The Myeloid Immune Signature of Enterotoxigenic *Bacteroides fragilis* Induced Murine Colon Tumorigenesis

by

Dr. med. univ. Erik Thiele Orberg

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Abstract

Enterotoxigenic *Bacteroides fragilis* (ETBF), a human commensal and candidate pathogen in colorectal cancer (CRC), is a potent initiator of IL-17-dependent colon tumorigenesis in Min mice.

In Chapters 2 and 3, we examined the role of IL-17 and ETBF on the differentiation of myeloid cells into myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM), which are known to promote tumorigenesis. The myeloid compartment associated with ETBF-induced colon tumorigenesis in Min mice was defined using flow cytometry and gene expression profiling. Cell sorted immature myeloid cells were functionally assayed for inhibition of T cell proliferation in order to delineate MDSC populations. A comparison of ETBF infection to that with other oncogenic bacteria (*Fusobacterium nucleatum* or pks+ *E. coli*) revealed a specific, ETBF-associated colonic immune infiltrate. ETBF-triggered colon tumorigenesis is associated with an IL-17-driven myeloid signature characterized by subversion of steady-state myelopoiesis in favor of the generation of protumoral monocytic (MO)-MDSC. Combined action of the *Bacteroides fragilis* enterotoxin bft and

ABSTRACT

IL-17 on colonic epithelial cells promoted the differentiation of MO-MDSC, which selectively upregulated *Arg1* and *Nos2*, produced NO and suppressed T cell proliferation. Evidence of a pathogenic inflammatory signature in humans colonized with ETBF may allow for the identification of populations at risk for developing colon cancer.

In Chapter 4, we describe targeting MDSC with the aim of suppressing tumorigenesis. CXCR2, an inflammatory chemokine receptor expressed on neutrophils and granulocytic (PMN)-MDSC, was a strong modifier of ETBF-Min tumorigenesis. Inhibition of CXCR2, via genetic knockout or a synthetic peptide antagonist, pepducin, significantly decreased recruitment of PMN-MDSC to the colon, increased numbers of M1 macrophages and was anti-tumoral. Specific inhibitors of MDSC targeting CXC receptors are under development and may emerge as a novel arm in immunotherapy.

ABSTRACT

Primary Reader

Dr. Drew Pardoll

Professor

Department of Oncology

The Johns Hopkins University School of Medicine

Secondary Reader

Dr. Franck Housseau

Assistant Professor

Department of Oncology

The Johns Hopkins University School of Medicine

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Dedication

This thesis is dedicated to my parents, Judith and Paulo for opening every door.

And to Katherine, in celebration of our partnership, our achievements together, and an auspicious future!

Contents

List of Figures

Chapter 1

Introduction

1.1 Colorectal cancer

This year, colorectal cancer is expected to cause 49,190 deaths in the United States. It is the third most common cancer, and ranks third in cancer-related deaths. American men and women have a lifetime risk of developing colon cancer of 4.7% and 4.4%, respectively.[1] While global mortality is decreasing, especially in the West, incidence rates are steadily climbings in developing nations in South America, Asia and Eastern Europe.

Colorectal cancer is in essence a genetic disease. Initiation and progression follow well-defined genetic and morphological pathways, with a long lead time of ten to forty years between the initial transformation event and cancer diagnosis. Normal colonic epithelium gives rise to small adenomas (polyps), which grow larger in size and

eventually progress to carcinomas.[2] Major genetic signaling pathways are impacted sequentially, and their disruption allows progression to the next, more dysplastic stage. The inactivation of tumor suppression genes involved in negative-feedback mechanisms that attenuate proliferative signaling constitutes a gateway mutation, and is followed by activation of oncogenes that allow for sustained proliferative signaling.[3] Key pathways in colon carcinogenesis are APC, Ras, PI3K/TP59/TGF-β, and mutations in one or more associated driver genes are frequently encountered.[4]

Genetic risk factors have been identified, such as germline mutations in the DNA repair genes MLH1, MSH2 or MSH6 in 'Lynch Syndrome', or the APC gene in 'Familial Adenomatous Polyposis'. However, upwards of 90% of all colon cancers arise sporadically in individuals with little to no genetic risk. Risk factors are diverse and loosely associated, encompassing obesity, physical inactivity, smoking and high intake of fat, alcohol or red meat.[5] A longstanding hypothesis is that intestinal microbes may contribute to colorectal cancer. This argument originated with insights into the microbial load in the human gut, which contains 10^{13} bacteria, thus outnumbering cells in the human body by 9 to 1. Interest in the contribution of the microbiome to colon cancer initiation and progression dramatically increased after next-generation sequencing enabled screening of bacterial species via 16S rRNA gene sequencing, shotgun metagenomic sequencing and other methods.[6] Seminal work by Eckburg et al. revealed an unimagined diversity within the human intestinal microbial flora, which contains 500 to 1000 individual bacterial species.[7,8] Furthermore, animal studies

have provided clues to the association between microbiota and cancer. Such studies observed an amelioration of chemically-induced tumorigenesis in germ-free mice or by a simple vivarium change, in which mice acquired a new microbiota.[9] Now, several models support the notion that specific bacterial pathogens, known as microbial 'drivers', themselves constitute or recruit a consortium of microbes to initiate colorectal cancer.[10–12]

The protective mucus layer lining the colon epithelium consists of two principal layers: a loose outer layer, which is densely inhabited by commensals and an inner, gel-like layer, that acts as a physical barrier. Beneath lies the intestinal epithelial layer, which creates a cell barrier separating host tissue from the external environment. Tight junctions and microvillar extensions on the apical surface of epithelial cells create an impermeable 'brush border', inhibiting microbial attachment and invasion.[13] Epithelial cells play a major role in maintaining mucosal immune homeostasis, through microbial recognition, the ability to discriminate between commensal and pathogenic bacteria as well as the regulation of immune cell functions of the gut-associated lymphoid tissues. Notably, commensals can themselves modulate intestinal epithelial-cell gene expression, i.e. inhibition of innate signaling pathways.[14] Bacteria that enter the inner layer are rapidly cleared by the host immune system: enterocytes release a multitude of inflammatory chemokines and cytokines leading to the induction of an immune response, e.g. IL-8, a neutrophil chemoattractant and CC chemokines MCP-1, MIP-1α, MIP-1β and RANTES, which attract monocytes,

eosinophils and T cells.[15] In inflammatory bowel disease, which if uncontrolled can predispose an individual to developing colon cancer, bacterial invasion and persistence in the inner mucus layer contribute to a state of chronic inflammation. Persistent bacterial infection with or without symptoms with a disruption of the inner mucus layer can result in pro-tumoral chronic inflammation.[16] Thus, possible mechanisms of bacteria-induced oncogenesis are considered to be:

1. chronic inflammation and

2. the production of oncogenic metabolites and toxins.[10]

To establish an association between exposure to a risk factor and subsequent disease, British epidemiologist Bradford-Hill established a set of criteria for causation.[7] These criteria were originally devised and successfully applied to establish a causal link between smoking and lung cancer. However, implementing them to support the notion of a microbial genesis of colorectal cancer has of yet been unsuccessful. Two key points as to why were highlighted in Eckburg's work. First, many of human commensal species are non-culturable. Second, two distinct communities exist: the mucosa-associated and intraluminal microbiome.[7,8] Further complication arises in that infection with a tumorigenic bacterium may be short-lived and transient, and may be no longer detectable at the time of cancer discovery.[11]

Three main models of host-microbe interactions exist to describe how microbiota can contribute to tumor initiation and progression. The first involves the loss of

the natural barrier function by colonic epithelial cells. This is the case in genetic or auto-immune diseases and results in the translocation of normally apathogenic commensal bacteria to submucosal layers, thus leading to an abnormal immune response through innate pathogen associated molecular patterns (PAMP)-sensing. PAMPs are conserved molecular patterns shared by microorganisms but not nonexistent in mammalian cells, and can be recognized by invariant, germline-encoded receptors expressed on epithelial and cells of the innate immune system.[17] The second model is characterized by the prevalence of specific pathobionts, sometimes also referred to as 'alpha-bugs'.[12,18] In a permissive genetic or environmental background, the presence of these pathogens alone is sufficient to promote tumorigenesis, in part through the expression of oncogenic virulence factors. The third and final model involves the establishment of a dysbiotic microbial community within the colon, in which an atypical mixture of normally apathogenic bacteria can promote tumorigenesis.[10]

Of the above models, there are significant efforts underway to better understand how chronic pathobiont colonization can act as a carcinogen. The sum of these efforts aim to identify culprit microbiota, which could allow for both early eradication and abrogation of tumorigenesis altogether, or, at the least, identification of patients at heightened risk of developing tumors. Chronic colonization with pathobionts such as *Enterotoxigenic Bacteroides fragilis*,[19] *pks+ E. coli*[20,21] and *Fusobacterium nucleatum*[22] may carry deleterious consequences, and is ultimately thought to foster tumor initiation and promotion through the mechanisms described next. First, colonization

leads to proliferation of the colonic epithelium through activation of proliferative signaling pathways, such as Wnt, NF-κB or Stat3. Second, the ensuing inflammatory response, mediated by both innate and adaptive effectors, can cause an inflammatory milieu that is rich in genotoxic mediators such as reactive oxygen species and reactive nitrogen species, resulting in DNA damage. In combination, hyperplasia and DNA mutations predispose colon cancer.[18]

Chronic infection with pathobionts can follow the driver-passenger model already eluded to.[11] Specifically, this represents a stepwise model for colon cancer progression following pathobiont infection. Initially, the normal epithelium becomes colonized with pathobionts. The mechanisms of and risk factors for persistent colonization are poorly understood and the course of infection differs from pathogen to pathogen. However, persistent, if transient, chronic infection is necessary. Over the course of infection epithelial cells become hyperproliferative and with changes in the local microenvironment — whether due to the inflammatory response or as a direct effect of pathobiont colonization — the composition of the local microbiome shifts, with the emergence of passenger bacteria. These can act as modifiers of the oncogenic process depending on the bacterial species, and may accelerate or suppress tumorigenesis. Once critical tumor suppressor pathways have been inactivated by mutations in the underlying genes, adenoma formation occurs, eventually progressing to carcinomas. At this stage, the driver bacteria may no longer be present, having been eliminated by adaptive immune responses or through competitive growth disadvantage versus

the new, modified passenger microbiome.

Forging a link between pathobiont colonization and tumorigenesis in humans faces many challenges:

- Infection can be asymptomatic and relatively short-lived.
- Detection is complicated by whether bacteria are mucosa-associated or intraluminal, the former necessitating a biopsy for conclusive diagnosis, the latter able to be detected in stool samples.
- Though many of the pathobionts identified to date are culturable, a large part of microbial species within the gut are not, requiring implementation of alternative methods for detection (sequencing, specific PCR).
- To achieve an oncogenic effect, many pathobionts require expression of oncogenic virulence factors. Confirmation of their absence or presence is required.
- The absence of driver bacteria in the adenoma and carcinoma stages of colon cancer, i.e. the window in which diagnosis is possible.

