as well as neoplastic disease of the colon. To dissect the role of MAZ in colitis and colon cancer, a novel model of transgenic epithelial-specific MAZ overexpressing mice was generated. RNA-sequence analysis from the transgenic mice found that MAZ regulates a novel repertoire of HIF-2α-independent genes. MAZ was an important driver of the inflammatory response in complementary models of chemically-induced and infectious colitis models. Moreover, MAZ expression significantly increased tumorigenesis in an inflammation-induced colon tumor model, suggesting MAZ was a novel link between inflammation and cancer of the colon. Mechanistically, a novel function for MAZ in the maintenance of STAT3 signaling both *in vivo* and *in vitro* was observed. This work delineates and important role for MAZ in the inflammatory response in colitis and colon cancer and suggests MAZ as a novel therapeutic target to dampen STAT3.

Methods

Animals and treatments

Mice expressing MAZ under control of the 12.4kb villin-promoter were generated (21). The mouse MAZ cDNA was subcloned from pET-16B plasmid into the Mlu1 and Kpn1 sites of the pUC12.4kb-villin promoter construct. The plasmid was then digested with Pme1 and used to generate *vilMAZ* mice in collaboration with the University of Michigan Transgenic Animal Core. *vilMAZ* mice were backcrossed to C57BL/6 mice for 5 generations. For all experiments, male and female mice aged 6 to 8-weeks were used. For dextran sulfate sodium (DSS)-colitis, *vilMAZ* and WT littermate controls were treated with 2.5% wt/vol DSS in drinking water for 7-days. For *Salmonella enterica* serovar Typhimurium (*S.* Typhi) colitis, mice were pretreated by oral gavage with streptomycin (7.5mg/mouse) and 24-hours later were orally gavaged with $1x10^7$ colony-forming units of *S.* Typhi. *Citrobacter rodentium*-colitis was induced with oral gavage of $1x10^7$ colony-forming units of *C. rod* in 6- to 8-week old mice WT mice. Azoxymethane (AOM)/DSS-induced tumorigenesis was induced in 6- to 8-week old *vilMAZ* and WT littermate controls. Mice were I.P. injected with AOM at (10mg/kg) then cycled on and off 2% DSS in their drinking water beginning 5-days following intial injection of AOM for 7-days with 14-days of regular drinking water interspersed between. Mice were sacrificed 100-days after AOM injection. For subcutaneous tumor growth, $1x10^6$ HCT116 cells were prepped in 37^oC 1x PBS and injected subcutaneously into the flanks of Nude mice. Nude mice were housed in autoclaved cages in facility specifically designed for housing immunodeficient mice and fed irradiated food and autoclaved water. Germ free mice were housed in the University of Michigan Germ Free core and treated with AOM/DSS as described above. Germ free mice were conventionalized with a single gavage of feces from WT mice. TLR2/4 double knockout mice were previously

described (22). All experiments were carried out using guidelines and approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Human tissue

Human IBD and colon cancer tissue was procured at the University of Michigan per Institutional Review Board approval and guidelines.

Cell culture and cell treatments

HCT116, MCA38, and human embryonic kidney (HEK) cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS and 1% anti-biotic/anti-mycotic. Cells were cultured at 37^0C and 5% CO_2 and 21% O_2. STAT3 response luciferase assay (SIE) was conducted by co-transfection of SIE luciferase plasmid with pCDNA MAZ1 or MAZ3 into cells using polyethylinimine (PEI) reagent. 24-hours after transfection, cells were treated with IL-6 (10ng/ml) for 12-hours. Standard luciferase assay was performed as previously described and normalized to ß-galactosidase activity (12). MAZ knockdown HCT116 cells were generated as previously described (11). For IL-6 treatments, cells were seeded in 6-well plates at a density of 3x10^5/well. 24 hours later, cells were treated with IL-6 (10ng/ml) for 30, 60, and 120 minutes. For JAK1/2 inhibition studies, HEK293T or HCT116 cells were transfected 500ng of pCDNA MAZ1 and MAZ3 for 24-hours then treated with 3uM Ruxolitinib or DMSO for 12-hours. For co-immunoprecipitation experiments, MAZ was co-transfected with human myc-JAK1 or myc-JAK2 expression construct for 48-hours. Cells were lysed and myc-immunoprecipitation was performed using Myc-TRAP beads as described in manufacturers protocol (Chromotek). For *S.* Typhi treatment, HCT116 and HT29 cells were seeded at a density

of 3x10^5 cells/well in a 6-well plate. 24-hours later cells were washed 3x with PBS then treated with 1x10^6 CFUs of *S.* Typhi for 4- and 8-hours. siRNA transfection was performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific) and 5μM MAZ or Ctrl siRNAs (Santa Cruz Biotechnology). Cell growth was monitored using 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent.

Histology and immunofluorescence

Colon tissue was excised, washed with cold PBS, and cut longitudinally. Tumors were sized and counted and then colon tissue was rolled and fixed in 10% formalin for 12-hours. Tissues were then embedded in paraffin. 5μm tissue sections were stained with hematoxylin and eosin and all histological analysis was done in a blinded manner as previously described (11). Inflammation scoring was based on crypt damage, inflammation, depth of injury, and percentage of tissue affected. Immunofluorescence of human ulcerative colitis was performed with frozen sections which were fixed with 10% formalin for 10-minutes, permeabilized with 0.5% Triton X-100 for 10-minutes, then blocked with 5% goat serum for 1-hour. Slides were then incubated with primary antibody for MAZ overnight (1:100, Abcam). Proliferation analysis was performed on paraffin embedded tissue slides following citrate buffer antigen retrieval using Ki67 antibody (Vector Laboratories).

Protein isolation and western blotting

Western blots were performed as previously described (11). Antibodies against MAZ (1:1000, Active Motif), GAPDH (1:1000, Santa Cruz Biotechnology), phospho-STAT3 (1:1000, Cell Signaling Technologies), total-STAT3 (1:1000, Cell Signaling Technologies), phosho-P38

(1:1000, Cell Signaling Technologies), total-P38 (1:1000, Cell Signaling Technologies), phospho-AKT (1:1000, Cell Signaling Technologies), total-AKT (1:1000, Cell Signaling Technologies), pJNK (1:1000, Cell Signaling Technologies), total-JNK (1:1000, Cell Signaling Technologies), phospho-JAK (1:1000, Cell Signaling Technologies), and total-JAK (1:1000, Cell Signaling Technologies) were used for blotting. Secondary horseradish peroxidase-conjugated antibodies (Cell Signaling Technologies) were used for protein detection.

Colon tumor enteroids

Patient-derived colon tumor and normal tissue enteroids were generated as previously described (23).

RNA isolation and qPCR analysis

RNA was isolated using isol-RNA lysis reagent and qPCR analysis was performed as previously described (24). Primers are listed in Supplemental Table 3.1. Expression analysis was normalized to β-Actin expression.

RNA-seq and RNA-seq data analysis

RNA sequencing was performed as previously described (10). Briefly, the TruSeq RNA library prep kit v2 (Illumina) was used to prepare RNA sequencing libraries The libraries were sequenced using single-end 50-cycle reads on a HiSeq 2500 sequencer (Illumina). The Flux high-performance computer cluster at the University of Michigan was used for computational analysis. RNA-seq read quality was assessed utilizing FastQC. Reads were aligned to a splice junction aware build of the mouse genome (mm10) using STAR(25) with the options

"outFilterMultimapNmax 10" and "sjdbScore 2". Differential expression testing between WT and *vilMAZ* colon samples was conducted with CuffDiff v 2.1.1 with the parameter settings "-compatible-hits-norm," and "–frag-bias-correct". We used UCSC mm10.fa and the GENCODE mouse M12 primary assembly annotation GTF as the reference genome and reference transcriptome, respectively. Genes were considered differentially expressed at a false-discovery rate-adjusted (FDR) *P* value of <0.05.

Statistical analysis

P-values were calculated by students t-test, paried t-test, or one-way ANOVA. Differential expression of mRNA expression with qPCR was calculated using deltadeltaCT method. Error bars represent standard error of the mean.

Results

Intestine epithelial MAZ regulates a novel repertoire of genes

MAZ is an important transcriptional cofactor in the hypoxic inflammatory response through direct interaction with HIF-2α and directing the pro-inflammatory HIF-2α transcriptional response (11). However, the precise functional role for MAZ in the progression of inflammation in colitis has not been assessed. To delineate the function of MAZ in the inflammatory progression of colitis a mouse model of intestinal epithelial-specific MAZ overexpressing transgenic mice was generated. cDNA for MAZ expressing a N-terminal FLAG-tag was cloned into the previously described 12.4kb *Villin*-promoter construct to drive MAZ expression to the intestine epithelium; hereby referred to as *vilMAZ* mice (21). MAZ and FLAG protein expression were verified from colon and small intestine lysates from WT and *vilMAZ*

mice (Fig. 4.1A). *Maz* transcript expression was 3-fold higher in colonic tissue and 12-fold higher in small intestine tissue from *vilMAZ* mice (Fig. 4.1B). Histologic analysis shows no observable differences in colonic architecture in untreated mice (Fig. 4.1C). Subcellular fractionation of colon tissue from WT and *vilMAZ* mice showed the vast majority of MAZ protein in *vilMAZ* mice was localized to the nucleus (Fig. 4.1D). No changes in expression of inflammatory cytokines *Tnfa* and *Il1β*, or colon lineage markers *Muc2*, *Tff3*, *Chga*, or *Cdh1* were observed (Fig. 4.1E). RNA-sequencing analysis was performed on colon tissue to determine genes differentially expressed in intestinal tissue of *vilMAZ* mice. Several novel genes including a number of inflammation-associated genes regulated by epithelial MAZ expression were identified (Fig. 4.1F). Interestingly, only 24% of significantly changed genes identified in *vilMAZ* RNA-seq corresponded with identified genes in RNA-seq from colon tissue of HIF-2α expressing animals (10) suggesting a repertoire of HIF-2α-independent target genes (Fig. 4.1G). qPCR validation of several targets confirmed MAZ expression significantly increases expression of *Gatsl3*, which encodes an arginine sensing protein, as well as the lncRNA *Fer1l4*. Moreover, MAZ expression decreased expression of the antimicrobial peptide *Ang4* and the zinc transporter *Slc30a10* (Fig. 4.1H). These results identify a novel repertoire of MAZ-regulated genes in colon epithelial cells, most of which do not overlap with genes directly regulated with HIF-2α.

MAZ potentiates the acute inflammatory response in colitis

MAZ protein is highly expressed in several inflammatory models including inflammatory arthritis (9). However, MAZ protein expression has never been analyzed in inflammatory disease of the colon. MAZ protein was significantly upregulated in mucosal biopsy specimens from patients with ulcerative colitis (UC) compared to normal patient controls (Fig. 4.2A). The vast

majority of MAZ protein in UC was localized to inflamed colon epithelial cells (Fig. 4.2B). Interestingly, increased MAZ protein expression was not observed in ileal biopsy specimens from Crohn's Disease (CD) (Fig. 4.2C). This suggests a potential role for colon epithelial MAZ in the inflammatory progression of colitis. The RNA-seq data demonstrates that MAZ regulates several genes known to be important inflammatory targets. An acute model of colitis was initiated by treatment of WT and *vilMAZ* mice with 2.5% dextran sulfate sodium (DSS) in the drinking water for seven days then changed back to regular drinking water for three days. Compared to WT animals, *vilMAZ* animals lost significantly more weight and failed to recover body weight (Fig. 4.2D). MAZ-expressing mice had moderately shorter colon lengths after DSS-colitis suggesting heightened inflammation (Fig. 4.2E). Histologic analysis shows a robust inflammatory cell infiltrate and disruption of colonic architecture and histological scoring confirmed that MAZ-expressing transgenic animals had robustly higher inflammation in DSS-induced colitis compared to WT mice (Fig. 4.2F & G). These data suggest that epithelial MAZ expression is sufficient to increase inflammation in acute DSS-colitis and that MAZ upregulation in human ulcerative colitis may potentiate disease progression.

MAZ is regulated by colon microbiota and sensitizes mice to bacteria-induced colitis

Microbial dysbiosis is now well appreciated as a critical driver of inflammation and disease progression in IBD (26). MAZ expression has previously been shown to be regulated by microbial products (27). To assess the role for pathogenic microbes in the regulation of MAZ protein in colitis, a *Salmonella enterica* serovar Typhimurium (*S.* Typhi) murine model of colitis was assessed. The *S.* Typhi model of murine colitis can recapitulate many of the aspects observed in human colitis (28). WT mice were pretreated with streptomycin and then inoculated

with *S.* Typhi. Robust MAZ protein expression is observed in a time-dependent manner at 5 and 10-days following inoculation (Fig. 4.3A).

To further delineate the role for pathogenic microbes in MAZ induction, we used the *Citrobacter rodentium* (*C. Rod*) model of colitis (29). In mice treated with *C. Rod* for 10-days, MAZ protein was also robustly induced (Fig. 4.3B). Both *S.* Typhi and *C. rod* induce robust colonic inflammation, therefore, it is possible the activation of MAZ in these tissues may be indirect through inflammatory mediators.