Taken together, these factors demonstrate that rather than rely on detection of the causative pathobionts themselves, reliable surrogates of chronic colonization are required. A promising approach is to take advantage of host immune responses. Often, these are long-lived and characteristic of infection with a specific pathogen. Early inflammatory signals skew immune responses towards distinct effector types, such

that different pathobionts are typically associated with different immune responses, and quality is reproducible in case of multiple infections with the same species. The observation that infection with a specific pathogen results in a specific immune response has been termed, 'immune signature' and overcomes many of the challenges associated with ascribing oncogenesis to a single pathobiont described earlier:

1. The interplay of pathogen-specific PAMPs and host pattern-recognition receptors polarizes the effector response.

2. Since pathobiont infection is often chronic, immune responses are durable and long-term, present even after driver bacteria no longer colonize the colon.

3. Characterization of immune responses is well-established and technically feasible and could be performed in parallel to pathology diagnostics upon collection of tissue, e.g. in endoscopy biopsies.

4. If major findings are reproducible in patients, immune signatures could serve as a prognostic marker for oncogenesis and could assist clinical decision-making.

5. Specific immune signatures may facilitate characterization of certain pathogens even if non-culturable and/or previously uncharacterized.

Immune signatures are already under development for use in cancer diagnostics as predictive biomarkers of metastasis[23] or to define protective immune responses in the skin.[24]

In microbially induced colorectal cancer, hypothetical scenarios could be as follows:

1. Recent infection with a pathobiont with evidence of malignancy-associated immune signature could lead to antibiotic therapy and efforts to normalize the microbiome and even provide passenger microbiota with tumor-suppressive activity.

2. Detection of malignant immune signature in adenomas discovered during routine screening colonoscopy could precipitate an aggressive screening regimen with frequent check-ups.

3. Established colorectal cancer with specific immune signatures may prove to be sensitive to immunotherapy and could guide therapeutic choices.

4. Epidemiological evidence of specific pathobiont-associated immune signature and colorectal cancer could meet Bradford-Hill criteria and would lead to a paradigm shift in the etiology of colorectal cancer and microbiota risk factors.

In this study, we present a novel mechanism: polarization of myeloid cells by a putative oncogenic human symbiote, Enterotoxigenic *Bacteroides fragilis* (ETBF), and the inflammatory cytokine IL-17, in Min (multiple intestinal neoplasia) mice. Subsequently, myeloid cells, specifically myeloid derived suppressor cells (MDSC), contribute to tumorigenesis. We identified a specific immune signature associated with distal colon tumors that arise in ETBF-colonized mice which may, with further

study, contribute to identifying patients at increased risk for developing colorectal cancer.

The murine model of ETBF-induced IL-17-mediated colitis with ensuing tumorigenesis is highly relevant to human biology and promises to unravel mechanisms of microbially triggered human colon cancer.[19] It provides several advantages:

1. ETBF is associated with acute colitis and colon cancer in humans.[25]
2. Th17 responses have been linked to poor outcome in human colorectal cancer.[26]
3. APC inactivation is a common mutation pattern in virtually all cases of colon cancer.
4. The ETBF-Min model mimics the dominant prevalence of distal colon cancers in humans.
5. The model also allows the role of IL-17 in promoting colon tumorigenesis to be addressed, which remains poorly understood and controversial.[27]

1.2 Enterotoxigenic *Bacteroides fragilis*

Enterotoxigenic *Bacteroides fragilis* (ETBF) is a human commensal and candidate pathogen in inflammation-induced human colorectal cancer. The genus *Bacteroides* is numerically among the most prominent in the intestinal microbiome.[28] The subspecies *B. fragilis* are gram-negative anaerobes that colonize the entire length of the

human colon and nonetheless grow in and benefit from nanomolar concentrations of oxygen.[29] *B. fragilis* has an unusually diverse gene repertoire of surface polysaccharides (PS), and encodes eight distinct capsular polysaccharides (A through H), with stable expression of a single type in colonized human hosts.[30] These play a major role in abscess formation,[31] bacterial-mucosal contacts,[32] mucosal immunity[33] and in modulating host-pathogen interactions.[34]

Interest in *B. fragilis* emerged in the 1970s when this subspecies was associated with bloodstream infections[35] and intra-abdominal abscesses.[36] Notably, *B. fragilis* was among the most common anaerobe isolated from clinical infections despite constituting less than 2% of the human microbiota.[37] A decade later, Myers et al. reported discovery of a *B. fragilis* strain capable of inducing inflammatory diarrhea in calves, foals, lambs and pigs.[38–41] Myers showed that inflammation was toxin-mediated by a heat-labile metalloprotease termed *B. fragilis* toxin or 'bft'.

In 1990, acknowledging the frequent involvement of zoonotic pathogens in human enteric diseases, Myers and Sack chose to investigate ETBF in children and performed a case-control study set in pediatric outpatient clinics in Whiteriver, Arizona. They found that ETBF colonization was twice as likely in patients with diarrhea than healthy controls (12% versus 6%).[42] Another study conducted with 728 Swedish men and women found a similar association between ETBF and diarrhea (27% in patients with community-acquired diarrhea vs 12% in outpatient controls). In 2008, a study by Sears and the International Centre for Diarrhoeal Disease Research expanded

upon this research, and showed that ETBF is associated with inflammatory diarrhea, characterized by the presence of fecal leukocytes as well as the inflammatory cytokines IL-8 and TNF-alpha.[43] Sears' findings strengthened earlier research by Prindiville et al., who showed an association of ETBF with inflammatory bowel disease[44] and where TNF is known to play a central role in the pathogenesis.[45]

The epidemiologic studies of ETBF in the 1990s and 2000s revealed that ETBF was widely present in the general population, detectable in the gut of 20-35% of children over one year of age and adults.[46] In some, infection leads to acute inflammatory diarrhea, though in most cases chronic asymptomatic colonization was observed. Researchers sought to combine these advancements in the understanding of ETBF biology – from of chronic colonization, high levels of inflammatory cytokines to emerging data on ETBF and inflammatory bowel disease – to establish a link to the initiation and promotion of colorectal cancer. In 2006, Toprak et al. investigated stool samples from colorectal cancer patients versus controls and found ETBF to be significantly increased in specimens from colorectal cancer patients.[47]

Ongoing pre-clinical studies show that the EBTF toxin bft has potent oncogenic activity. In cell culture, bft increases barrier permeability, chloride ion secretion and leads to cleavage of E-cadherin, an integral cell-to-cell adhesion protein.[48,49] Bft binding to a putative cell surface receptor on colonic epithelial cells is thought to mediate cleavage of E-cadherin resulting in release of β-catenin.[50] Increased cytosolic β-catenin triggers Wnt signaling and can promote cell growth through expression of

c-Myc. Additional oncogenic effects include:

1. disruption of epithelial barriers,
2. activation of NF-κB signaling pathways and production of IL-8 as well as TNF-alpha,[51]
3. upregulation of Cox2 resulting in increased levels of PGE2[52] and
4. induction of spermine polyamine metabolism with production of H2O2 that results in apoptosis and DNA damage.[53]

The *Bacteroides fragilis* enterotoxin bft is both necessary and sufficient to cause chronic inflammation in mice. Histology and immunohistochemistry revealed an inflammatory response characterized by hyperplasia, Stat3 activation, reactive oxygen species production and DNA damage.[54] ETBF expresses the toxin bft, while a closely related species of *B. fragilis*, termed nontoxigenic *B. fragilis* (NTBF), does not.[55] Studies comparing infection in mice demonstrated no differences in regards to colonization. However, NTBF infection was asymptomatic, with little to no inflammation and no significant induction of colon tumorigenesis. Importantly, NTBF does not encode the bft toxin. Asymptomatic infection was observed as well in mice inoculated with genetically modified ETBF lacking the bft toxin (EBTF Δbft), adding further emphasis to the toxin's potency as virulence factor.

In light of these observations, researchers have sough to demonstrate that ETBF is a potent initiator of colon tumorigenesis in mice. Infection leads to uniform col-

onization of the murine colon and results in persistent chronic inflammation with oncogenic transformation. In C57/BL6 mice, mice develop IL-17A-dominant colitis and the infection lasts over a year.[55] In Min mice ($APC^{\Delta 716}$) infection results in rapid tumorigenesis: microadenomas occur within days and polyposis present within weeks.[19]

The Min strain is heterozygous for a mutant APC (adenomatous polyposis coli) allele, encoding a protein truncated at amino acid residue 716 of 2845 ($APC^{\Delta 716}$).[56] APC is a classic tumor suppressor gene; its role is to form a cytosolic complex responsible for binding and targeting β-catenin for proteosomal degradation. Min mice typically develop multiple adenomas in the small intestine, but colon tumors in the large intestine are exceedingly rare. However, upon single exposure with ETBF, multiple colonic polyps, located predominantly in the distal colon, quickly become detectable. Remarkably, the murine ETBF Min model mirrors several critical features of human colorectal cancer including altered APC/β-catenin signaling, a predominant distal localization of colon tumors and accurate reproduction of the pathogenic role of overt IL-17.

Progression follows the same steps characteristic of human colorectal cancer: APC loss of heterozygosity (LOH) is detected in a majority of early microadenomatous lesions and virtually all colon tumors.[56,57] LOH is considered a gatekeeper event in early tumorigenesis as APC is a tumor suppressor gene and negative regulator of the Wnt pathway. As part of a multi-protein 'destruction complex', APC binds to and

phosphorylates β-catenin, leading to ubiquitin-mediated degradation.[58] However, as a consequence of APC LOH, cytoplasmic β-catenin translocates to the nucleus with subsequent activation of transcription of Wnt target genes. It is unclear how and whether ETBF contributes directly to APC LOH. Rapid onset DNA damage to colon epithelial cells occurs *in vivo* in response to bft, as evident by activation of H2A histone family, member X (H2AX).[53] *In vitro*, the bft-induced polyamine catalyst spermine oxidase can trigger production of reactive oxygen species, DNA damage, and hyperplasia. Nonetheless, the timeline and mechanisms of LOH in Min mice — thought to occur within days of challenge with ETBF — remains only insufficiently understood.

Under steady state, cytoplasmic β-catenin is sequestered by E-cadherin, an intracellular adhesion protein of the zonula adherens, and localized to cell-cell contacts. However, bft disrupts cell-cell contacts, as shown by increased barrier permeability with active secretion of chloride ions in colonic epithelial cell monolayers.[59] ETBF's bft-mediated disruption of epithelial tight junctions in the colonic mucosa leads to increased β-catenin signaling in the absence of functional APC.[55] However, data does not support direct cleavage of E-cadherin by ETBF, bft acts instead via a putative epithelial cell-surface receptor, precipitating shedding of the E-cadherin extracellular domain and presenilin-1/γ-secretase processing of the intracellular fragment.[60] As a result of enhanced nuclear β-catenin signaling, the oncogene c-Myc is upregulated, thereby contributing to hyperproliferation.[61] Presence of ETBF in the intestinal

mucosa is accompanied by widespread and sustained Stat3 activation, in both epithelial as well as immune cells. Persistent Stat3 activation constitutes a pro-tumoral signaling pathway, increasing tumor cell proliferation, survival and invasion while suppressing anti-tumor immunity.[62] In many cancers, persistent Stat3 activation is mediated by the inflammatory pathways IL-6 and IL-17.[63]

Within a few days of ETBF infection, microadenomas become visible by microscopy in colons of Min mice. After 4 to 8 weeks, tumors are macroscopically visible. In the subsequent weeks and prior to cancerous transformation of the numerous colon polyps, euthanasia of the mice becomes necessary due to lethargy, likely due to maldigestion secondary to bowel obstruction. Although tumorigenesis is nearly always restricted to the distal colon (thus replicating the phenotype of polyp distribution in human Familial Adenomatous Polyposis patients), ETBF colonizes the entire colon uniformly.[64] Genetic knockout of Stat3 or of the Stat3-dependent cytokine IL-17, can abrogate ETBF tumorigenesis in Min mice.[19,65] Consistent with these observations, Housseau, Sears and Wu recently demonstrated that colonization of Min mice with ETBF triggered IL-17-dependent colon tumorigenesis mediated in large part by redundant innate and adaptive sources of IL-17.[19,65]

1.3 IL-17

Infection with ETBF triggers a potent Th17 mediated inflammation and treatment with αIL-17 antibody abrogates ETBF-induced tumor formation.[19] IL-17 is required: IL-17A$^{-/-}$ Min mice infected with EBTF are immune to tumorigenesis, and both colom microadenomas as well as tumors are drastically reduced at 12 weeks post-infection. Similarly, ETBF-infected IL-17RA$^{-/-}$ Min display lower tumor burden as compared to IL-17RA-sufficient controls. An experiment in which bone marrow of IL-17RA$^{+/+}$ donors was transplanted into IL-17RA$^{-/-}$ Min showed comparable results: reduction of microadenomas and absence of tumors in the colon of ETBF-colonized mice.[65]

A study by Chae et al. confirmed the critical role of IL-17 in colon tumorigenesis.[66] Herein, intestinal tumorigenesis was significantly reduced in IL-17$^{-/-}$ Min mice, and microadenomas in the colon are virtually absent. Of importance is their finding that IL-17$^{-/-}$ Min mice seem resistant to APC LOH, suggesting that a mechanism downstream of IL-17 inflammation is necessary for APC LOH.

Although rare, healthy Min mice can develop sporadic microadenomas in the colon associated with APC LOH, despite absence of ETBF. A possible explanation is that other commensal bacteria induce IL-17 production in the colon, albeit at lower levels. This hypothesis can be applied to a report by Dove et al., in which intestinal tumorigenesis was assessed in germ-free wildtype and Min mice.[67] Germ-free mice trended to have less adenomas in their colon as compared to their conventional coun-

terparts. In summary, against the backdrop of chronic inflammation, IL-17 is a potent pro-tumoral cytokine when its levels are sustained.

Regulatory T cells (Treg) are recognized as a protective factor in Min mice tumorigenesis, and adoptive transfer of wildtype $CD4^+CD25^+$ cells to syngeneic Min mice succeeds in inhibiting the development of 90% of intestinal tumors.[68,69] Although Treg are potent suppressors of Min tumorigenesis, Treg generated in germ-free mice have impaired function,[70] which may explain why the aforementioned study with germ-free Min mice was inconclusive. These studies however, help explain the baseline occurrence of microadenomas in Min mice, in which a subclinical infection with commensals can elicit low level IL-17 response that is controlled by Treg. Importantly, while such a process is sufficient to induce microadenomas, adenomas seldomly arise, suggesting that, in addition to APC LOH, further transformations must occur in colonic epithelial cells for tumorigenesis to progress. More recently, the notion that Treg are protective in the setting of colon carcinogenesis has been challenged by findings of Geis et al., who showed that Treg promote the earliest stages of immune carcinogenesis via enhancement of IL-17 production, while dampening IFN-γ production.[71]

IL-17 can contribute to tumorigenesis via multiple mechanisms: activation of Stat3 and NF-κB as well as expression of VEGF and IL-6.[72] Another is the G-CSF dependent recruitment of immature myeloid cells (IMC):[73] IL-17R knockout mice exhibit tumor resistance, linked to lower levels of G-CSF and reduced recruitment of IMC to

the tumor microenvironment. Recently, IL-17 was shown to orchestrate the selective accumulation of CXCR2 myeloid-derived suppressor cells (MDSC) at inflammatory sites and the TME via promotion of CXCR2 ligand expression by stressed epithelial and tumor cells. He et al. found that tumor growth inhibition in IL-17R-deficient mice or via IL-17 neutralizing antibody treatment was due to reduced MDSC infiltration and increased numbers of intra-tumoral cytotoxic CD8 T cells. In a model of carcinogen-induced *de novo* tumorigenesis, Wang et al. showed that IL-17 activated STAT3 through IL-6, and an IL-6-STAT3-dependent pathway regulated the expression of several inflammatory mediators (e.g. CXCL1, S100A8, S100A9, Cox2 and IL-1β).[74] These cytokines have in common the ability to act specifically on immature myeloid cells (IMC) and may contribute to shifting steady-state myelopoiesis towards the generation of MDSC.[75]

Together with T reg, MDSC mobilization to tissues in response to inflammatory stimuli represents a homeostatic mechanism designed to limit collateral damage.[76] Cancer, via its associated inflammatory microenvironment and cytokine milieu, can alter the steady-state maturation and differentiation of mobilized IMC, including monocytes (MO), macrophages (MΦ), dendritic cells (DC) and polymorphonuclear (PMN) cells. This can result in the generation of procarcinogenic myeloid cell populations, the most important of which are MDSC. In the context of ETBF infection, immature myeloid cells (IMC) are recruited to the inflamed colon lamina propria, persist in the tissue, and accumulate in tumors where — as a result of the surround-

ing microenvironment and tumor-secreted factors — differentiate to MDSC as well as tumor-associated macrophages (TAM).

1.4 Myeloid-derived suppressor cells

Myeloid derived suppressor cells originate in the bone marrow from hematopoietic stem cells, specifically the common myeloid progenitor cell. Under homeostatic conditions, the bone marrow generates progenitor cells that go on to differentiate into macrophages, dendritic cells and neutrophils in the periphery. In cancer and other chronic pathologic conditions, these cells fail to differentiate and remain in an immature state, and are thus referred to as IMC. Hallmarks of MDSC are as follows:

1. Myeloid origin,
2. Immature state and
3. Immune suppressive.

Despite being phenotypically and functionally heterogeneous, MDSC are derived from a common myeloid lineage and share a capacity for immune modulation. The two main subsets of MDSC are defined as granulocytic (PMN) and monocytic (MO) MDSC. In contrast to IMC, which are not immunosuppressive at steady state, MDSC respond to inflammatory cytokines such as IFN-γ, IL-4 and IL-13, as well as various danger signals or TLR ligands. Their activation triggers the deployment of immunosuppressive effector functions, including nitric oxide (NO), reactive oxygen species

(ROS), and arginase 1 (Arg1), a potent metabolic enzyme which acts as a T cell immunosuppressant by depleting arginine in the local milieu. Several transcription factors critical to MDSC function have been identified, the most prominent of which are proteins of the STAT family as well as NF-κB.[77] Nonetheless, available data suggest the existence of other, as yet unrecognized, signaling pathways and transcriptional regulators critical to the immunosuppressive activity of MDSC.[78]

Historically, these cells were termed 'null cells' due to lack of expression of CD3, CD56, CD19 and CD13, or designated natural suppressors, in recognition of their capacity to inhibit lymphocyte numbers and T lymphocyte activity.[79] In mice, the lack of specific markers meant the defining characteristic of these cells was their suppressive function. Null cells drew interest as they were discovered in mice to deplete lymphoid populations,[80] and to occur in close association with tumors[81].[82] Researchers soon recognized several factors that could exacerbate myeloid suppressor cell numbers:

1. the abnormal induction of hematopoiesis, such as by injection of cyclophosphamide,[83]

2. co-culture with tumor secretions[84] and

3. the exogeneous supply of GM-CSF[85] or G-CSF.[86]

Importantly, reduction of NS cell numbers, achieved through administration of IFN-γ and TNF-α,[81] could diminish tumor recurrence and metastasis. Similarly, tumor

growth could be inhibited by antibody-mediated depletion of granulocytes.[82]

In the mid 1990s, lineage-negative myeloid populations were first documented[87],[88] and an inverse relationship between T cell function and MDSC was uncovered in cancer patients.[89] Ectopic production of GM-CSF in patients with head and neck carcinoma was associated with increased myeloid cell suppressive activity, recurrence and metastasis.[89] Host tumor burden often strongly correlated with the increased number of suppressive cells in circulation[9091] and solid tumor resection could successfully reduce myeloid cell numbers, thus restoring immunity.[92] In a murine mammary adenocarcinoma model, reduction of suppressor cells achieved objective responses, decreased tumor burden and led to improved survival.[93] Unlike in mouse models, where tumor burden could be reduced by manipulation of myeloid cell numbers, few clinical studies have shown improved overall survival in response to targeting myeloid suppressor cells.[94] We now know that the number of circulation MDSC has the likeness of a biomarker and can predict: disease progression and poor prognosis,[94] tumor angiogenesis[95] and osteoporosis.[96,97] A better understanding of the biology of these suppressor cells will be necessary to produce therapeutic strategies that will translate into improved clinical outcomes.