To determine if bacteria directly activated MAZ, colon cancer-derived cell lines HCT116 and HT29 were infected with *S.* Typhi *in vitro*. MAZ protein was increased as early as 4-hours and was sustained through 8-hours after infection (Fig. 4.3C). To address if basal maintenance of MAZ protein is microbiota-dependent, germ free (GF) and conventionalized (Conv) mice were assessed. Compared to GF colon tissue, microbiota conventionalization upregulated MAZ protein in colon tissue suggesting that microbiota are critical for basal expression of MAZ (Fig. 4.3D). Futhermore *S.* Typhi was sufficient to promote robust MAZ expression in WT mice but not toll-like receptor (TLR)2/4 double KO mice (Fig. 4.3E) suggesting that LPS-dependent TLR signaling is a key-mediator of MAZ upregulation in colitis. To assess the role for MAZ in bacterial-derived colitis, WT and *vilMAZ* mice were treated with *S.* Typhi for 10-days. *vilMAZ* mice had significantly enhanced inflammation in bacterial colitis compared to WT mice, suggesting activation of MAZ in bacterial-colitis is a critical step in the inflammatory response (Fig. 4.3F & G).

MAZ expression increases colitis-associated colon tumorigenesis

Inflammation and cancer of the colon are linked (30). Our data show that MAZ expression is sufficient to increase inflammation in models of acute colitis. To model inflammation-dependent colon tumorigenesis, an AOM/DSS colon tumor model was assessed (Fig. 4.4A) (31). Compared to WT mice, *vilMAZ* mice develop significantly more colon tumors and had higher tumor burden in the inflammation-induced AOM/DSS model (Fig. 4.4B-D). Moreover, tumors from *vilMAZ* mice had a higher proportion of actively proliferating cells (Fig. 4.4E). This observation suggests an important role for activation of epithelial MAZ in the transition from chronic inflammation to colon cancer.

We next assessed the requirement for microbiota in MAZ protein induction in inflammation induced colon tumors. GF and SPF mice were subjected to AOM/DSS-induced tumorigenesis. SPF mice demonstrated a robust MAZ expression in colon tumors compared to adjacent normal colon tissue, MAZ expression in GF mice was dramatically reduced in the adjacent normal colon tissue, compared to SPF mice, and the increased tumor-associated MAZ expression was ablated (Fig. 4.4F). Collectively, these data suggest microbial-mechanisms are essential for MAZ activation in inflammation-induced colon tumors and that this axis increases disease severity.

MAZ protein is highly expressed in human colon cancer and has cell autonomous roles in colon cancer growth.

MAZ has an important role in the induction of inflammation-driven colon cancer *in vivo*. Moreover, increased MAZ protein expression is observed in tumor tissue compared to adjacent normal colon tissue in ten colorectal cancer (CRC) patients (Fig. 4.5A). MAZ protein expression was assessed in human normal tissue or colon tumor enteroids. Colon tumor enteroids are

derived from primary patient colon tumor epithelial cells and maintain three-dimensional structure and cell polarity (23). Interestingly, MAZ protein was highly induced in patient-derived colon tumor enteroids compared to normal colon enteroids suggesting MAZ is upregulated in epithelial cells of colon cancer patients (Fig. 4.5B). Oncomine data analysis suggests MAZ mRNA is decreased in human colon tumors compared to normal colon (Fig. 4.5C). The Cancer Genome Atlas (TCGA) analysis shows significant decrease of MAZ mRNA in human colon cancer relative to normal colon tissue (Fig. 4.5D). qPCR analysis of matched patient normal and colon tumor tissue shows that 7/10 patients have a relative decrease in the tumor MAZ mRNA (Fig. 4.5E). Collectively, these data suggest that in human colon cancer, novel mechanisms promoting the activation of MAZ protein are established independent of MAZ transcripts to promote tumorigenesis and tumor growth.

MAZ protein was expressed in varying degrees across a panel of several different human colon cancer cell lines (Fig. 4.5F). To determine if MAZ is important for colon cancer growth, siRNAs that robustly reduced expression of MAZ in cell lines were tested (Fig. 5G). In HCT116, SW480, and HT29 cells, siRNA-mediated MAZ knockdown significantly reduced colon cancer cell growth *in vitro* (Fig. 4.5H). Furthermore, HCT116 cells with shRNA-mediated MAZ knockdown grow significantly slower than control shRNA (shCtrl) expressing HCT116 cells in subcutaneous tumor model in Nude mice (Fig. 4.5I). These data suggest MAZ has important functions for regulating colon cancer growth independent of the enhanced inflammatory response in the AOM/DSS model.

MAZ expression induces STAT3 activation in acute colitis

No direct regulator of MAZ-mediated cell growth was identified in the RNA-sequence analysis. To address mechanisms by which MAZ expression enhances colon cancer growth colon tissue from WT and *vilMAZ* mice were screened to analyze pathways that are important in driving colon tumorigenesis in both sporadic and CAC models (Fig. 4.6A). Epithelial MAZ expression correlated with heightened STAT3 phosphorylation (Fig. 4.6A). STAT3 is an inflammation-induced transcription factor that is an essential driver of tumorigenesis in CAC and sporadic colon cancer (17, 32). To determine if MAZ-induced STAT3 activation was cell autonomous, MAZ transcript variants 1 and 3 (MAZ1 & MAZ3) were expressed in HEK cells. Western blot analysis shows both MAZ1 and MAZ3 expression robustly induced STAT3 phosphorylation (Fig. 4.6B). To delineate if this led to heightened STAT3 transcriptional activity, STAT3-responses element luciferase reporter (SIE) was assessed. In colon-cancer HCT116 cells, expression of MAZ1 and MAZ3 dramatically potentiated IL-6-induced STAT3 transcriptional activity (Fig. 4.6C). These data suggested that MAZ-induced STAT3 activation was independent of enhanced inflammation in colitis. Interestingly, no difference in STAT3 activation was observed in tumor tissue from WT or *vilMAZ* mice. However, colon tumor induced MAZ expression in WT mice was relatively equivalent to the observed MAZ protein expression in tumors of *vilMAZ* mice (Fig. 4.6D). Furthermore, knockdown of MAZ in HCT116 cells dramatically reduced STAT3 phosphorylation in response to IL-6 at all time points assessed (Fig. 4.6E). This also led to decreased STAT3 transcriptional response as MAZ knockdown significantly blunted SIE luciferase activity in response to IL-6 (Fig. 6F). In colon cancer, IL-6 and IL-11 are key cytokines in STAT3 activation and tumor progression (33). However, bacterial products also regulate STAT3 signaling, specifically products derived from *Salmonella* (34). Treatment with *S.* Typhi in MAZ knockdown cells led to significantly reduced STAT3 activation

compared to control cells (Fig. 4.6G). Additionally, siRNA knockdown of MAZ in mouse colon cancer cell line MCA38 and HEK cells also show reduced STAT3 responsiveness to IL-6 (Fig. 4.6H & I). These results delineate an essential role for MAZ in regulation of STAT3 activation in colon cancer cells in response to both cytokine and bacterial stimuli. Moreover, the data suggests MAZ serves as a link between inflammation-induced STAT3 signaling and colon cancer.

MAZ regulates STAT3 signaling in a JAK-dependent manner

STAT3 is activated in response to extracellular cytokines whose receptors promote the activation of Janus Kinases (JAKs) through the adaptor protein GP130 (35). Under normal circumstances, JAK-mediated STAT3 activation was rapidly inhibited by various negative feedback mechanisms. To assess the requirement for JAK in MAZ-dependent STAT3 activation, the JAK1/2 inhibitor Ruxolitinib was assessed. In HCT116 and HEK cells, expression of both MAZ1 and MAZ3 were sufficient to induce STAT3 phosphorylation. However, in cells treated with Ruxolitinib, the increased STAT3 phosphorylation was completely ablated (Fig. 4.7A & B). MAZ knockdown decreased phosphorylated JAK in colon cancer cells suggesting MAZ is a novel regulator of JAK activation (Fig. 4.7C). Interestingly, MAZ knockdown did not decrease mRNA expression of any of the major components of the STAT3 signaling cascade (Fig. 4.7D). Furthermore, no direct interaction of MAZ with either JAK1 or JAK2 could be detected by co-immunoprecipitation *in vitro* (Fig. 4.7E & F).

Discussion

MAZ was discovered more than two decades ago, however, relatively little is known about the function of MAZ in normal physiology or the disease state. MAZ was first described as a factor binding to the *cMYC* promoter (36). Since then several MAZ direct target genes have been described. Our previous work has shown an essential role for MAZ in the hypoxic progression of colitis and MAZ is a direct protein interactor of HIF-2α (12). HIF-2α transcription of the pro-inflammatory cytokine *Tnfa* was MAZ-dependent (11). Moreover, HIF-2α dependent expression of Caveolin-1, which increased acute inflammation in colitis through decreased epithelial barrier integrity, was MAZ-dependent (12). Our previous data suggested that MAZ was an important HIF-2α cofactor and directed HIF-2α to promoters of inflammatory target genes. Using a novel mouse model of intestine epithelial specific MAZ expression, our current data suggest HIF-2α-independent functions of MAZ in the inflammatory progression of colitis and colon cancer. A novel repertoire of MAZ regulated genes and pathways have been identified.

MAZ was highly active in inflammatory foci and was highly expressed in inflamed tissues of rheumatoid arthritis patients. Transgenic MAZ expressing mice have previously been shown to potentiate the inflammatory response in an infectious model of arthritis (3). Mechanistically, MAZ regulates transcription of *mmp1* and *mmp9* in inflamed joint tissue (37, 38). MAZ expressing animals have heightened susceptibility to develop serum amyloidosis, a condition associated with chronic inflammation due to rheumatoid arthritis and Crohn's disease (39). Consistent with the previous data, we have shown that intestine epithelial MAZ expression is sufficient to enhance the acute inflammatory response in complementary models of infectious and chemically induced colitis. Previous work has identified MAZ regulation through

inflammatory stimuli including IL-6, IL-1B, and LPS (40, 41). In monocytic cells, direct LPS treatment is sufficient to drive MAZ expression (27). Moreover, these studies have identified inflammation-dependent phosphorylation events which regulate MAZ transcriptional potential (42). In the current study, we propose that pathogenic microbes are key regulators of MAZ protein expression. We find that MAZ is robustly induced in two separate bacterial models of colitis. MAZ protein was also induced in GF mice when colonized with microbiota from SPF mice. Pathogenic bacteria can directly induce MAZ protein in colon cancer cells independent of an underlying inflammatory response. Furthermore, microbiota are essential for MAZ protein expression in colon tumors. Microbial dysbiosis is critical in activating inflammation in human colitis and human colon cancer (43). Thus, it is perhaps possible that the increased MAZ protein we observed in human ulcerative colitis patients is in part due to microbial dysbiosis and direct activation by colon microbiota. Increased MAZ expression observed in colitis may serve to enhance the inflammatory response through HIF-2α-dependent as well as independent mechanisms and provide a critical link between chronic inflammation and colon cancer.

Several mechanisms for MAZ-dependent regulation of tumor growth and progression have been proposed. In hepatocellular carcinoma, MAZ was shown to regulate EMT signature genes to promote tumor invasion (4). In breast cancer, MAZ was shown to transcriptionally regulate expression of both *HRAS* and *KRAS* to direct tumor angiogenesis (44). Additionally, MAZ can directly drive angiogenesis in triple negative breast cancer cells through direct transcriptional regulation of *VEGF* (7). In prostate cancer, MAZ can regulate tumor cell growth through activation of androgen receptor expression (5). Furthermore, in pancreatic cancer MAZ is a regulator of *KRAS* transcription and therapeutic targeting of MAZ interaction with *KRAS* promoter using decoy oligonucleotides shows efficacy in decreasing tumor growth (45). This

method also showed efficacy in preclinical bladder cancer models (46). In the current study, using novel epithelial specific transgenic MAZ overexpressing mice, MAZ expression is sufficient to enhance tumorigenesis in an inflammation-induced model. Furthermore, MAZ was shown to have cell autonomous regulation of cell growth in colon cancer cell both *in vitro* and *in vivo*. Recently, our work suggested an important role for MAZ in the inflammatory progression of colon cancer by regulating HIF-2α-mediated expression of the potent neutrophil chemokine *Cxcl1* (10). In the current study, we have discovered a novel HIF-2α-independent function for MAZ in the regulation of STAT3 signaling.

Persistent STAT3 activation is observed in human colon cancer and epithelial expression of STAT3 is a crucial link between inflammation and cancer of the colon. Intestine epithelial specific STAT3 knockout mice develop very few tumors in CAC (17). Conversely, transgenic mice with a constitutive activation of intestinal epithelial STAT3 develop significantly more tumors in the same model (17). STAT3 activation is transient and negatively regulated by various intracellular proteins including SOCS3. In tumors STAT3 is persistently elevated through unknown mechanisms. Previous studies have suggested intra-tumoral iron as an important mediator of STAT3 maintenance in colon tumors (47). Furthermore, the negative TLR signaling regulator IRAK-M has been shown to maintain STAT3 signaling through increased STAT3 protein stability, linking microbial sensing to STAT3 maintenance (19). MAZ was found to be sufficient to increase STAT3 phosphorylation in acute colitis *in vivo* as well as in cell lines. Moreover, MAZ was essential for the activation of STAT3 in response to both cytokine and bacterial stimuli. MAZ protein was highly upregulated in human colon cancer, suggesting that MAZ upregulation in colon cancer is a necessary event to maintain STAT3 activation at high levels. Recent evidence suggests MAZ is a key regulator of both AKT signaling pathways in

pancreatic cancer cells (48). However, no changes to AKT activation were detected in the *vilMAZ* mice (Fig. 6A). Our combination of *in vivo* and *in vitro* data suggest MAZ may provide a novel therapeutic target to dampen colon cancer associated inflammatory responses through reduction of STAT3 signaling and may have efficacy for treatment of patients. Interestingly, MAZ did not transcriptionally alter any key components of the JAK/STAT3 signaling pathway and no known direct regulators of this pathway were discovered in RNA-sequence data. Moreover, MAZ did not directly interact with JAK1 or JAK2, suggesting novel mechanisms integrating MAZ and JAK/STAT3 signaling. Interestingly, JAKs have several post-translational modifications including phosphorylation, acetylation, and ubiquitylation, most of which are not well understood or characterized in their functional regulation of JAK activity (49). Further investigation aimed at understanding how MAZ may regulate JAK activity and protein modifications will be important to address how MAZ modulates the activation of STAT3.

Funding Information: This work was supported by NIH grants (CA148828 and DK095201 to Y.M.S., ES028802 to J.A.C.), the University of Michigan Gastrointestinal Peptide Center (Y.M.S.), a pilot grant from the University of Michigan GI Spore (CA130810 to Y.M.S.), D.T. was supported by NIH Grant (F30CA213664).

References

1. Siegel RL, Miller KD, Jemal A. 2018. Cancer statistics, 2018. CA: A Cancer Journal for Clinicians 68:7-30.
2. Terzić J, Grivennikov S, Karin E, Karin M. 2010. Inflammation and Colon Cancer. Gastroenterology 138:2101-2114.e5.
3. Ray A, Kumar D, Shakya A, Brown CR, Cook JL, Ray BK. 2004. Serum Amyloid A-Activating Factor-1 (SAF-1) Transgenic Mice Are Prone to Develop a Severe Form of Inflammation-Induced Arthritis. The Journal of Immunology 173:4684.
4. Luo W, Zhu X, Liu W, Ren Y, Bei C, Qin L, Miao X, Tang F, Tang G, Tan S. 2016. MYC associated zinc finger protein promotes the invasion and metastasis of hepatocellular carcinoma by inducing epithelial mesenchymal transition. Oncotarget 7:86420-86432.
5. Jiao L, Li Y, Shen D, Xu C, Wang L, Huang G, Chen L, Yang Y, Yang C, Yu Y, Sun Y. 2013. The prostate cancer-up-regulated myc-associated zinc-finger protein (MAZ) modulates proliferation and metastasis through reciprocal regulation of androgen receptor. Medical Oncology 30:570.
6. Zhu X, Luo W, Liang W, Tang F, Bei C, Ren Y, Qin L, Tan C, Zhang Y, Tan S. 2016. Overexpression and clinical significance of MYC-associated zinc finger protein in pancreatic carcinoma. OncoTargets and therapy 9:7493-7501.
7. Ray A, Dhar S, Ray BK. 2011. Control of VEGF Expression in Triple-Negative Breast Carcinoma Cells by Suppression of SAF-1 Transcription Factor Activity. Molecular Cancer Research 9:1030.
8. Ray BK, Murphy R, Ray P, Ray A. 2002. SAF-2, a Splice Variant of SAF-1, Acts as a Negative Regulator of Transcription. Journal of Biological Chemistry 277:46822-46830.
9. Ray A, Dhar S, Shakya A, Ray P, Okada Y, Ray BK. 2009. SAF-3, a novel splice variant of the SAF-1/MAZ/Pur-1 family, is expressed during inflammation. FEBS Journal 276:4276-4286.
10. Triner D, Xue X, Schwartz AJ, Jung I, Colacino JA, Shah YM. 2017. Epithelial Hypoxia-Inducible Factor 2α Facilitates the Progression of Colon Tumors through Recruiting Neutrophils. Molecular and Cellular Biology 37:e00481-16.
11. Xue X, Ramakrishnan S, Anderson E, Taylor M, Zimmermann EM, Spence JR, Huang S, Greenson JK, Shah YM. 2013. Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice. Gastroenterology 145:831-841.
12. Xie L, Xue X, Taylor M, Ramakrishnan SK, Nagaoka K, Hao C, Gonzalez FJ, Shah YM. 2014. Hypoxia-Inducible Factor/MAZ-Dependent Induction of Caveolin-1 Regulates Colon Permeability through Suppression of Occludin, Leading to Hypoxia-Induced Inflammation. Molecular and Cellular Biology 34:3013-3023.
13. Yu H, Pardoll D, Jove R. 2009. STATs in cancer inflammation and immunity: a leading role for STAT3. Nature Reviews Cancer 9:798.
14. Dang Eric V, Barbi J, Yang H-Y, Jinasena D, Yu H, Zheng Y, Bordman Z, Fu J, Kim Y, Yen H-R, Luo W, Zeller K, Shimoda L, Topalian Suzanne L, Semenza Gregg L, Dang Chi V, Pardoll Drew M, Pan F. Control of T_H17/T_{reg} Balance by Hypoxia-Inducible Factor 1. Cell 146:772-784.

15. Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, Lehr H-A, Hirth S, Weigmann B, Wirtz S, Ouyang W, Neurath MF, Becker C. 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. The Journal of Experimental Medicine 206:1465-1472.
16. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, Ripke S, Lee JC, Jostins L, Shah T, Abedian S, Cheon JH, Cho J, Dayani NE, Franke L, Fuyuno Y, Hart A, Juyal RC, Juyal G, Kim WH, Morris AP, Poustchi H, Newman WG, Midha V, Orchard TR, Vahedi H, Sood A, Sung JY, Malekzadeh R, Westra H-J, Yamazaki K, Yang S-K, The International Multiple Sclerosis Genetics C, The International IBDGC, Barrett JC, Alizadeh BZ, Parkes M, Bk T, Daly MJ, Kubo M, Anderson CA, Weersma RK. 2015. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nature genetics 47:979-986.
17. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli Ö, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-Mediated Stat3 Activation in Enterocytes Regulates Cell Survival and Cell-Cycle Progression during Colitis-Associated Tumorigenesis. Cancer Cell 15:91-102.
18. Grivennikov S, Karin E, Terzic J, Mucida D, Yu G-Y, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. 2009. IL-6 and STAT3 are required for survival of intestinal epithelial cells and development of colitis associated cancer. Cancer cell 15:103-113.
19. Kesselring R, Glaesner J, Hiergeist A, Naschberger E, Neumann H, Brunner Stefan M, Wege Anja K, Seebauer C, Köhl G, Merkl S, Croner Roland S, Hackl C, Stürzl M, Neurath Markus F, Gessner A, Schlitt H-J, Geissler Edward K, Fichtner-Feigl S. IRAK-M Expression in Tumor Cells Supports Colorectal Cancer Progression through Reduction of Antimicrobial Defense and Stabilization of STAT3. Cancer Cell 29:684-696.
20. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. 2001. Cis3/Socs3/Ssi3 Plays a Negative Regulatory Role in Stat3 Activation and Intestinal Inflammation. The Journal of Experimental Medicine 193:471-482.
21. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. 2002. cis Elements of the Villin Gene Control Expression in Restricted Domains of the Vertical (Crypt) and Horizontal (Duodenum, Cecum) Axes of the Intestine. Journal of Biological Chemistry 277:33275-33283.
22. Zeng MY, Cisalpino D, Varadarajan S, Hellman J, Warren HS, Cascalho M, Inohara N, Núñez G. 2016. Gut Microbiota-Induced Immunoglobulin G Controls Systemic Infection by Symbiotic Bacteria and Pathogens. Immunity 44:647-658.
23. Dame MK, Jiang Y, Appelman HD, Copley KD, McClintock SD, Aslam MN, Attili D, Elmunzer BJ, Brenner DE, Varani J, Turgeon DK. 2014. Human colonic crypts in culture: segregation of immunochemical markers in normal versus adenoma-derived. Laboratory investigation; a journal of technical methods and pathology 94:222-234.
24. Xue X, Shah YM. 2013. Hypoxia-inducible factor-2α is essential in activating the COX2/mPGES-1/PGE(2) signaling axis in colon cancer. Carcinogenesis 34:163-169.

25. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15-21.
26. Matsuoka K, Kanai T. 2015. The gut microbiota and inflammatory bowel disease. Seminars in Immunopathology 37:47-55.
27. Ray BK, Ray A. 1997. Involvement of an SAF-like Transcription Factor in the Activation of Serum Amyloid A Gene in Monocyte/Macrophage Cells by Lipopolysaccharide. Biochemistry 36:4662-4668.
28. Barthel M, Hapfelmeier S, Quintanilla-Martínez L, Kremer M, Rohde M, Hogardt M, Pfeffer K, Rüssmann H, Hardt W-D. 2003. Pretreatment of Mice with Streptomycin Provides a Salmonella enterica Serovar Typhimurium Colitis Model That Allows Analysis of Both Pathogen and Host. Infection and Immunity 71:2839-2858.
29. Koroleva EP, Halperin S, Gubernatorova EO, Macho-Fernandez E, Spencer CM, Tumanov AV. 2015. Citrobacter rodentium-induced colitis: A robust model to study mucosal immune responses in the gut. Journal of Immunological Methods 421:61-72.
30. Triner D, Shah YM. 2016. Hypoxia-inducible factors: a central link between inflammation and cancer. The Journal of Clinical Investigation 126:3689-3698.
31. Robertis MD, Massi E, Poeta ML, Carotti S, Morini S, Cecchetelli L, Signori E, Fazio VM. 2011. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. Journal of Carcinogenesis 10:9.
32. Phesse TJ, Buchert M, Stuart E, Flanagan DJ, Faux M, Afshar-Sterle S, Walker F, Zhang H-H, Nowell CJ, Jorissen R, Tan CW, Hirokawa Y, Eissmann MF, Poh AR, Malaterre J, Pearson HB, Kirsch DG, Provero P, Poli V, Ramsay RG, Sieber O, Burgess AW, Huszar D, Vincan E, Ernst M. 2014. Partial inhibition of gp130-Jak-Stat3 signaling prevents Wnt–β-catenin–mediated intestinal tumor growth and regeneration. Science Signaling 7:ra92.
33. Putoczki Tracy L, Thiem S, Loving A, Busuttil Rita A, Wilson Nicholas J, Ziegler Paul K, Nguyen PM, Preaudet A, Farid R, Edwards KM, Boglev Y, Luwor Rodney B, Jarnicki A, Horst D, Boussioutas A, Heath Joan K, Sieber Oliver M, Pleines I, Kile Benjamin T, Nash A, Greten Florian R, McKenzie Brent S, Ernst M. Interleukin-11 Is the Dominant IL-6 Family Cytokine during Gastrointestinal Tumorigenesis and Can Be Targeted Therapeutically. Cancer Cell 24:257-271.
34. Lu R, Wu S, Zhang Y-g, Xia Y, Zhou Z, Kato I, Dong H, Bissonnette M, Sun J. 2016. Salmonella Protein AvrA Activates the STAT3 Signaling Pathway in Colon Cancer(). Neoplasia (New York, NY) 18:307-316.
35. Yu H, Lee H, Herrmann A, Buettner R, Jove R. 2014. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. Nature Reviews Cancer 14:736.
36. Bossone SA, Asselin C, Patel AJ, Marcu KB. 1992. MAZ, a zinc finger protein, binds to c-MYC and C2 gene sequences regulating transcriptional initiation and termination. Proceedings of the National Academy of Sciences 89:7452.
37. Ray A, Bal BS, Ray BK. 2005. Transcriptional Induction of Matrix Metalloproteinase-9 in the Chondrocyte and Synoviocyte Cells Is Regulated via a Novel Mechanism: Evidence for Functional Cooperation between Serum Amyloid A-Activating Factor-1 and AP-1. The Journal of Immunology 175:4039.