There are two distinct subsets of myeloid-derived suppressor cells: (1) monocytic (MO) and (2) granulocytic or polynuclear (PMN). MO-MDSC are usually characterized by high expression of Arg1 and production of nitric oxide as well as of suppressive cytokines, i.e. IL-10 and TGF-β. Like monocytes, they express surface

markers such as F4/80, CD115, 7/4 (Ly6B) and CD192 (CCR2). They suppress in an antigen-unspecific fashion and can give rise to tumor associated macrophages through Hif1-α signaling triggered by hypoxia in the tumor bed.[98] PMN-MDSC are more-closely associated with the production of reactive oxyges species, such as $O2^{-}$, H_2O_2 and peroxynitrates (PNT). In contrast to the MO effector products, these are short-ranged and short-lived, such that PMN-MDSC require cell-to-cell contact and suppresses antigen-specific $CD8^{+}$ T cell in an antigen specific manner.[99]

In mice, MDSC were initially characterized as $CD11b^{+}$ $Gr\text{-}1^{+}$ cells, while lacking markers typical of terminally differentiated macrophages and dendritic cells.[100,101] The two distinct subsets can be separated by differential expression of the Ly6C and Ly6G markers:[102]

MO-MDSC $CD11b^{+}$ $LyC6^{high}$ $Ly6G^{-}$

PMN-MDSC $CD11b^{+}$ $Ly6C^{low}$ $Ly6G^{+}$

Human MDSC characterization remains controversial due to their heterogeneity. MDSC subsets have been identified on the basis of CD14 (MO) or CD15 (PMN) and CD33 co-expression within the HLA-DR^{-} and $lineage^{-}$ compartment (i.e. $CD3^{-}$, $CD19^{-}$, $CD56^{-}$, $CD13^{-}$ with CD33 as an exception).[103] Since their discovery, the nature of MDSC has been open to question: Debate arises on if they are a distinct, evolutionary population or if they represent a transient state, arrested in development and prevented from progressing to mature innate effector cells, such as neutrophils

and macrophages[104]? Notably, the phenotype of MO and PMN-MDSC, based on the expression of Ly6C and Ly6G is the same of inflammatory monocytes and of neutrophils, respectively.[105,106] As Youn et al. pointedly state:

> "MDSC are not simply the result of myeloid precursor cells expanding under pathological conditions. MDSC is a functional definition of immature myeloid cells that have acquired potent immune suppressive activity and other non-immunological functions."[104]

MDSC arise during long-term chronic pathological conditions, such as chronic inflammation, infection, or cancer. In contrast, acute bacterial infections do not result in the expansion of MDSC. When MDSC enter the tumor microenviroment, they undergo differentiation to tumor-associated macrophages (TAM). Narita et al. demonstrated that spleen-derived MDSC differentiated into suppressor macrophages under the influence of tumor cell-derived factors obtained from CMC-1 carcinoma culture supernatant, while concomitantly increasing their inhibitor activity.[107] These findings were confirmed by adoptive transfer of MDSC to tumor-bearing mice, in which differentiation into TAM with potent immunosuppressive activity was observed.[108] The concern regarding nomenclature was addressed in a 2007 letter to The Journal of Cancer Research, in which leading experts in the field called for the universal adoption of the MDSC term, making the following argument:

> "In the literature, these cells have been called immature myeloid cells or myeloid suppressor cells (MSC). Although both of these names reflect the biology of cells, neither term is entirely accurate. The name immature myeloid cell implies that these cells are normal myeloid precursors. [...] The name MSC implies that these cells include populations of mature myeloid cells, such as macrophages or dendritic cells, capable of displaying some immunosuppressive features under certain circumstances."[109]

Tumor-secreted factors affecting MDSC maturation include VEGFA,[101] GM-CSF,[110] FMS,[111] Kit ligand,[112] G-CSF[113] and M-CSF.[114] Many of these factors can act on two levels, the first is cellular accumulation and expansion, the second is activation. These two processes are interconnected and regulated by overlapping transcription factors: Stat3, IRF8, CEBP-β, Stat1, Stat6 and NfKB.[77] Stat3 plays a major role in MDSC biology, including: prevention of apoptosis,[62] promotion of proliferation[115] and upregulation of the pro-inflammatory proteins S100A8 and S100A9. These, in turn, inhibit DC differentiation,[116] promote recruitment of MDSC to tumor sites, boost their accumulation and enhance their suppressive activity.[117] NADPH oxidase, a multi-protein complex consisting of the subunits p47phox and gp91phox, are also downstream of Stat3, and its activation results in increasing ROS levels, which translates to enhanced inhibitory activity of MDSC.[118] Signaling through Stat1 increases iNOS expression[108] while Stat6 controls Arg1[119] and TGF-β expression,[120] all of which are critical to MDSC-mediated immune suppression. TLR family proteins, which signal through a set of common adapters, MyD88 and TRIF, and subsequent activation and nuclear translocation of the Nf-κB transcription factor, are important for mobilization of MDSC to tumor sites.[121]

Another level of regulation occurs through cytokines released by activated T cells, such as IFN-γ, IL-4, IL-10 and IL-13.[122] These act in concert with tumor-derived pro-inflammatory factors, such as IL-1-β, IL-6, S100A8 and S100A9 to divert the maturation of immature myeloid cells first to MDSC then to immunosuppressive

TAM.[123] Lastly, chemoattractants play a crucial role in the recruitment of immature myeloid cells to the TME. CCL2 and CCL12 can bind to CCR2 expressed on MO-MDSC as well as on macrophages, while members of the CXCL chemokine ligand family (1, 5, 6, 8, 12) can bind to receptors such as CXCR2 on the surface of PMN-MDSC.[124]

The main pro-tumoral mechanisms employed by MDSC are as follows:

1. Enhancing tumor cell survival,
2. Angiogenesis,
3. Tumor invasion,
4. Metastasis.

Enhancing tumor cell survival is most potently achieved by suppressing anti-tumor immune responses and by inactivation of apoptosis. Four principal immunomodulatory mechanisms have been described, which are mediated by specific key proteins: Nox2 (NADPH oxidase), Arg1 and Nos2. The first involves the depletion of nutrients required by lymphocytes to sustain effector functions. Arg1 reduces interstitial levels of L-arginine.[125] Amino acids essential to the metabolism of effector T cells L-cysteine[126] and tryptophan are consumed or sequestered from local inflammatory sites through transporters or enzymes such as Xc- and indoleamine 2,3-dioxygenase. The result is a downregulation of the T cell receptor due to loss of the TCR-ζ chain, and proliferative arrest of T cells.

The second is the generation of oxidative stress. Nox2 or Cybb, a subunit of NADPH oxidase, can catalyze the production of ROS. Combined and cooperative action of NADPH, Arg1 and Nos2 can lead to the generation of reactive nitrogen species such as oxygen radicals (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO). The result is a loss of the TCR-ζ chain,[127] nitration or nitrosylation of TCR, CD8 or CD3, resulting in desensitization and reduced TCR signaling.[128] Nitrosylation also interferes with IL-2 receptor signaling.[129]

A third mechanism of inhibition of T cell responses employed by MDSC is interference with T cell migration, trafficking and viability. The metalloproteinase ADAM67, which is expressed by MDSC, can cleave CD62L on naive T cells, thus preventing their migration to draining lymph nodes and the transition to effector state. Galectin 9, an S-type-lectin which is expressed by MDSC, can bind to the immune checkpoint TIM3 on murine T cells and induce apoptosis,[130] though there is controversy as to whether this interaction translates in humans.[131] Membrane-bound TGF-β binds to inhibitory NK cell receptors (NKp30).[132]

Lastly, MDSC secrete inhibitory cytokines such as IL-10 and TFG-β, leading to suppression through fostering *de novo* Treg development and expanding Treg recruitment.[133] High levels of IL-10 also act on tumoricidal M1 macrophages, which are skewed to the protumoral M2 phenotype leading to reduction of IL-12 levels and thus providing a feed-forward mechanism for continued aggregation of M2 and TAM.[134]

1.5 Pro-tumoral inflammation

Commensals lining the gut epithelium interact with innate receptors and impact the development, basal activation, maintenance and regulation of local inflammation and immunity.[135] This intricate, usually symbiotic relationship can be disrupted by changes in the local microbial community (termed dysbiosis), which alters the predominant species (bloom of specific taxa) and modifies the composition of the microbial community (changes in diversity).[136] Dysbiosis has been directly associated with colon cancer in human metagenomic studies.[22,137,138] Furthermore, novel evidence compellingly suggests that the gut microbiota exert profound influence on the response to cancer immunotherapy and chemotherapy by affecting the differentiation of myeloid cells.[139] As such, it is highly likely that the reverse is true, and pathobionts and dysbiosis can directly influence myeloid cells to advance initiation and promotion of colorectal cancer.

Principal inflammation-induced oncogenic mechanisms include:

1. Direct mutagenesis through DNA damage secondary to reactive oxygen and reactive nitrogen species, i.e. as shown by p53 mutations through oxidative damage in colitis-associated cancer.[140]

2. Inactivation of mismatch repair genes, resulting in enhanced inflammation-induced mutagenesis and subsequent activation of further tumor-suppressor genes.[141]

3. Production of growth factors that can confer a stem cell-like phenotype upon epithelial cells, stimulating proliferation and resulting in hyperplasia, the most important of which are : Stat3, NF-κB and TNF-α. Stat3 is associated with stem cell reprogramming and renewal.[142] NF-κB increases Wnt/β-catenin signaling in colonic crypts.[143] Similarly, macrophage-derived TNF-α has been shown to promote Wnt signaling.[144]

Notably, many of these oncogenic transcription factors can be switched on by bacterial pattern-associated molecular patterns binding to host cells via cognate toll like receptors.[145] In turn, innate responders, such as myeloid cells and macrophages, can secrete pro-tumoral cytokines that are prominently involved in tumor initiation and progression. Early production of IL-6 and IL-11 originating from MDSC potentily induced Stat3.[146] In colitis-associated cancer (CAC), MDSC contributed to early stage hyperplasia and inhibition of apoptosis in epithelial cells.[147] Disruption of NF-κB signaling in a mouse model of CAC blocked production of IL-6, thus reducing tumorigenesis.[148] There is strong evidence that myeloid derived early pro-inflammatory factors IL-6, IL-11, TNF-α[149] and IL-1-β[150] play a leading role in tumorigenesis. TAM secreted IL-23 is another potent inflammatory cytokine, driving IL-17 and IL-22 production by Th17 T cells. TAM-derived IL-23 is upregulated by Stat3, PGE2, ATP and lactic acid production by shifting the reciprocally regulated pro-tumoral IL-23/ anti-tumoral IL-12 axis towards the former.[151]

In light of this data, we hypothesized that ETBF inflammation and the ensuing

high-levels of IL-17 production[19152153] act to disrupt normal myelopoiesis and result in the accumulation of procarcinogenic MDSC in the TME. Our findings suggest that in contrast to the non-colitogenic *Fusobacterium nucleatum*,[22] or the T cell-independent *pks+ Escherichia coli*,[20] ETBF oncogenesis requires the coordinated action of its toxin, bft and an IL-17-driven inflammatory response to orchestrate the recruitment of myeloid cells to the TME, as well as their differentiation and activation into immunosuppressive MDSC, specifically, iNOS^{hi} MO-MDSC. We propose that ETBF-triggered colon tumorigenesis is characterized by a specific immune signature combining IL-17-driven colitis and altered myeloid differentiation into MO-MDSC.

Taken together, myeloid cells can be differentially programmed by cues originating from the interplay between host and microbiome. These can be anti-tumoral or pro-tumoral, depending on the microbial community, and the presence of specific driver bacteria. Understanding the mechanisms between colonization with a specific pathobiont, the mobilization and activation of MDSC and how these events are linked to tumor initiation and progression will contribute to discovering the aetiology of human colorectal cancer, and may give rise to new tools and therapies for colon cancer prevention and treatment.