38. Ray A, Kuroki K, Cook JL, Bal BS, Kenter K, Aust G, Ray BK. 2003. Induction of matrix metalloproteinase 1 gene expression is regulated by inflammation-responsive transcription factor SAF-1 in osteoarthritis. Arthritis & Rheumatism 48:134-145.
39. Ray A, Shakya A, Kumar D, Benson MD, Ray BK. 2006. Inflammation-Responsive Transcription Factor SAF-1 Activity Is Linked to the Development of Amyloid A Amyloidosis. The Journal of Immunology 177:2601.
40. Ray A, Yu G-Y, Ray BK. 2002. Cytokine-Responsive Induction of SAF-1 Activity Is Mediated by a Mitogen-Activated Protein Kinase Signaling Pathway. Molecular and Cellular Biology 22:1027-1035.
41. Ray BK, Ray A. 1997. Induction of Serum Amyloid A (SAA) Gene by SAA-activating Sequence-binding Factor (SAF) in Monocyte/Macrophage Cells: EVIDENCE FOR A FUNCTIONAL SYNERGY BETWEEN SAF AND Sp1. Journal of Biological Chemistry 272:28948-28953.
42. Ray A, Fields AP, Ray BK. 2000. Activation of Transcription Factor SAF Involves Its Phosphorylation by Protein Kinase C. Journal of Biological Chemistry 275:39727-39733.
43. Sears CL, Garrett WS. 2014. Microbes, Microbiota and Colon Cancer. Cell host & microbe 15:317-328.
44. Ray A, Ray BK. 2015. Induction of Ras by SAF-1/MAZ through a feed-forward loop promotes angiogenesis in breast cancer. Cancer Medicine 4:224-234.
45. Cogoi S, Zorzet S, Rapozzi V, Géci I, Pedersen EB, Xodo LE. 2013. MAZ-binding G4-decoy with locked nucleic acid and twisted intercalating nucleic acid modifications suppresses KRAS in pancreatic cancer cells and delays tumor growth in mice. Nucleic Acids Research 41:4049-4064.
46. Miglietta G, Gouda AS, Cogoi S, Pedersen EB, Xodo LE. 2015. Nucleic Acid Targeted Therapy: G4 Oligonucleotides Downregulate HRAS in Bladder Cancer Cells through a Decoy Mechanism. ACS Medicinal Chemistry Letters 6:1179-1183.
47. Xue X, Ramakrishnan SK, Weisz K, Triner D, Xie L, Attili D, Pant A, Győrffy B, Zhan M, Carter-Su C, Hardiman KM, Wang TD, Dame MK, Varani J, Brenner D, Fearon ER, Shah YM. 2016. Iron Uptake via DMT1 Integrates Cell Cycle with JAK-STAT3 Signaling to Promote Colorectal Tumorigenesis. Cell metabolism 24:447-461.
48. Maity G, Haque I, Ghosh A, Dhar G, Gupta VG, Sarkar S, Azeem I, McGregor D, Choudhary A, Campbell DR, Kambhampati S, Banerjee SK, Banerjee S. 2018. The MAZ transcription factor is a downstream target of the oncoprotein Cyr61/CCN1 and promotes pancreatic cancer cell invasion via CRAF–ERK signaling. Journal of Biological Chemistry doi:10.1074/jbc.RA117.000333.
49. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. 2015. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Research 43:D512-D520.

Figures

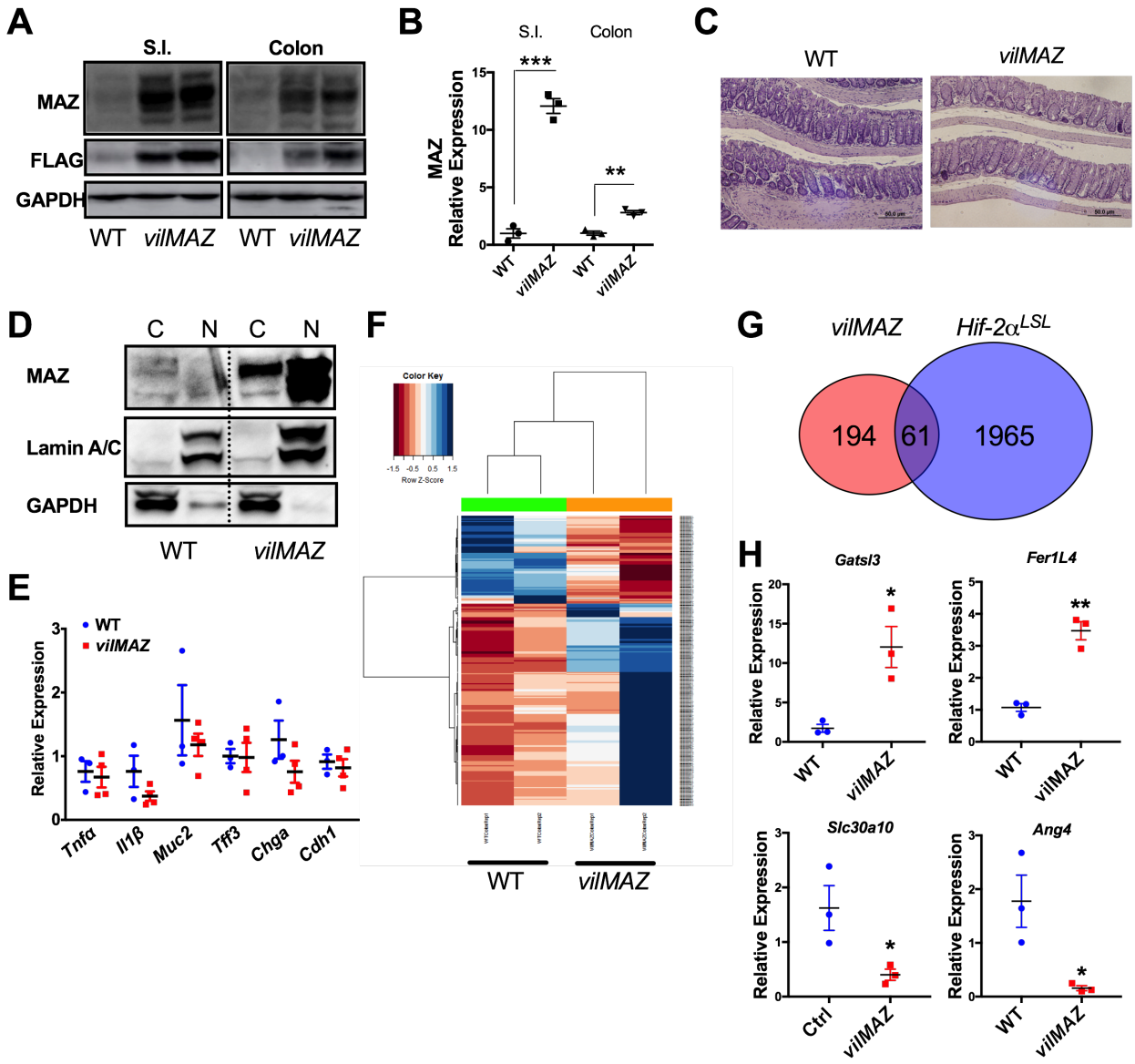

Figure 4.1. Generation of intestine-epithelial specific MAZ transgenic mice. (A) Western blot analysis of MAZ and FLAG expression in colon and small intestine (S.I.) extracts from WT and *vilMAZ* mice. (B) qPCR analysis of MAZ transcript and (C) representative images of hematoxylin and eosin (H&E) staining from colon tissue of WT and *vilMAZ* mice. (D) Western analysis of MAZ protein expression in cytosolic (C) and nuclear (N) extracts from colon tissue of WT and vilMAZ mice. Lamin A/C and GAPDH are nuclear and cytosolic markers respectively. (E) qPCR analysis of indicated genes from colon tissue of WT and *vilMAZ* mice. (F) Heat map of RNA-seq data WT and *vilMAZ*. (G) Venn diagram of genes identified as overlapping with HIF-2α RNA-seq and *vilMAZ*-independent genes. (H) qPCR confirmation of several MAZ target genes identified in (F) from WT and *vilMAZ* colon tissue. Statistical analysis was performed with Student's *t* test. The error bars represent standard error. *** p=0.001; ** p<0.01; * p<0.01

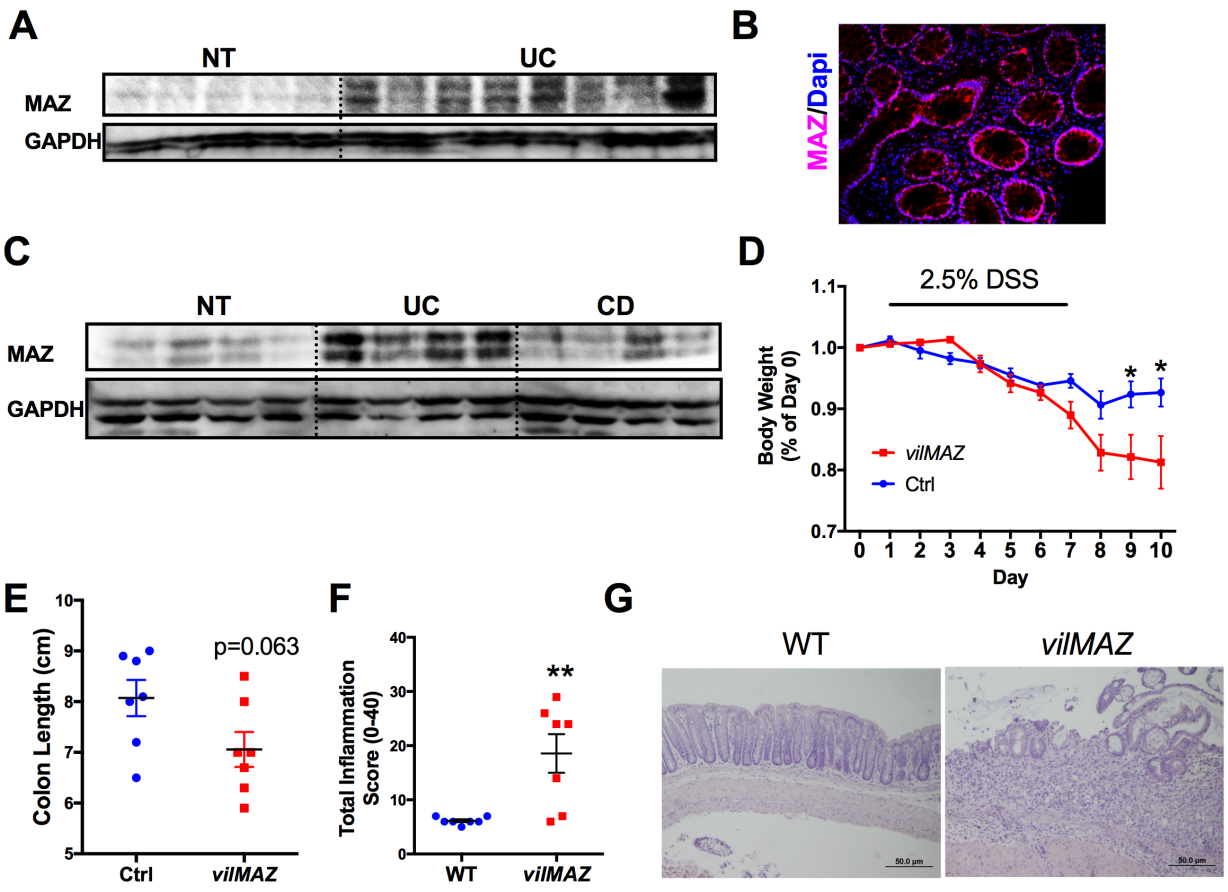

Figure 4.2. MAZ expression enhances the acute inflammatory response in colitis. (A) Western blot analysis of MAZ expression in human ulcerative colitis (UC) and normal tissue biopsy samples. Each lane represents a different patient. (B) Immunofluorescence of MAZ in human UC frozen tissue section. (C) Western blot analysis of MAZ expression in human Normal, UC, and Crohn's Disease (CD) biopsy specimens. (D) Body weight (E) colon length (F) histologic inflammation score and (G) H&E analysis from WT and *vilMAZ* mice treated with 2.5% DSS. Statistical analysis was performed with Student's *t* test. The error bars represent standard error. ** $p<0.01$; *$p<0.05$

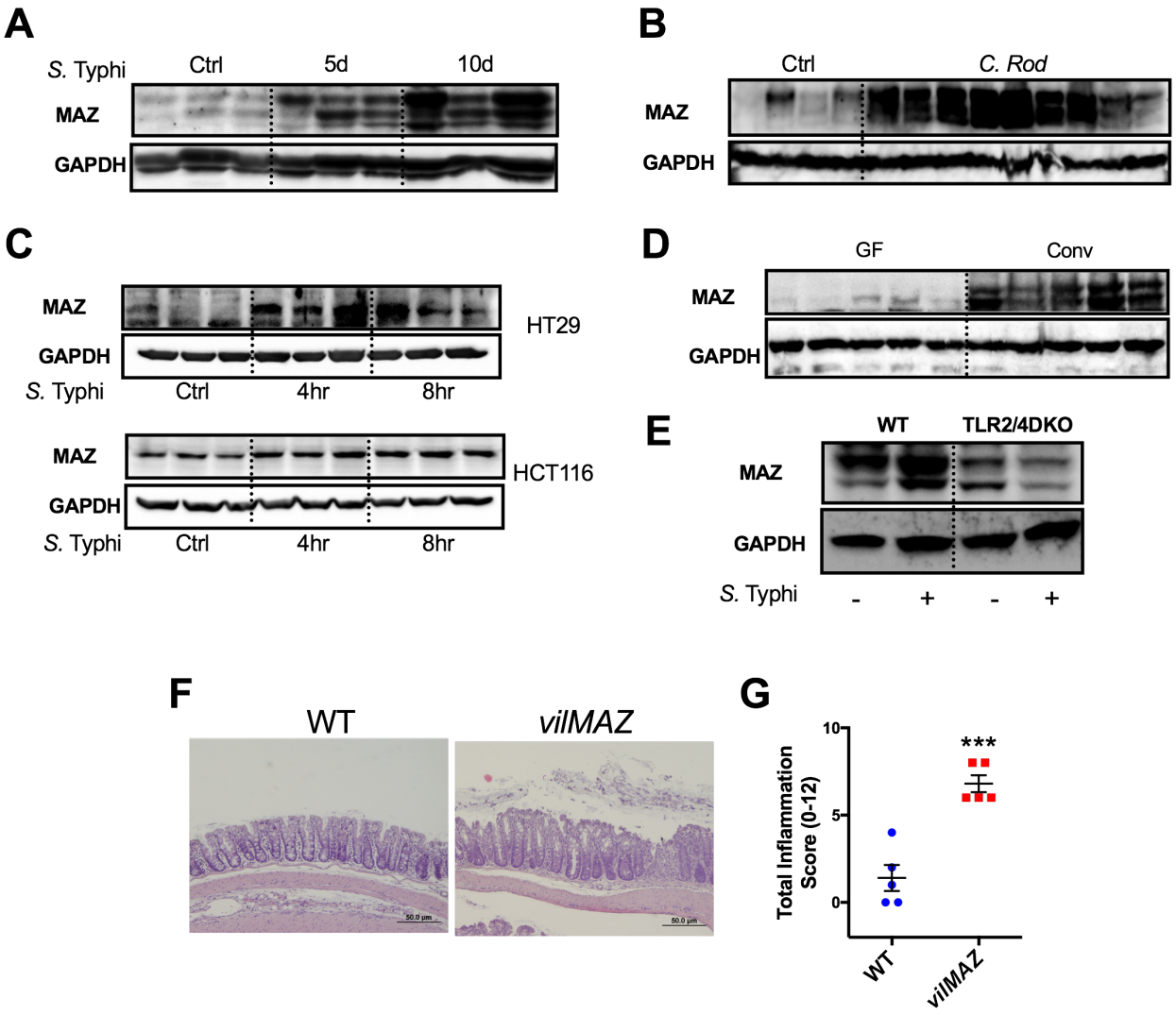

Figure 4.3. MAZ is regulated by microbiota and increases inflammation in bacterial-driven colitis. (A) Western blot analysis of MAZ expression in mice treated with *S.* Typhi colitis for 5-days and 10-days. (B) Western analysis of MAZ expression in mice treated with *C. rod* colitis for 10-days. (C) Western blot analysis of MAZ expression in HT29 and HCT116 cells treated with *S.* Typhi for 4-hours and 8-hours. (D) Western blot analysis of MAZ expression in colon tissue from germ free mice and germ free (GF) mice reconstituted with microbiota (Conv). (E) Western blot analysis of MAZ expression in colon tissue from WT and TLR2/4DKO mice treated with and without *S.* Typhi colitis for 10-days. (F) H&E and (G) total inflammation score of WT and *vilMAZ* mice treated with *S.* Typhi colitis for 10-days. Statistical analysis was performed with Student's *t* test. The error bars represent standard error. *** $p<0.001$

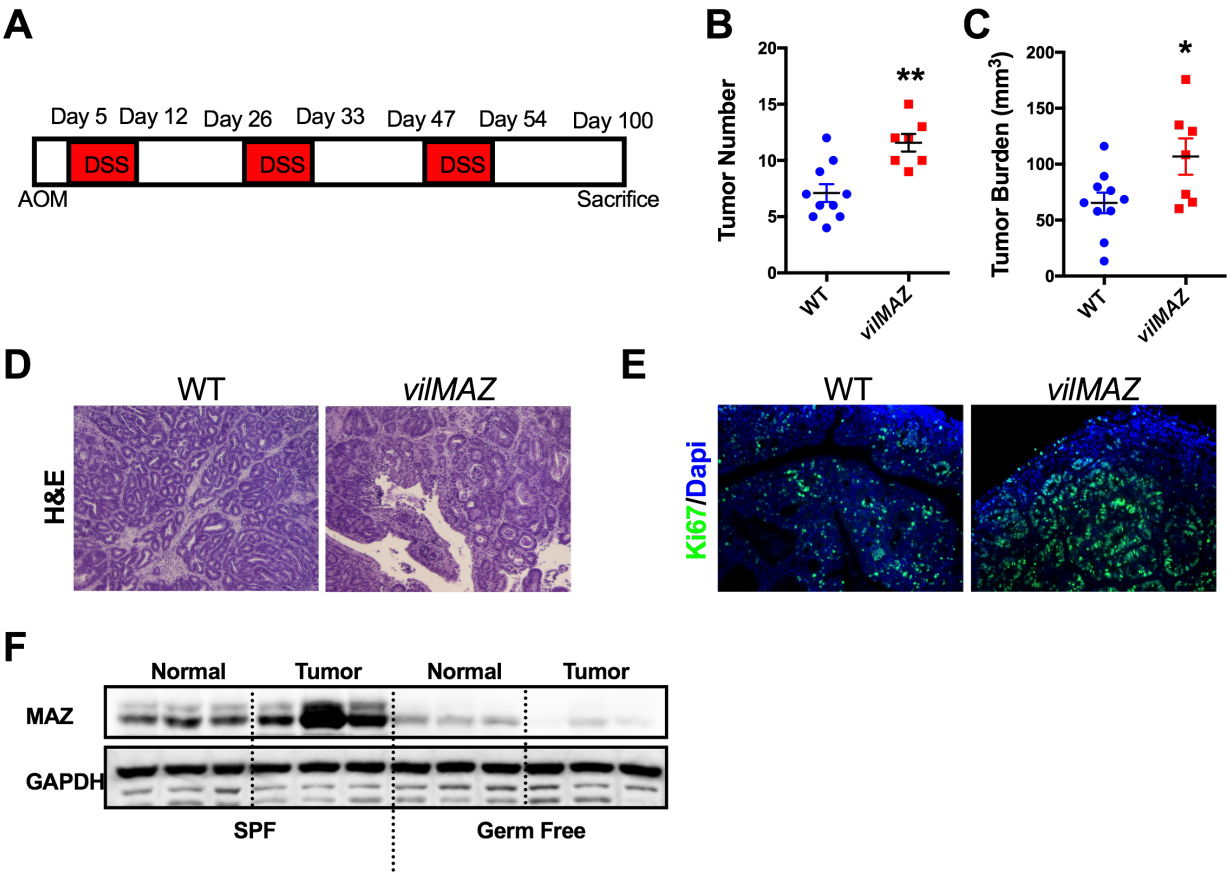

Figure 4.4. MAZ expression increases colitis-associated colon tumorigenesis. (A) Schematic of AOM/DSS model. (B) Tumor number (C) tumor burden (D) and H&E analysis of WT and *vilMAZ* animals treated with AOM/DSS colon tumorigenesis. (E) Representative immunofluorescence staining of Ki67 in indicated tumor tissue. (F) Western blot analysis of MAZ expression in tumor and adjacent normal tissue from specific pathogen free (SPF) and germ free (GF) mice with AOM/DSS-induced colon tumorigenesis. Statistical analysis was performed with Student's *t* test. The error bars represent standard error. **p<0.01; *p<0.05

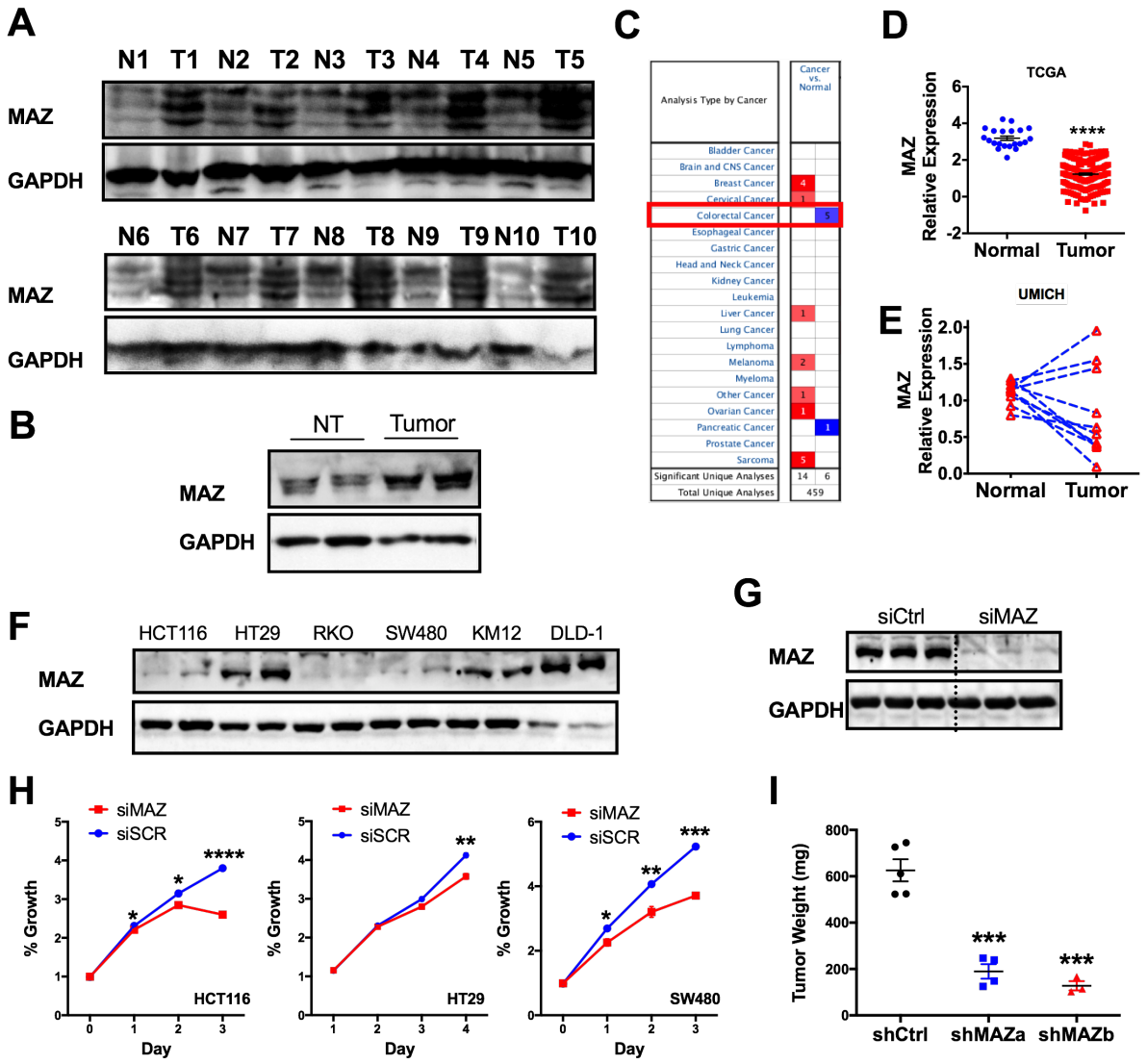

Figure 4.5. MAZ is important for human colon cancer growth. (A) Western blot analysis of MAZ expression in human colon tumor (T) biopsies compared to adjacent normal tissue (N). (B) Western blot analysis of MAZ expression normal colon tissue (NT) (n=2) and colon tumor enteroids (n=2). (C) Oncomine database analysis of MAZ expression in human colon cancer relative to normal colon controls in independent microarrays. (D) MAZ gene expression from The Cancer Genome Atlas. (E) MAZ expression in a matched set of 10 colon tumors and adjacent normal. (F) Western blot analysis of MAZ expression in indicated human colon cancer cell lines assessed in duplicate. (G) Western blot analysis of MAZ expression in HEK cells treated with Ctrl or MAZ-targeting siRNAs. (H) MTT assay of cell growth in HCT116, HT29, & SW480 cells treated with Ctrl or MAZ targeting siRNAs. (% of Day 0). (I) Tumor weight of 28-days after subcutaneous injection of HCT116 cells stably transfected with control shRNA (shCtrl) or two different MAZ shRNAs (shMAZa or shMAZb) into nude mice. Statistical analysis was performed with Student's *t* test. The error bars represent standard error. ****p<0.0001; *** p<0.001; **p<0.01; *p<0.05