Chapter 2

Results

2.1 ETBF specifically promotes the accumulation of immature monocytic cells in colon tumors of Min mice

$CD11b^+$ myeloid cells accumulated progressively over time in the distal colon of ETBF Min mice and at 3 months made up 77.6 ± 5.4% (mean ± SEM, n=3 experiments) of the tumor-infiltrating $CD45^+$ leukocytes (Figure 2.1A) or 6.5 ± 1.5% of all viable epithelial and hematopoietic cells. Thus, myeloid cells constituted the overwhelming majority of hematopoietic cells that infiltrated colon tumors in Min mice after 3 months of ETBF colonization. Among $CD45^+$ leukocytes, three main groups of $CD11b^+$ myeloid cells comprised the ETBF colon TME: macrophages (MΦ)

(Gr-1^{-}, 25 ± 7.1%), monocytic (MO)-IMC (Gr-1^{low} or Ly6C^{hi}Ly6G^{-}, 11.8 ± 1.7%) and granulocytic (PMN)-IMC (Gr-1^{hi} or Ly6C^{low}Ly6G^{+}, 45.3 ± 7.9%) populations (all mean ± SEM, n=3 experiments, representative flow cytometry plots are shown in Figure 2.1B). CD4^{+} T cells and $\gamma\delta^{+}$ T cells, the predominant sources of protumoral IL-17[19,65] , made up only 1% and 0.1% of all CD45^{+} cells, respectively (Figure 2.1). When comparing myeloid cell infiltration in the distal colon between C57BL/6 wildtype (WT) and Min mice, we noticed that IMC were readily detectable in both strains at 1 week following infection (Figure 2.1C). However, with persistent ETBF colonization, IMC – especially PMN-IMC – accumulated in higher numbers in Min mice, while in WT, myeloid populations regressed by 8 weeks. To assess whether these observed differences were due to differential Apc function in Min versus WT mice, we addressed whether the Apc mutation affected the hematopoietic cell compartment in Min mice. For this purpose, lethally irradiated Min mice were reconstituted with WT or Min bone marrow (BM), challenged with ETBF and assessed for tumor numbers. As displayed in Figure 6.1, we found no significant difference between WT and Min BM-engrafted recipient Min mice, implying that Apc heterozygosity in the hematopoietic compartment does not impact tumorigenesis. This result suggests that the local myeloid environment is shaped by Apc loss of heterozygosity (LOH) in colonic epithelial cells (CEC) in conjunction with ETBF colonization of the colon.

We previously demonstrated that ETBF stably colonizes the colon, but not the small intestine (SI), yielding markedly induced colon tumorigenesis in Min mice, lo-

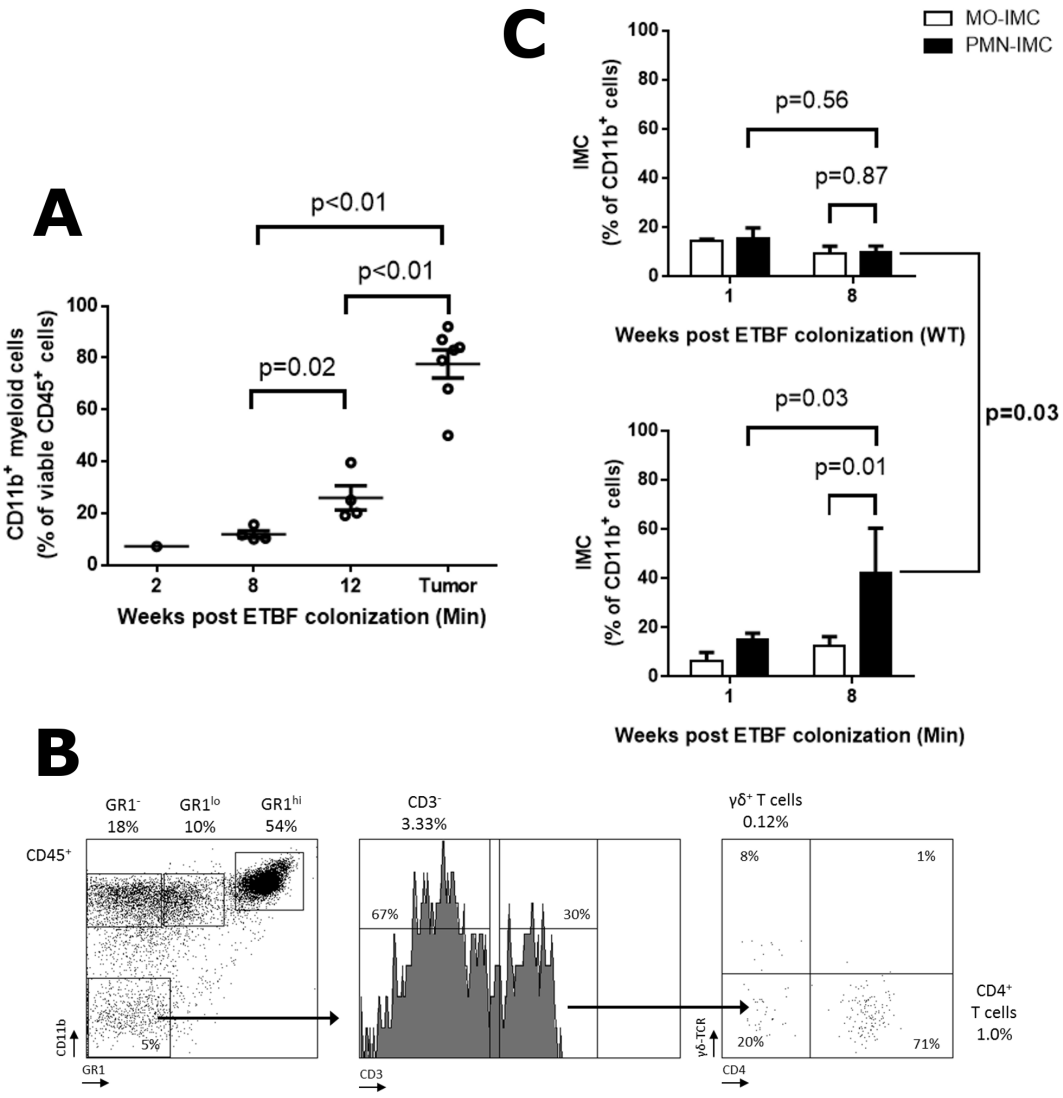

Figure 2.1: Myeloid cells are the predominant leukocytic population infiltrating ETBF-induced colon tumors in Min mice

A, Myeloid cells from enzymatically digested grossly normal distal colon tissue (weeks 2, 8 and 12 after ETBF colonization) and distal colon tumors (12 weeks post ETBF) were analyzed by CD45/CD11b staining and flow cytometry. Results are expressed as percent (%) of $CD11b^+$ cells among viable $CD45^+$ leukocytes. Aggregate data of n=7 independent experiments with pooled colon or tumors samples from 3-4 mice/experiment.

B, Flow cytometry of ETBF-triggered colon tumors in Min mice. Percent $CD45^+$ cells are indicated. Representative plots of n=3 or more experiments with 3-4 mice/experiment.

C, Proportion of $Ly6C^{hi}Ly6G^-$ monocytic immature myeloid cells (MO-IMC) (white bars) and $Ly6C^{lo}Ly6G^+$ granulocytic (PMN)-IMC (black bars) as percent of $CD11b^+$ isolated from distal colon of WT (top) or Min (bottom) mice at the time points indicated. Of note, IMC in distal colon tissue of sham Min mice were below the limit of detection. Aggregate data of n=4 independent experiments with pooled colon samples from 3 mice/group.

calized predominantly to the distal colon (Figure 6.1).[19] In contrast, SI tumorigenesis, a characteristic of parental Min mice, is unchanged upon ETBF colonization.[19] We examined the myeloid compartment present in SI (ETBF-independent) and colon (ETBF-dependent) tumors as well as normal adjacent tissue by flow cytometry and microscopic analysis. We distinguished MΦ ($CD11b^{+}F4/80^{+}$MHC-$II^{+}Gr\text{-}1^{-}CD11c^{-/low}SSC^{int}$), dendritic cells (DC, $CD11c^{hi}$MHC-$II^{hi}SSC^{low}$), mast cells (MC, CD11b-Fc$\epsilon$$RI^{+}CD117$(c-kit)$^{+}$), MO-IMC ($CD11b^{+}Gr\text{-}1^{hi}F4/80^{hi}CD11c^{-/low}$MHC-$II^{-/+}SSC^{hi}$) and PMN-IMC ($CD11b^{+}Gr\text{-}1^{hi}F4/80^{-}CD11c^{-}$MHC-$II^{-}SSC^{hi}$) (Figure 2.2A–C). FSC/SSC analysis (Figure 2.2B) and Wright-Giemsa staining performed on PMN-IMC and MO-IMC sorted from ETBF-induced colon tumors confirmed their distinct morphologies (Figure 2.2C). Notably, although developing concomitantly in ETBF-colonized Min mice,[19] the SI- and colon tumor-associated tumor microenvironment (TME) had distinct myeloid infiltrates (Figure 2.2D). MC were present in SI tumors but sparse in colon tumors ($3.5 \pm 2.3\%$ in SI versus $0.3 \pm 0.2\%$ in colon, $p<0.05$, mean $\pm$ SEM) and PMN-IMC were significantly more common in colon tumors than in SI tumors ($8.9 \pm 3.9\%$ in colon versus $1.2 \pm 0.6\%$ in SI, $p<0.05$, mean $\pm$ SEM). The normal tissue adjacent to colon tumors was characterized by a robust population of $CD11c^{hi}$MHC-II^{hi} DC (29.7% of viable cells in normal vs. 5.8% in ETBF tumors, Figure 2.2A).