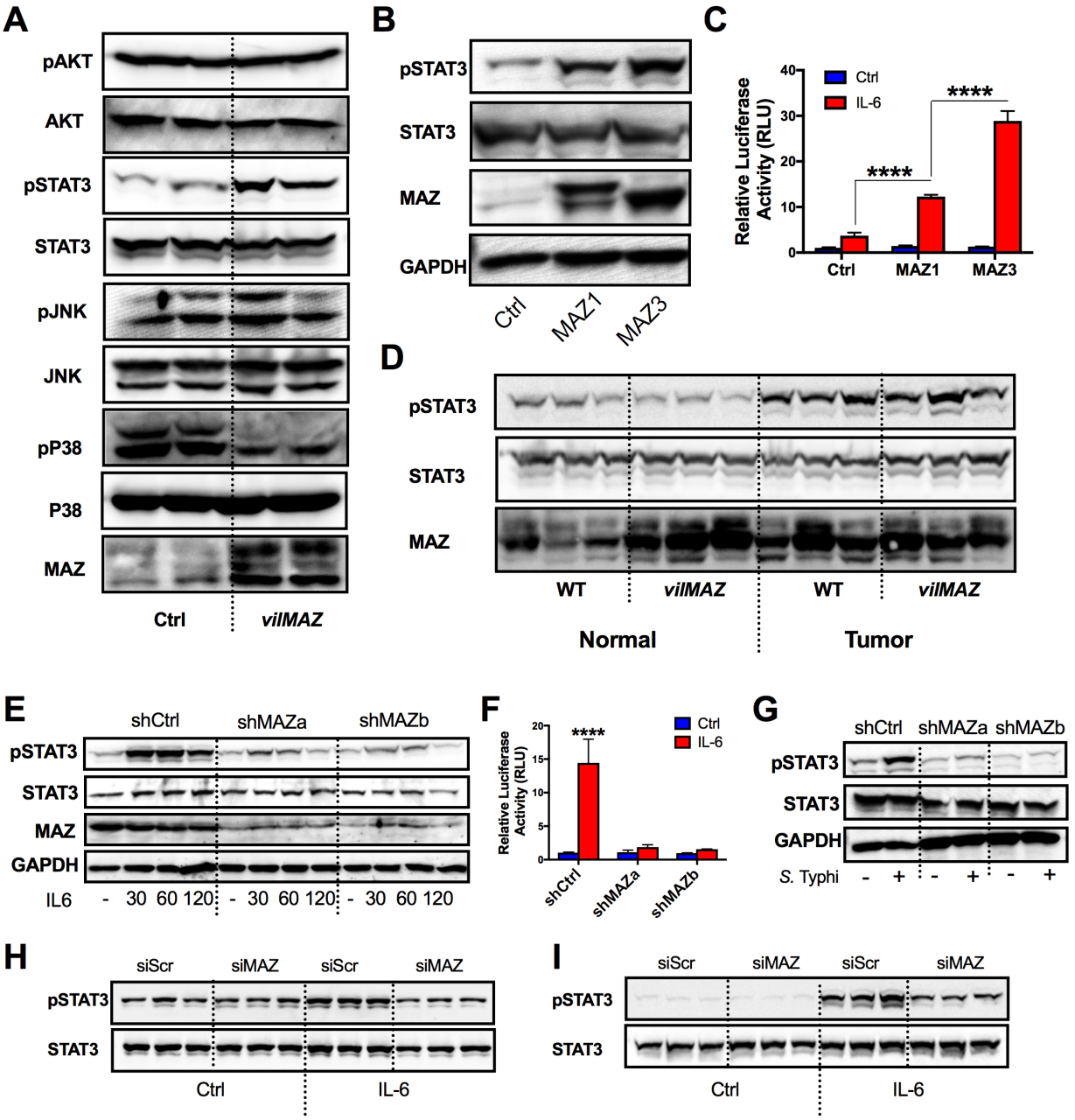

Figure 4.6. MAZ is a novel regulator of STAT3 signaling. (A) Western blot analysis of phosphorylated AKT, STAT3, JNK, and P38 in WT and *vilMAZ* mice treated with 2.5% DSS for 7-days. (B) Western blot analysis of pSTAT3 in HEK cells expressing MAZ transcript variants 1 & 3. (C) STAT3 luciferase activity analysis of HCT116 cells expressing MAZ transcript variants 1 & 3 treated with PBS or IL-6 (10ng/ml) for 12-hours. Data shown as fold change over control ****p<0.0001. (D) Western blot analysis of pSTAT3 expression in tumor and adjacent normal tissue from WT and *vilMAZ* mice. (E) pSTAT3 analysis in HCT116 cells expressing MAZ shRNAs treated with IL-6 (10ng/ml) for 30, 60, and 120 minutes. (F) STAT3 luciferase activity analysis of HCT116 cells expressing MAZ shRNAs treated with IL-6 (10ng/ml) for twelve hours. Data shown as fold change over control. ****p<0.0001. (G) pSTAT3 western blot analysis in HCT116 cells expressing MAZ shRNAs treated with *S.* Typhi for 4-hours. Phosphorylated STAT3 western blot analysis in (H) MCA38 cells or (I) HEK cells transfected

with MAZ siRNAs for 24-hours then treated with IL-6 (10ng/ml) for 60-minutes. Statistical analysis was performed with one-way ANOVA.

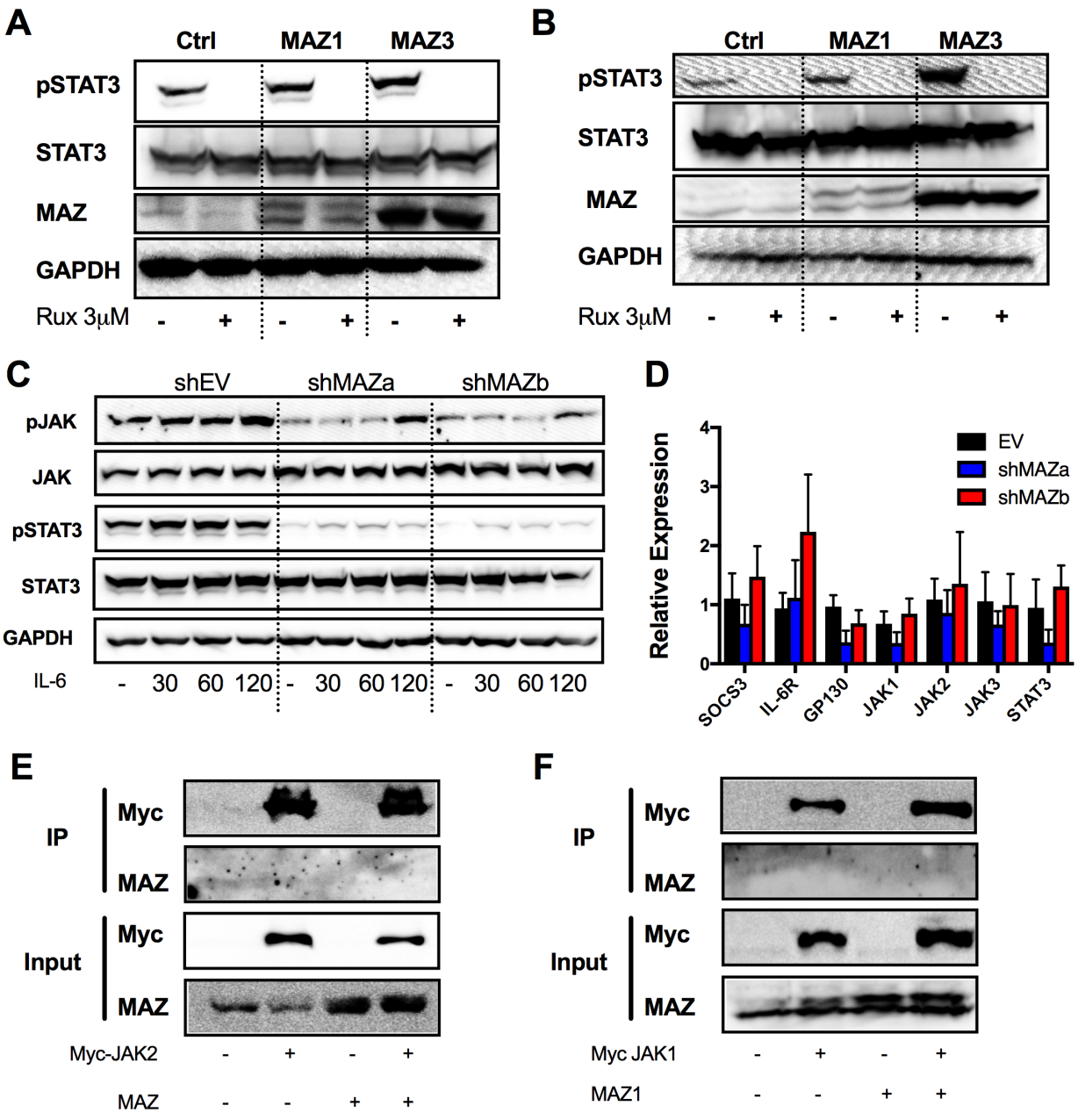

Figure 4.7. MAZ regulates JAK-mediated STAT3 activation. Western blot analysis of (A) HCT116 and (B) HEK cells expressing MAZ transcript variants 1 & 3 pretreated with Ctrl or the JAK inhibitor for 12-hours. (C). pJAK and pSTAT3 western blot analysis in HCT116 cells expressing MAZ shRNAs treated IL-6 (10ng/ml) for 30, 60, and 120 minutes. (D) qPCR analysis of STAT3 signaling component genes in MAZ knockdown HCT116 cells. (E) Western blot analysis of myc-immunoprecipitation from HEK cells expressing myc-JAK1 and MAZ or (F) myc-JAK2 and MAZ.

Table 3.1 Primer list

Mouse Primers	Forward (5'-3')	Reverse (5'-3')
β-Actin	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
Maz	ACCACCTGAACCGACATAAGCT	GGCACACAGGGCACTGGTA
Fer1l4	CCGTGTTGAGGTGCTGTTC	GGCAAGTCCACTGTCAGATG
Slc30a10	TGTGGTCATCACGGCTATCAT	ATGTAGCACTGCCAGTTACAC
Ang4	ACAACAAAGGACATGGGCTC	TCTCCAGGAGCACACAGCTA
Gatsl3	CACGGAGCCACTGTGAGATA	ACACTCTCATGGTGGATGAGG
Tnfα	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTCTGTCTAC
Il1β	AAGAGCTTCAGGCAGGCAGTATCA	TGCAGCTGTCTAGGAACGTCA
Tff3	GCACCATACATTGGCTTGG	AGAGCCCTCTGGCTAATGCT
Muc2	CCTGAAGACTGTCGTGCTGT	GGGTAGGGTCACCTCCATCT
Chga	GTCTCCAGACACTCAGGGCT	ATGACAAAAGGGGACACCAA
Cdh1	AAAAGAAGGCTGTCCTTGGCC	GAGGTCTACACCTTCCCGGT

Human Primers	Forward (5'-3')	Reverse (5'-3')
β-Actin	TATTGGCAACGAGCGGTTCC	GGCATAGAGGTCTTTACGGATGT
hMAZ	TCGGCTTATATTTCGGACCA	CATTGGACAAACCTCACCAGT
hJAK1	GAATGACGCCACACTGACTG	GATGACAAGATGTCCCTCCG
hJAK2	CCATTCCCATGCAGAGTCTT	CAGGCAACAGGAACAAGATG
hJAK3	GGACAAGAGGCTGCATGAAC	CTTCGAAAGTCCAGGGTCC
hGP130	CGGACAGCTTGAACAGAATGT	ACCATCCCACTCACACCTCA
hSTAT3	CTGCTCCAGGTACCGTGTGT	CCTCTGCCGGAGAAACAG
hIL6R	ACTGGTCAGCACGCCTCT	GGGACCATGGAGTGGTAGC
SOCS3	GAGCCAGCGTGGATCTG	GGCTCAGCCCCAAGGAC

Chapter 5

Conclusions and Future Directions

Epithelial HIF-2α is a well characterized activator of the intestinal inflammatory response in the setting of colitis. HIF-2α directly targets expression of a battery of cytokines and chemokines to promote mucosal inflammatory responses. In addition to its role in colitis, the work in this thesis provides convincing evidence of the role of the colon epithelial hypoxic response and HIF-2α activation as critical regulators of inflammation-induced colon tumorigenesis. Disruption of intestinal epithelial HIF-2α significantly reduces colon tumorigenesis in complementary inflammation-induced tumor models. Moreover, constitutive activation of HIF-2α in mice with epithelial deletion of *Vhl* enhanced colon tumorigenesis, suggesting HIF-2α is both an essential and sufficient regulator of colitis-associated colon tumorigenesis in mice.