Subsequent comparison of the myeloid environment in colon tumors of ETBF-colonized versus sham Min mice provided evidence of altered myeloid differentiation in direct response to ETBF colonization (Figures 2.3A,B). Both the rare, sporadically-

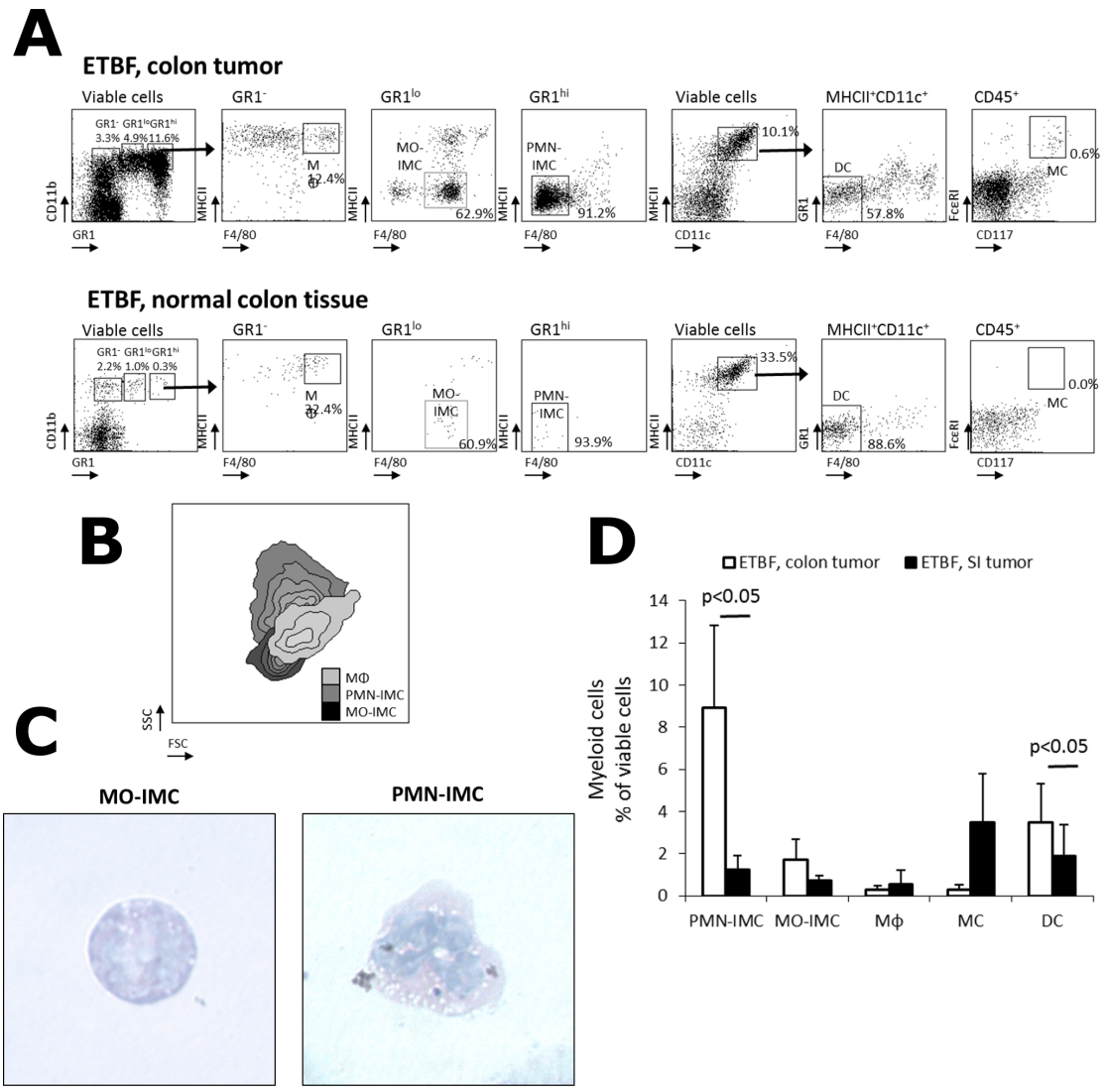

Figure 2.2: Characterization of the myeloid compartment in ETBF-colonized Min mice
A, Flow cytometry of distal colon tumors (top) as well as adjacent grossly normal colon tissue (bottom) isolated from 3 month ETBF-colonized Min mice. $CD11b^{hi}Gr\text{-}1^{lo}MHC\text{-}II^{lo}F4/80^{-/low}$ MO-IMC; $CD11b^{+}Gr\text{-}1^{hi}MHC\text{-}II^{lo}F4/80^{-/low}$ PMN-IMC; $CD11b^{-}FcRI^{+}CD117^{+}$ mast cells (MC); $CD11b^{+}Gr\text{-}1^{-}F4/80^{+}$ macrophages (MΦ); $CD11c^{hi}MHCII^{hi}Gr\text{-}1^{-}F4/80^{-}$ dendritic cells (DC) are shown. In normal colon tissue, only MΦ and DC populations are readily detectable since only few MO- and PMN-IMC are normally present in the lamina propria. Representative plots of n=3 or more experiments with 3-4 mice/experiment.
B, Representative forward scatter (FSC) and side scatter (SSC) of the predominant myeloid populations, i.e.: PMN-IMC ($CD11b^{hi}Gr\text{-}1^{hi}$), MO-IMC ($CD11b^{hi}Gr\text{-}1^{lo}$) and MΦ ($CD11b^{hi}Gr\text{-}1^{-}F4/80^{+}$). Representative plot of n=3 or more experiments with 3-4 mice/experiment.
C, Wright-Giemsa staining of cytospin-fixed MO-IMC (left) and PMN-IMC (right), FACS-sorted as $Ly6C^{hi}Ly6G^{-}$ MO-IMC and $Ly6C^{lo}Ly6G^{+}$ PMN-IMC, respectively. Representative images of n=2 independent samples from one cell sorting experiment with tumor samples from 2-3 mice.
D, Proportions of myeloid cell subsets in ETBF-triggered distal colon tumors versus ETBF-independent small intestine (SI) tumors as defined by the gating strategy outlined in A. Bars represent mean ± SEM of n=3-5 independent experiments with 3-4 mice/experiment for colon and SI tumors, respectively.

occurring colon tumors in sham Min mice and the abundant colon tumors in ETBF Min mice were highly infiltrated by PMN-IMC (Gr-1^{hi}; 84% versus 70% of $CD11b^+$ cells in sham and ETBF tumors, respectively), which in grossly normal colon tissues constituted only a minor population (3% and 15% of $CD11b^+$ cells in sham and ETBF-colonized Min mice, respectively) (Figure 2.3A,B). Combined with the time-dependent attrition of myeloid cells from the distal colon of WT mice colonized with ETBF (Figure 2.1C), this result suggests a likely ETBF-independent but Apc LOH dependent signal affecting myeloid cell differentiation in the TME of Min mice.

Sporadic, but not ETBF-induced tumors, were highly infiltrated by inflammatory MΦ (Inf-MΦ) expressing high levels of F4/80 and MHC-II (23% vs. 6% of $CD11b^+$Gr-1^{lo} cells, respectively). MO-IMC, on the other hand, accumulated markedly in ETBF but to a lesser extent in sporadic colon tumors (83% vs. 57% of $CD11b^+$Gr-1^{lo}MHC-$II^-F4/80^-$ cells, respectively, Figure 2.3A). Furthermore, Inf-MΦ isolated from sporadic colon tumors overexpressed Arg1 and Fizz1 (though not Ym1), hallmarks of protumoral M2 MΦ (Figure 2.3C),[154] whereas ETBF tumor-derived MO-IMC strongly upregulated Nos2 (encoding iNOS) but not Arg1 mRNA (x10 and less than x2, respectively) compared to MO-IMC isolated from sporadic colon tumors (Figure 2.3D). Gene expression profiling revealed that sparse Inf-MΦ (6%; Figure 2.3A)) in ETBF-Min tumors expressed H2-ab1 (encoding MHC-II), Csf1r (encoding M-CSF), Il12b and Emr1 (encoding F4/80) (Figure 6.2), indicating a terminally differentiated state but with lower expression of M2 markers compared to those in sporadic colon tumors

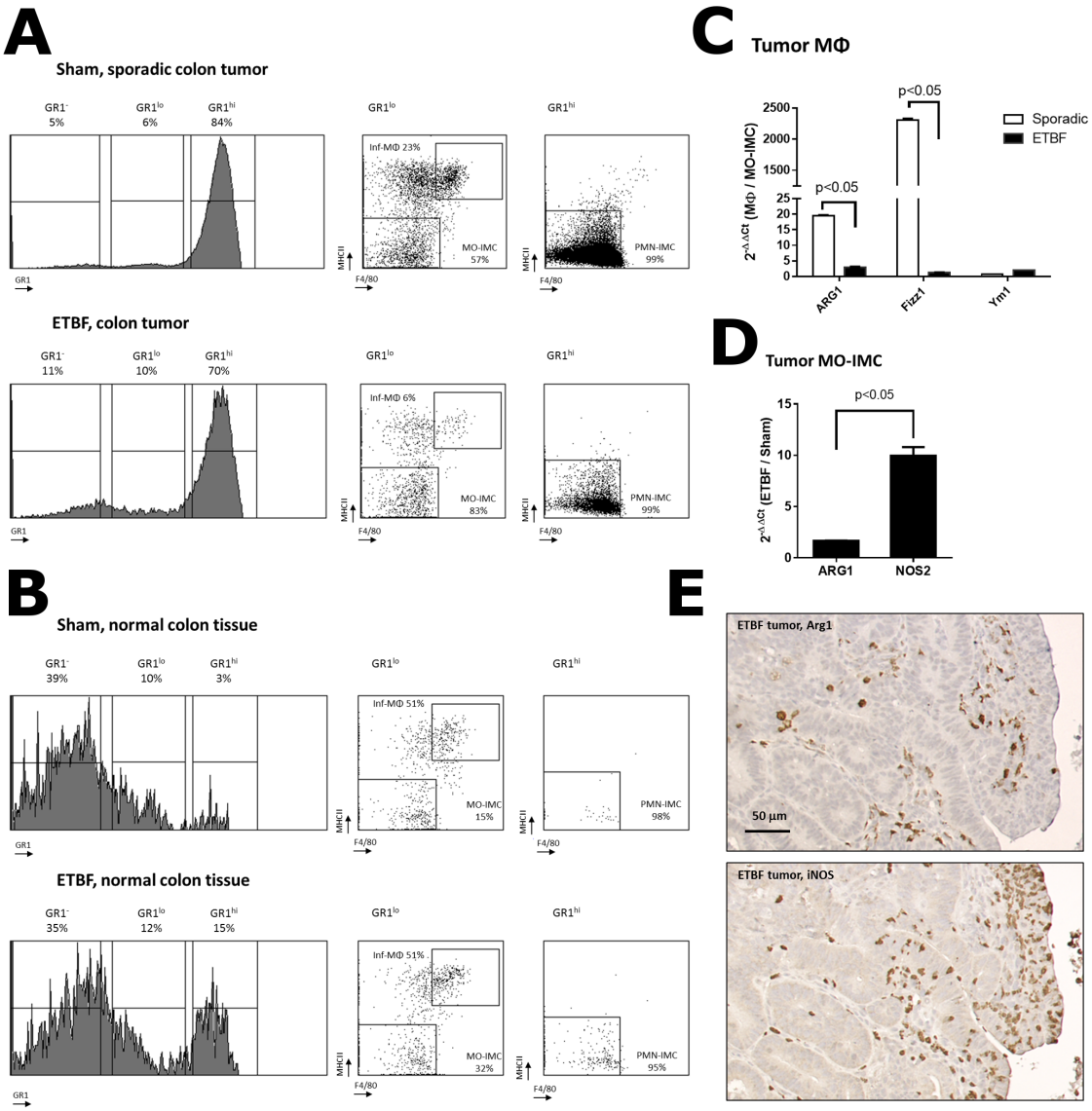

Figure 2.3: ETBF colonization promotes specific accumulation of MO-IMC to the colon TME

A, B, Flow cytometry analysis of myeloid cells in sporadic (sham) or ETBF-induced colon tumors (A) compared to normal colon tissue (B) in Min mice. Histograms represent Gr-1 expression on viable CD11b+ cells (% $CD11b^+$ indicated). Dot plots represent F4/80 and MHC-II staining in $CD11b^+Gr\text{-}1^{low}$ (left) and $CD11b^+Gr\text{-}1^{hi}$ cells (right). $Gr\text{-}1^{low}$ encompasses inflammatory MΦ ($MHC\text{-}II^{hi}F4/80^{hi}$) and MO-IMC ($MHC\text{-}II^{low\text{-}int}F4/80^{int}$) cell types whereas $Gr\text{-}1^{hi}$ consists predominantly of PMN-IMC ($MHC\text{-}II^{low}F4/80^{low}$). Representative plots of n=5 independent experiments with 2-4 mice/group. Additional representative flow cytometry plots are shown in Fig S8.
C, Arg1, Fizz1 and Ym1 expression in Inf-MΦ versus MO-IMC FACS-sorted from sporadic or ETBF tumors. Bars represent fold increase of gene expression in inflammatory MΦ com-pared to MO-IMC, RQ (2-ΔΔCt(MΦ/MO-IMC)). Aggregate data of n=2 independent samples from one cell sorting experiment with tumor samples from 2-3 mice/group.
D, Arg1 and Nos2 expression in MO-IMC FACS-sorted from ETBF-induced colon tumor versus MO-IMC from sporadic (sham) colon tumors. Bars represent fold increase of gene expression in ETBF compared to sham colon tumors, RQ (2-ΔΔCt(ETBF/Sham)). Aggregate data of independent samples from one cell sorting experiment with tumor samples from 2-3 mice/group. E, IHC expression of Arg1 (left) and iNOS (right) in ETBF-induced colon tumors. Scale bar: 50 m. Representative images of n=3 or more independent experiments with 3-4 mice/experiment.

(Figure 2.3C). Moreover, ETBF-associated MO-IMC upregulated genes such as Ido1, Mmp9, S100a8/9, bearing resemblance to the transcriptional activity of MDSC (Figure 6.2). Lastly, IHC demonstrated that ARG1 and iNOS proteins did not co-localize in colon tumors of ETBF-colonized Min mice (Figure 2.3E) and therefore may delineate different myeloid effectors (M2-like MΦ versus MO-IMC). Taken together, these findings demonstrate that the specific accumulation of iNOS-expressing MO-IMC is a unique feature of the inflammatory microenvironment of colon tumors induced by ETBF. In SI tumors (ETBF-independent) (Figure 2.2D) as well as sporadic colon tumors (no ETBF colonization) (Figure 2.3A), levels of MO-IMC are comparatively low.

2.2 Intratumoral but not peripheral MO-IMC are immunosuppressive in ETBF Min mice

We sought to determine if the distinct population of ETBF colon tumor-associated iNOS^{hi} MO-IMC displayed an immunosuppressive phenotype in accordance with their gene expression profile (Figure 2.3D). Although both MO- and PMN-IMC were detected in the spleen, blood and colon tumors of ETBF tumor-bearing Min mice (Figure 2.4A), only colon tumor-associated MO-IMC were immunosuppressive when

assayed in vitro for inhibition of OVASIINFEKL-specific $CD8^+$ T cell (OT-1) proliferation (40.9 ± 4.5% of inhibition, mean ± SEM, n=3 mice/group; Figure 2.4B). Comparatively, PMN-IMC exhibited only very modest immunosuppressive activity on OT-1 cell proliferation (12.8 ± 3.9%, as above). Thus, the distinct MO-IMC population associated with ETBF colon tumors resemble MO-MDSC and will henceforth be named as such.

We next chose to compare the transcriptomes of splenic MO-IMC and intratumoral MO-MDSC (3 month post ETBF colonization) to probe the mechanism behind the $CD8^+$ T cell suppression we observed. The expression of genes of interest was quantified using Taqman qPCR. As shown in Figure 2.4C, when compared to splenic MO-IMC from ETBF-colonized mice, intratumoral ETBF MO-MDSC exhibited a higher expression of Arg1 (x129,960) and Nos2 (x110), known to be central to arginine metabolism and Ido1 (x442), which mediates the degradation of tryptophan. These metabolic pathways are all well established as key players involved in the suppression of T cell responses.[155–157] Il10 (x258), Cd274 (PD-L1, x50) and Tgfb1 (x3) – known to contribute to inhibiting anti-tumor immunity in the TME[133] – were also highly expressed in ETBF tumor MO-MDSC. Il23a, a promoter of Stat3 activation and Th17 differentiation, was strongly upregulated (x2,867). Finally, intratumoral ETBF MO-MDSC overexpressed Stat3 (x3), Vegfa (x9) or Mmp9 (x5), genes involved in tumor growth and angiogenesis. Intratumoral PMN-IMC also displayed an immunosuppressive transcriptome (Figure 6.3). However, despite stark differences

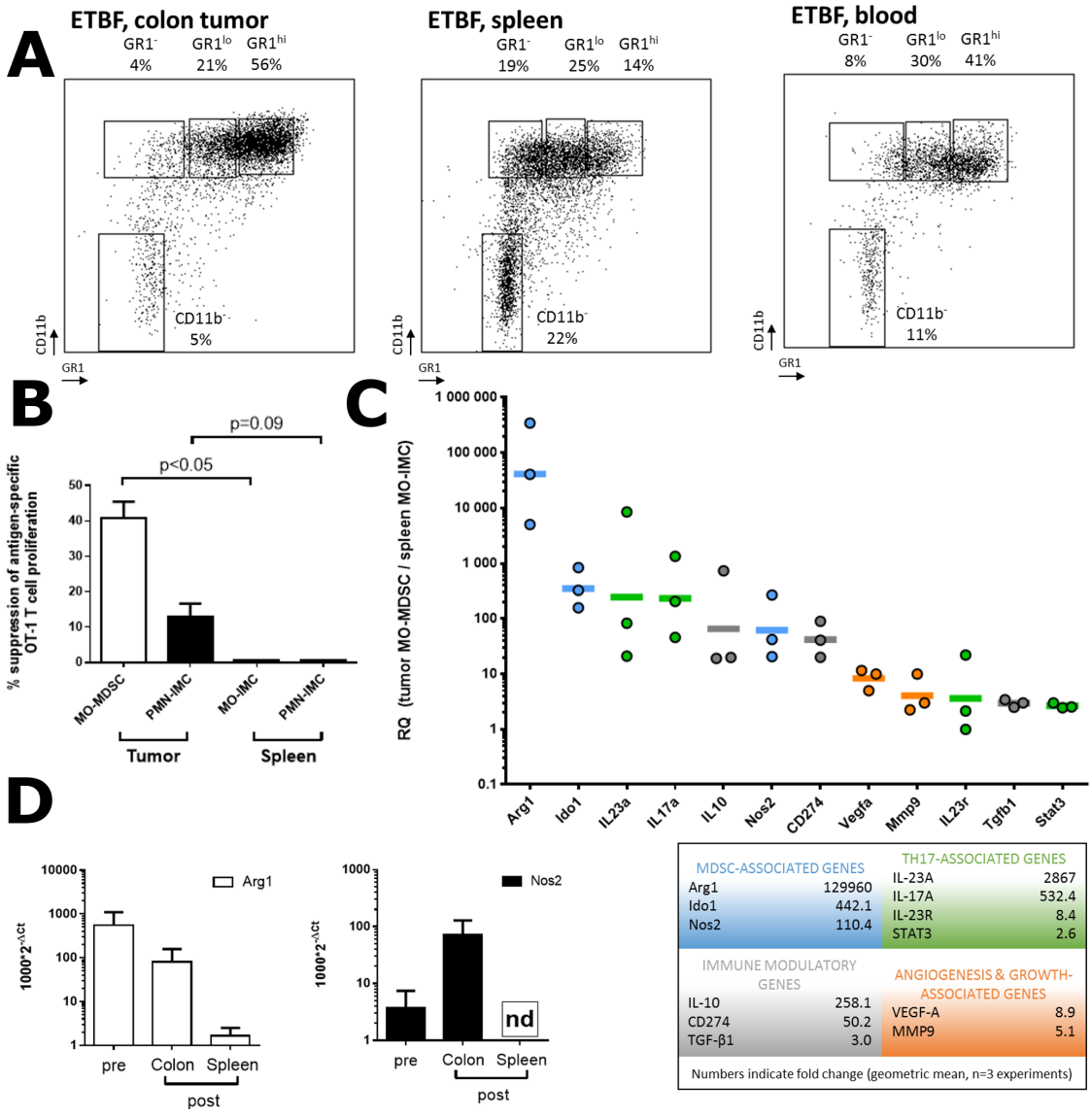

Figure 2.4: Colon tumor-infiltrating myeloid cells are myeloid-derived suppressor cells
A, Flow cytometry analysis of tumor, spleen and blood-derived myeloid cells in ETBF-colonized Min mice. Percent CD45 are indicated. Representative plots of n=3 or more experiments.
B, MO-MDSC and PMN-IMC (in tumors) and IMC (in spleen) were cell-sorted from ETBF Min mice. Inhibition of proliferation of CFSE-labeled OVA-specific transgenic $CD8^+$ T cells (OT-1) by MDSC (T cell:IMC ratio 10:1) was measured by dilution of CFSE using flow cytometry. Independent samples from one cell sorting experiment with n=3 mice.
C, Gene expression array in MO-MDSC (in tumors) or MO-IMC (in spleen) of 3 month ETBF Min mice. Select genes are grouped according to biological function. Lines represent geometric mean of fold increase of gene expression (RQ) in tumor MDSC versus spleen IMC. Dots rep-resent RQ values from individual cell sorting experiments. Aggregate data of n=3 independent experiments with pooled tumor samples of 3-4 mice/experiment.
D, In vitro derived MO-IMC were adoptively transferred into Min mice with established colon tumors (2 months post ETBF colonization). 7 days after transfer, MO-IMC were harvested from spleen or colon tumors and Nos2 and Arg1 gene expression was analyzed. Ct values were normalized with CtGapdh (ΔCt=Ct-CtGapdh) and bars represent 2-ΔCt. Aggregate data of n=2 independent experiments with 2-3 mice/experiment. nd=not detected.

in their in vitro inhibitory capacities (Figure 2.4B), a direct comparison of TME-resident MO-MDSC and PMN-IMC showed no major differences in transcriptional programs (Figure 6.4), with the exception of the genes CCR2 (chemokine receptor mediating myeloid trafficking) and Il6 (protumoral cytokine via Stat3 activation), which were both upregulated only in MO-MDSC. Collectively, these results support the conclusion that ETBF induces a distinct population of immunosuppressive, protumoral MO-MDSC in the colon TME, which are predicted to play a key role in ETBF-induced inflammatory carcinogenesis.