Our previous work has shown that HIF-2α can modulate the colon tumor inflammatory response via distinct pathways. HIF-2α is a direct transcriptional regulator of cyclooxygenase-2 (COX-2) and microsomal prostaglandin e synthase (mPGES) to increase tumor-associated inflammation and inflammatory prostaglandin production (1). Moreover, HIF-2α is essential for colon tumor uptake of iron and intra-tumoral iron increases tumor associated inflammation and oncogenic cytokine production (2). The work in this thesis extends this data to show that HIF-2α can directly modulate the tumor immune microenvironment through recruitment of PMNs. Deletion of intestinal epithelial HIF-2α led to significant reduction of colon tumor-associated

PMNs in complementary models of colitis-associated colon cancer. Furthermore, expression of epithelial HIF-2α is sufficient to increase PMN recruitment into colon tissue and colon tumors. Mechanistically, this was downstream of HIF-2α transcription of the potent PMN chemokine *Cxcl1*.

CXCL1 is highly expressed in sites of inflammation and several solid tumor through unknown mechanisms. This thesis shows that the colon tumor epithelium is an important and major source of CXCL1 in a HIF-2α-dependent manner. Deletion of epithelial HIF-2α significantly reduced colon tumor CXCL1 mRNA and protein abundance. The *Cxcl1* proximal promoter contains several hypoxia response elements (HREs) and HIF-2α activation of the *Cxcl1* promoter is HRE-dependent. Inhibition of the CXCL1 receptor, CXCR2, using inhibited tumorigenesis in a HIF-2α-driven tumor model. These data demonstrate an essential role for epithelial HIF-2α in modulating the tumor microenvironment and suggest targeting HIF-2α therapeutically may show benefit in colon cancer patients. Our work also extends previous data showing that epithelial HIF-1α does not play a significant role in inflammation-induced colon cancer. This is consistent with our prior work which showed hyper-activation of HIF-1α did not promote colon tumorigenesis (3). Taken together, the work in this thesis to extend upon the function of HIF-2α in colitis-associated colon cancer and identifies HIF-2α as an important link between inflammation and cancer. Furthermore, this work defines HIF-2α as a master regulator of *Cxcl1* expression and PMN recruitment into colon tumors. With the development of novel HIF-2α-specific inhibitors, future studies in our lab will be aimed at modulating HIF-2α activity in preclinical murine colitis and colon cancer models with an impetus toward clinical development of these drugs.

HIF-based therapeutics in cancer

Due to the highly complex role of HIF in tumorigenesis, HIF-based therapies need to be assessed on an individual tumor basis. The best-characterized approach to alter HIF signaling is through inhibiting PHDs, which leads to activation of HIF signaling (4). PHD inhibitors have been assessed in models of inflammation-induced tissue injury and were shown to be effective and safe (5-7). Through a decrease in inflammation-induced tissue injury, PHD inhibitors may have a beneficial role in several cancers. Recent work has clearly demonstrated that PHD inhibitors can selectively activate HIF-1α (8). This suggests the possibility of finding novel agents that may target each isoform. However, there are concerns about therapeutic activation of HIF signaling, as most data suggest that HIF pathways lead to enhanced tumor progression. Currently, there are several drugs that inhibit HIF-1α and many of them are in clinical trials (9). Most of the compounds that are in clinical trials were originally discovered as targeting other pathways. 2-methoxyestradiol, a metabolite of estradiol, can decrease tumor growth through inhibition of HIF-1α but simultaneously can decrease angiogenesis and disrupt microtubules (10). Cardiac glycosides, including Digoxin, can robustly reduce tumor growth through inhibition of HIF-1α (11). Furthermore, several topoisomerase inhibitors have been shown to decrease tumor growth through HIF-1α inhibition (12, 13). However, no HIF-1α specific inhibitors have been discovered.

Selective inhibition of HIF-2α can be achieved through targeting the iron response element in the 5'-UTR of HIF-2α (14). Additionally, through structural analysis of HIF-2α, a ligand-binding cavity located within its PAS-B domain, which contains a β-sheet that mediates interaction with ARNT. This cavity is not present on HIF-1α. (15). No endogenous ligands are known for HIF-2α, but this cavity has been targeted for drug development and several promising

highly specific small molecule inhibitors have been identified as efficaciously disrupting HIF-2α heterodimerization with ARNT and blocking DNA binding and transcription of target genes in cultured cells (16). These inhibitors have shown efficacy in preclinical models of renal cell carcinoma, which is driven by HIF-2α (17, 18). Our data in colon cancer suggest inhibition of HIF-2α may dampen tumor growth and progression through several mechanisms. HIF-2α has cell-autonomous effects in colon cancer cells through hyper-activation of iron uptake via divalent metal transporter 1 (Dmt1) (2). Interestingly, our lab has recently discovered a unique HIF-2α-dependent mechanism of colon tumor iron trapping through a local hepcidin axis (Andrew Schwartz, unpublished data). These data in addition to the data in this thesis show HIF-2α can inhibit cell-autonomous growth and decrease tumor-associated inflammation. The current available reagents coupled with recent HIF structural analysis provide a framework to specifically regulate HIF-1α and HIF-2α and provide clinical tools to alter tumor pro-tumor inflammatory response or anti-tumor-immune response in both colitis and colon cancer (19).

Neutrophils and colon cancer

The data in this thesis extends previous work suggesting that PMNs are critical drivers of tumor growth and progression. However, most studies rely upon PMN trafficking receptor inhibitors or antibody-mediated PMN depletion in established tumors. Inhibition of PMNs using CXCR2 blocking peptide suggested that PMNs play an important role in tumorigenesis in hypoxic-driven and established tumors (20). The primary contribution of PMNs to the induction of colon tumors is less clear. Our studies suggest that complete PMN ablation prior to the induction of colon tumorigenesis using *LysM*-Cre-driven *Mcl1* deletion dramatically enhances colon tumor growth and progression. In the AOM/DSS colitis-associated colon cancer model,

greater than 60% of PMN-deficient animals developed highly invasive adenocarcinoma. This was able to be recapitulated in sporadic colon tumor models by generating PMN-deficient chimeric *Cdx2*-CreERT2; $Apc^{fl/fl}$ mice. Strikingly, we observed invasive adenocarcinoma in this model at 14-days after colon tumor induction. Mechanistically, PMN-deficiency increased colon tumor-associated bacteria, genetic instability, and heightened inflammatory response through IL-17 expression and NF-*k*B signaling. Furthermore, antibiotics treatment was able to reverse the increased tumor growth and progression in PMN-deficient mice in the AOM/DSS model of colitis-associated colon cancer. These studies suggest an important role for PMNs in the restriction of tumor-associated microbes to decrease colon tumor progression. This thesis suggests divergent and even opposing functions of PMNs in colon cancer and suggest more study into PMN heterogeneity and kinetics of PMN are warranted.

Limitations to our studies include the *LysM*-Cre used for *Mcl1* deletion. Cre expression downstream of the *LysM* promoter is activated in all myeloid cells, therefore although only PMNs are depleted in this model, off target effects cannot be completely excluded. However, the PMN-specific *Mrp8*-Cre and the myeloid *LysM*-Cre phenocopied each other in a sporadic colon tumor model suggesting this effect was due specifically to PMN depletion and not off target effects in other myeloid cells. Other genetic models of PMN depletion have been generated, including the *Csf3r* knockout mouse which fails to develop PMNs (21).

Our data suggest that temporal regulation of PMNs may be critical to exert either pro- or anti-tumor effects. Interestingly, human studies of early stage lung cancer have suggested that PMNs from early stage tumors are T-cell stimulatory and as lung cancers progress PMNs become less T-cell stimulatory (22). In mouse-models of colon cancer, inhibition of PMNs after the initiation of colon tumorigenesis suggests PMNs promote tumor growth and progression.

However, in human colon cancer, it has been proposed that early PMN infiltration into colon tumors is associated with positive prognosis (23). Future studies should be aimed at identifying temporal evolution of intra-tumoral PMNs in colon cancer. This could be done using Cre-dependent Diphtheria Toxin Receptor (DTR) expressing mice for PMN depletion (24). *Mrp8*-Cre expressing mice can be crossed to mice expressing DTR down-stream of a *loxp*-STOP-*loxp* cassette that has been previously described. For these experiments three groups in which PMNs are depleted with Diphtheria Toxin prior to AOM/DSS-induced tumorigenesis, PMN depletion after the third cycle of DSS, and a group in which PMNs are depleted after induction of colon tumorigenesis. Moreover, temporal control of PMN depletion could be achieved by generation of a tamoxifen-inducible *LysM*-Cre or *Mrp8*-Cre mouse. These studies and mouse models will more precisely define the dependence of PMNs during different stages of colon tumorigenesis.

Depletion of PMNs prior to the initiation of colon tumorigenesis increases colon tumor progression through limiting microbiota-dependent responses. However, other anti-tumor mechanisms of PMNs in early stage colon cancer are largely undefined. Previous studies have suggested that PMNs in early stage lung cancers acquire antigen presenting cell (APC) functions and can activate anti-tumor T-cell responses (25). It has also been reported that PMNs in acute colitis acquire APC functions suggesting PMNs may inhibit initiation of colon tumorigenesis through activation of T-cell responses (26). It would be interesting to examine the contribution of PMN-APCs in the initiation of colitis-associated colon tumorigenesis. For these studies, previously described mice with floxed major histocompatibility complex II (*MhcII*) alleles are being crossed to *Mrp8*-Cre mice (27). Coupling AOM/DSS as well as sporadic colon cancer models may provide clues into the role of PMN-dependent antigen presentation in the initiation of colon tumorigenesis.

MAZ and regulation of STAT3 signaling

Our lab has suggested that the cellular microenvironment can modulate the hypoxic response and HIF-2α target gene specificity. For example, low iron status activates intestinal epithelial HIF-2α. In this setting, HIF-2α activates transcription of numerous genes related to iron absorption but not inflammatory target genes. Current work in our lab suggest this effect is in part mediated by SMAD3/4 signaling (Nupur Das, unpublished data). Interestingly, in the inflamed state epithelial HIF-2α activates transcription of inflammatory target genes but not iron absorption. We have previously identified Myc-associated zinc finger (MAZ) as a direct HIF-2α interacting protein (28). MAZ is a cys2-his2 type zinc finger transcription factor that is highly upregulated in chronic inflammatory diseases and several human cancers. Although its expression is induced in several models, very little is known of its function in inflammation and cancer. We have shown that MAZ is crucial for HIF-2α-dependent inflammatory target gene specificity in colitis and colon cancer. We first identified MAZ as an essential factor for HIF-2α-dependent transcription of *Tnfα* (29). Interestingly, although HIF-2α directly binds to and regulates activation of the *Tnfα* promoter, no HIF-2α binding site is present in the *Tnfα* proximal promoter. Deletion of a MAZ binding site in the *Tnfα* proximal promoter completely ablated HIF-2α-dependent *Tnfα* induction (29). This thesis extends on our previous data to show that MAZ is also important for HIF-2α-dependent *Cxcl1* activation. However, the precise functional role and HIF-2α-independent functions of MAZ are not known.

The work in this thesis demonstrates that MAZ is highly induced in human ulcerative colitis and colon cancer. To delineate the function of MAZ, we generated a novel mouse model of intestine epithelial-specific MAZ expression. MAZ expression dramatically enhanced

susceptibility to colitis and complementary models of chemically-induced and bacterial colitis (DSS & *S*. Typhi). Moreover, MAZ expression enhanced the susceptibility to colitis-associated colon tumorigenesis in the AOM/DSS model and was important for the growth of human colon cancer cells both *in vitro* and *in vivo*. Mechanistically, we have identified an important role for MAZ in the regulation of oncogenic STAT3 signaling. STAT3 is an inflammation-induced transcription factor that is absolutely essential for the initiation and maintenance of colon tumorigenesis (30). STAT3 is highly activated and maintained in human colon cancer through unknown mechanisms. We show that both *in vivo* and *in vitro* MAZ promotes increased activation of STAT3. Moreover, knockdown of MAZ in colon cancer cells reduces responsiveness of STAT3 to inflammatory stimuli. These data suggest targeting MAZ may provide a novel therapeutic avenue to dampening STAT3 activation in colon cancer.

To address more mechanistically the role of MAZ in inflammation and cancer we have used CRISPR/CAS9 to generate full body knockout mice. Guide RNAs targeting the first exon of MAZ and founders were identified that were heterozygous for an 11-base pair deletion of exon 1 (Fig. 5.1A). Interestingly, MAZ protein expression downregulated but is not completely lost in these mice, suggesting hypomorphic expression (Fig. 5.1B). This is perhaps the result of alternative splicing. MAZ has an upstream exon used in alternative splicing, which bypasses MAZ exon 1, may bypass the deletion of MAZ in our knockout mice. Interestingly, basal STAT3 phosphorylation in the small intestine and colon of these mice is reduced relative to WT littermate controls suggesting that these mice still may provide an effective model for the study of MAZ in colitis and colon cancer (Fig. 5.1C). Additionally, the lab is generating mice with floxed *Maz* alleles. These mice will be crossed to intestine epithelial *Villin*-Cre expressing mice to be used for further analysis of MAZ in colitis and colitis-associated colon tumor models.

The exact mechanism of MAZ-dependent STAT3 activation is unknown. The work in this thesis suggests MAZ activates STAT3 in a JAK-dependent mechanism. However, MAZ does not transcriptionally regulate expression of JAK1, JAK2, or JAK3 or have direct interaction with JAK1 or JAK2 suggesting a novel mechanism of JAK regulation. Future experiments in the lab including screening for novel MAZ protein interactors using BioID, a method which allows identification of protein interactors by conjugating a protein of interest to a biotin ligase which biotynylates interacting proteins (31). Furthermore, the lab is investigating genome wide MAZ binding sites using MAZ chromatin immunoprecripitation (ChIP). Further delineation of the mechanism by which MAZ regulates the STAT3 signaling cascade may provide insight into mechanisms maintaining high STAT3 phosphorylation in colon cancer.

Final thoughts

The data in this thesis clearly shows that epithelial HIF-2α is essential in inflammation-induced colon tumorigenesis. The present work demonstrates that PMN inhibition in established tumors can therapeutically decrease tumor growth. Interestingly, evidence suggest that HIF-2α expression in immune cells is also important in colon tumorigenesis (32). Therefore, this work combined with previous work provides rationale for the continued development of HIF-2α-specific inhibitors in the treatment of colon cancer. The data in this thesis also challenges the role of intra-tumoral PMNs in colon cancer. We show that inhibition of PMNs in established tumors reduces tumor progression. However, we also show that depletion of all PMNs prior to initiation of tumorigenesis increases colon tumor growth and progression in complementary inflammation-induced and sporadic colon tumor models. Our data suggest an important role for intra-tumoral PMNs in restricting microbe-dependent tumor responses. Therefore, more evaluation of PMN

evolution and heterogeneity in colon cancer must be considered before initiating clinical trials in human colon cancer patients. This thesis also delineates a novel function for the HIF-2α interacting protein MAZ in the regulation of oncogenic STAT3 signaling. Therefore, targeting MAZ may be effective in dampening the pro-inflammatory HIF-2α response as well as reduce STAT3 signaling in colon cancer.

References

1. Xue X, and Shah YM. Hypoxia-inducible factor-2α is essential in activating the COX2/mPGES-1/PGE(2) signaling axis in colon cancer. *Carcinogenesis.* 2013;34(1):163-9.
2. Xue X, Ramakrishnan SK, Weisz K, Triner D, Xie L, Attili D, et al. Iron Uptake via DMT1 Integrates Cell Cycle with JAK-STAT3 Signaling to Promote Colorectal Tumorigenesis. *Cell metabolism.* 2016;24(3):447-61.
3. Xue X, Ramakrishnan SK, and Shah YM. Activation of HIF-1α does not increase intestinal tumorigenesis. *American Journal of Physiology - Gastrointestinal and Liver Physiology.* 2014;307(2):G187-G95.
4. Rabinowitz MH. Inhibition of Hypoxia-Inducible Factor Prolyl Hydroxylase Domain Oxygen Sensors: Tricking the Body into Mounting Orchestrated Survival and Repair Responses. *Journal of Medicinal Chemistry.* 2013;56(23):9369-402.
5. Robinson A, Keely S, Karhausen J, Gerich ME, Furuta GT, and P.Colgan S. Mucosal Protection by Hypoxia-Inducible Factor (HIF) Prolyl Hydroxylase Inhibition. *Gastroenterology.* 2008;134(1):145-55.
6. Cummins EP, Seeballuck F, Keely SJ, Mangan NE, Callanan JJ, Fallon PG, et al. The Hydroxylase Inhibitor Dimethyloxalylglycine Is Protective in a Murine Model of Colitis. *Gastroenterology.* 2008;134(1):156-65.e1.
7. Eckle T, Brodsky K, Bonney M, Packard T, Han J, Borchers CH, et al. HIF1A Reduces Acute Lung Injury by Optimizing Carbohydrate Metabolism in the Alveolar Epithelium. *PLoS Biology.* 2013;11(9):e1001665.
8. Keely S, Campbell EL, Baird AW, Hansbro PM, Shalwitz RA, Kotsakis A, et al. Contribution of epithelial innate immunity to systemic protection afforded by prolyl hydroxylase inhibition in murine colitis. *Mucosal Immunol.* 2014;7(1):114-23.
9. Wigerup C, Påhlman S, and Bexell D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacology & Therapeutics.* 2016;164:152-69.
10. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, et al. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell.* 2003;3(4):363-75.
11. Zhang H, Qian DZ, Tan YS, Lee K, Gao P, Ren YR, et al. Digoxin and other cardiac glycosides inhibit HIF-1α synthesis and block tumor growth. *Proceedings of the National Academy of Sciences.* 2008;105(50):19579-86.
12. Beppu K, Nakamura K, Linehan WM, Rapisarda A, and Thiele CJ. Topotecan Blocks Hypoxia-Inducible Factor-1α and Vascular Endothelial Growth Factor Expression Induced by Insulin-Like Growth Factor-I in Neuroblastoma Cells. *Cancer Research.* 2005;65(11):4775-81.
13. Pencreach E, Guérin E, Nicolet C, Lelong-Rebel I, Voegeli A-C, Oudet P, et al. Marked Activity of Irinotecan and Rapamycin Combination toward Colon Cancer Cells In vivo and In vitro Is Mediated through Cooperative Modulation of the Mammalian Target of Rapamycin/Hypoxia-Inducible Factor-1α Axis. *Clinical Cancer Research.* 2009;15(4):1297-307.
14. Zimmer M, Ebert BL, Neil C, Brenner K, Papaioannou I, Melas A, et al. Small molecule inhibitors of HIF-2a translation link its 5'-UTR Iron-Responsive Element (IRE) to oxygen sensing. *Molecular cell.* 2008;32(6):838-48.

15. Scheuermann TH, Tomchick DR, Machius M, Guo Y, Bruick RK, and Gardner KH. Artificial ligand binding within the HIF2α PAS-B domain of the HIF2 transcription factor. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(2):450-5.
16. Scheuermann TH, Li Q, Ma H-W, Key J, Zhang L, Chen R, et al. Allosteric Inhibition of Hypoxia Inducible Factor-2 with Small Molecules. *Nature chemical biology.* 2013;9(4):271-6.
17. Chen W, Hill H, Christie A, Kim MS, Holloman E, Pavia-Jimenez A, et al. Targeting renal cell carcinoma with a HIF-2 antagonist. *Nature.* 2016;539:112.
18. Cho H, Du X, Rizzi JP, Liberzon E, Chakraborty AA, Gao W, et al. On-target efficacy of a HIF-2α antagonist in preclinical kidney cancer models. *Nature.* 2016;539:107.
19. Wu D, Potluri N, Lu J, Kim Y, and Rastinejad F. Structural integration in hypoxia-inducible factors. *Nature.* 2015;524(7565):303-8.
20. Triner D, Xue X, Schwartz AJ, Jung I, Colacino JA, and Shah YM. Epithelial Hypoxia-Inducible Factor 2α Facilitates the Progression of Colon Tumors through Recruiting Neutrophils. *Molecular and Cellular Biology.* 2017;37(5).
21. Blaisdell A, Crequer A, Columbus D, Daikoku T, Mittal K, Dey SK, et al. Neutrophils Oppose Uterine Epithelial Carcinogenesis via Debridement of Hypoxic Tumor Cells. *Cancer cell.* 2015;28(6):785-99.
22. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *The Journal of Clinical Investigation.* 2014;124(12):5466-80.
23. Wikberg ML, Ling A, Li X, Öberg Å, Edin S, and Palmqvist R. Neutrophil infiltration is a favorable prognostic factor in early stages of colon cancer. *Human Pathology.* 2017;68:193-202.
24. Buch T, Heppner FL, Tertilt C, Heinen TJAJ, Kremer M, Wunderlich FT, et al. A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nature Methods.* 2005;2:419.
25. Singhal S, Bhojnagarwala PS, O'Brien S, Moon EK, Garfall AL, Rao A, et al. Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen Presenting Cell Features (Hybrid TANs) in Early-Stage Human Lung Cancer. *Cancer cell.* 2016;30(1):120-35.
26. Ostanin DV, Kurmaeva E, Furr K, Bao R, Hoffman J, Berney S, et al. Acquisition of Antigen-Presenting Functions by Neutrophils Isolated from Mice with Chronic Colitis. *Journal of Immunology (Baltimore, Md : 1950).* 2012;188(3):1491-502.
27. Cho KW, Morris DL, DelProposto JL, Geletka L, Zamarron B, Martinez-Santibanez G, et al. An MHC Class II Dependent Activation Loop Between Adipose Tissue Macrophages and CD4(+) T cells Controls Obesity-Induced Inflammation. *Cell reports.* 2014;9(2):605-17.
28. Xie L, Xue X, Taylor M, Ramakrishnan SK, Nagaoka K, Hao C, et al. Hypoxia-Inducible Factor/MAZ-Dependent Induction of Caveolin-1 Regulates Colon Permeability through Suppression of Occludin, Leading to Hypoxia-Induced Inflammation. *Molecular and Cellular Biology.* 2014;34(16):3013-23.
29. Xue X, Ramakrishnan S, Anderson E, Taylor M, Zimmermann EM, Spence JR, et al. Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice. *Gastroenterology.* 2013;145(4):831-41.

30. Grivennikov S, Karin E, Terzic J, Mucida D, Yu G-Y, Vallabhapurapu S, et al. IL-6 and STAT3 are required for survival of intestinal epithelial cells and development of colitis associated cancer. *Cancer cell.* 2009;15(2):103-13.
31. Roux KJ, Kim DI, Burke B, and May DG. *Current Protocols in Protein Science*. John Wiley & Sons, Inc.; 2001.
32. Imtiyaz HZ, Williams EP, Hickey MM, Patel SA, Durham AC, Yuan L-J, et al. Hypoxia-inducible factor 2α regulates macrophage function in mouse models of acute and tumor inflammation. *The Journal of Clinical Investigation.* 2010;120(8):2699-714.

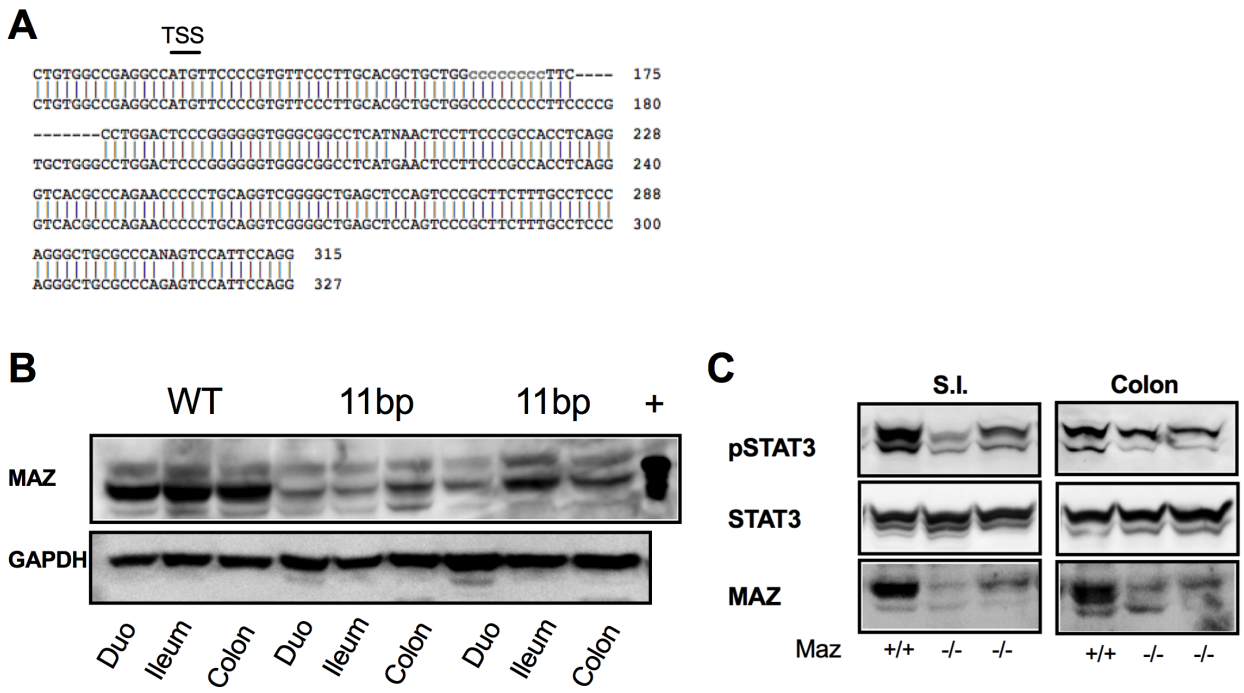

Figure 5.1 Generation of full-body MAZ knockout mice. (A) Sanger sequencing analysis of 11-bp deletion in Exon 1 of MAZ. + = MAZ positive control. **(B)** Western blot analysis of MAZ expression in duodenum, ileum, and colon tissue of one WT mouse and two separate 11-bp MAZ knockout mice. **(C)** pSTAT3 western blot analysis in small intestine (S.I.) and colon tissue from indicated mice.