As our research group recently reported, we found that *Il17a* was expressed in intratumoral ETBF MO-MDSC but not splenic MO-IMC (Figure 2.4C).[65] We ruled out contamination by Il17a derived from T cells (including $CD4^+$, $CD8^+$, $\gamma\delta^+$, NKT cells) by flow cytometry (Figure 6.5) and Cd3e qPCR (data not shown). However, although Il17a gene expression appeared to selectively delineate intratumoral MDSC (especially MO-MDSC) (Figure 6.6A), IL-17 protein was not detected by intracellular cytokine staining.[65] Nonetheless, *Il17a* expression by MO-MDSC correlates with their selective activation in the TME: when comparing gene expression 7 days after ETBF colonization in MO-MDSC and MO-IMC harvested from Min or WT mice respectively, we found IL-17 to be differentially regulated between groups, and highly expressed only in Min (x146, Figure 6.6B). T cells isolated from Min versus WT mice displayed only modestly greater IL-17 expression (x5). Additional transcription factors that typically regulate *Il17a* transcription, such as *Rorc*, *Il23r* and *Irf4*,

were expressed in colon lamina propria-infiltrating MO-MDSC 7 days after ETBF colonization at levels comparable to those found in infiltrating T cells (Figure 6.6B), with the exception of Rorc, which was markedly increased only in MO-MDSC isolated from Min distal colon (x596). *IL17a* and *Rorc* expression, together with that of *Nos2*, may constitute activation markers specifically identifying ETBF-associated tumor MO-MDSC.

To further confirm the ability of the ETBF-associated TME to polarize IMC towards MO-MDSC, we adoptively transferred WT CD45.1$^+$ BM-derived MO-IMC into 2-month ETBF-colonized or sham-inoculated CD45.2$^+$ Min mice. We compared the gene expression profile of CD45.1$^+$ BM-derived MO-IMC prior to and 7 days after transfer, when they were cell-sorted from spleen and colon tumors of the recipient mice. Nos2 was strongly upregulated in adoptively transferred cells isolated from colon tumors, but not spleen, of the ETBF Min recipients. Arg1 expression declined compared to pre-transfer BM-derived MO-IMC (Figure 2.4D), consistent with data in Figure 2.3D.

In summary, our findings demonstrate that the ETBF-associated colon TME in Min mice uniquely promotes the differentiation to and recruitment of iNOS^{hi} MO-MDSC (also marked by Il17a and Rorc expression), which are characterized by the capacity to suppress T cell responses as well as to provide tumor growth, pro-angiogenesis and pro-inflammatory factors.

2.3 Endogenous IL-17 mobilizes and activates MDSC during de novo ETBF-induced colon tumorigenesis

Next, we sought to identify tumor-associated signals accounting for MO-MDSC recruitment and their activation in ETBF-induced colon tumors. Since we previously demonstrated that IL-17 is critical to ETBF-triggered colon carcinogenesis, and the accumulation of protumoral MDSC in the TME was recently attributed to IL-17,[19,152,153,158] we postulated that intratumoral IL-17 was, at least in part, responsible for the selective accumulation and activation of pro-carcinogenic iNOS^{hi} MO-MDSC in the colon tumors of ETBF-colonized Min mice. To test this hypothesis, we demonstrated that IL-17R-expressing MO-MDSC (Figure 2.4C) sorted from colon tumors of ETBF Min mice could be activated in response to recombinant IL-17 (rIL-17). Crucially, the ETBF TME is IL-17-rich and purified MO-MDSC are likely pre-conditioned, therefore the response to exogenous IL-17 may be suppressed. Notwithstanding, ex vivo treatment of purified MO-MDSC with rIL-17 induced significant upregulation of Nos2 and modest upregulation of Arg1, genes involved in T cell immunosuppression (Figure 2.5A). In addition, we observed a trend to upregulate Stat3, which is strongly associated with protumoral activity, as well as proinflammatory genes such as Cybb (encoding NOX2), Ptgs2 (encoding COX2) or Stat1, in

response to rIL-17 treatment. As shown in Figure 2.4C and Figure 6.2, many of these genes were also overexpressed in vivo in ETBF tumor-derived MO-MDSC. In contrast, splenic MO-IMC isolated from ETBF-colonized Min mice were unable to upregulate Arg1 or Nos2 in response to rIL-17, despite comparable *IL-17ra* mRNA expression (data not shown), suggesting the notion that additional ETBF-driven mucosal (i.e. CEC or TME-derived) signals act in conjunction with IL-17 to promote protumoral and immunosuppressive MDSC. Consistent with this idea, the upregulation of IL-10 and IL-23 gene transcription in vivo together with that of Stat3 underscored the likely contribution of paracrine feedback signaling on the expression of STAT3 (i.e. STAT3 activation is downstream of the IL-10 and IL-23 receptors), and suggests that STAT3 may serve as an important signal transducer of IL-17-mediated MDSC activation.[63,74]

Further, we found that the enhanced transcriptional activity of Nos2 correlated with increased nitric oxide protein expression. rIL-17 increased in vitro iNOS activity as measured by the production of nitric oxide (NO) in the culture supernatant of LPS-conditioned MO-MDSC (Figure 6.7). Spleen-derived MO-IMC were unable to produce NO upon in vitro stimulation.

Finally, using ETBF-colonized mixed BM chimeric Min mice — generated by reconstituting lethally irradiated Min mice with a 1:1 mixture of CD45.1^{+}IL-17R$^{+/+}$ and CD45.2^{+}IL-17R$^{-/-}$ BM – we showed that CD45.1^{+}IL-17R^{+} MO-MDSC (i.e. sensitive to endogenous IL-17) sorted from colon tumors 10 weeks after ETBF inoculation exhibited a higher expression of Nos2, Arg1, Il23a, Ptgs2 (Cox2), Cd274 (PD-L1)

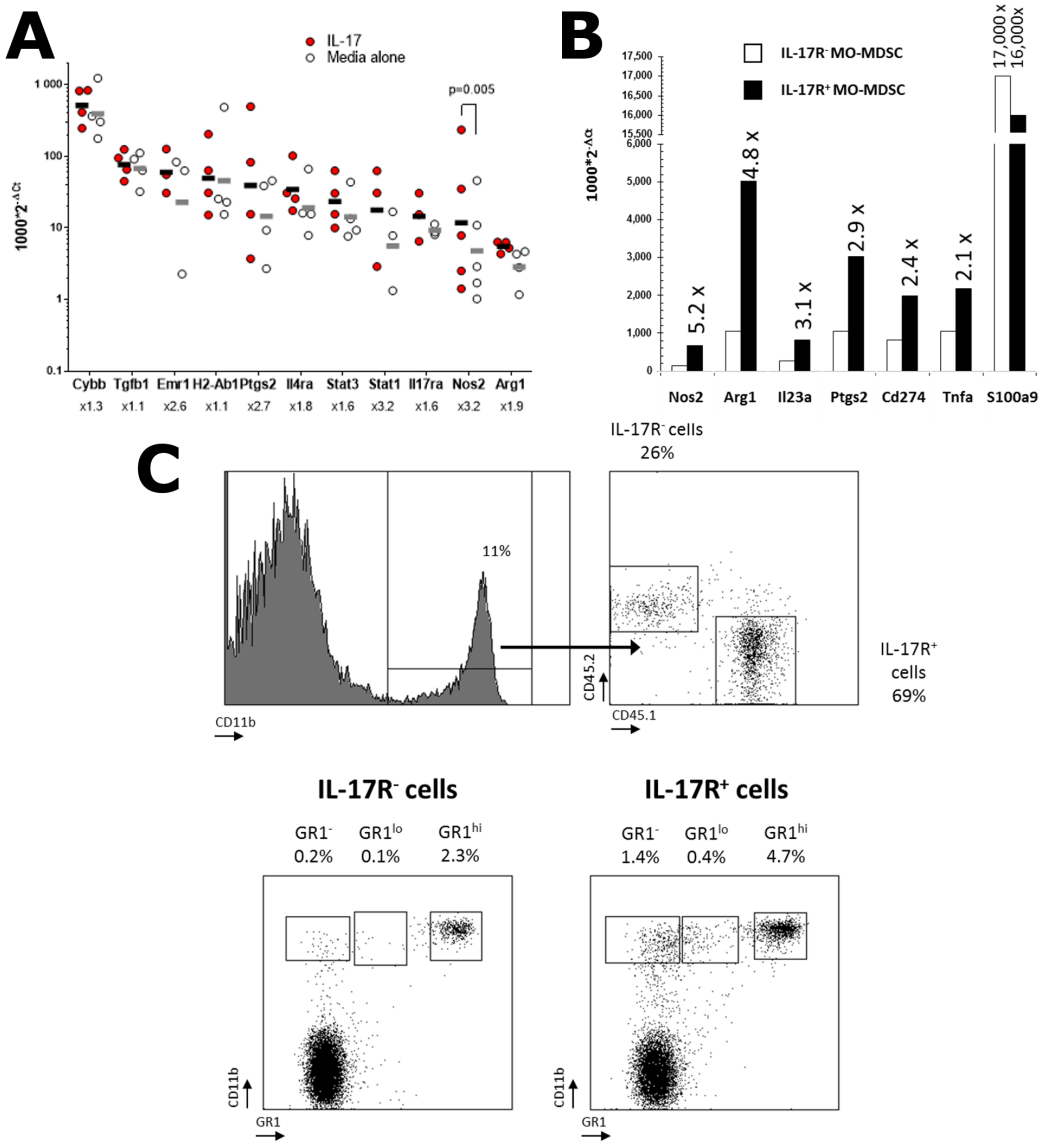

Figure 2.5: IL-17-induced protumoral transcriptomes in tumor-infiltrating MO-MDSC
A, Tumor MO-MDSC sorted from ETBF Min colon tumors were incubated overnight with rIL-17 (10 ng/ml) and gene expression was assessed by qPCR. Lines represent geometric mean of 2-ΔCt in MO-MDSC cultured with IL-17 compared to medium alone. Dots represent 2-ΔCt values from individual cell sorting experiments. Fold increase of gene expression are indicated below gene labels. Increase of Nos2 expression in IL-17-treated cells was significant with p=0.0055 (ratio paired t test). Aggregate data of n=3-4 independent experiments with 2-3 mice/experiment.
B, Ex vivo gene expression in CD45.2+IL-17R- MO-MDSC (white bars) and CD45.1$^+$IL-17R$^+$ MO-MDSC (black bars) sorted from colon tumors of 2 month ETBF-colonized [CD45.2$^+$IL-17R$^-$/CD45.1$^+$IL-17R$^+$] mixed bone marrow (BM) chimera Min mice. Bars represent 2-ΔCt. Fold increase of gene expression between CD45.1$^+$IL-17R$^+$ vs. CD45.2$^+$IL-17R$^-$ MDSC are indicated above the black bars. Representative data of independent samples from one cell sorting experiment with n=2 mice.
C, Flow cytometry of myeloid populations infiltrating colon tumors of one month ETBF-colonized mixed BM chimera [CD45.2$^+$IL-17R$^-$/CD45.1$^+$IL-17R$^+$] Min mice. Percentages and phenotype of CD45.2$^+$ (IL-17R$^{-/-}$) versus CD45.1+ (IL-17R+/+) myeloid populations were compared. CD11b and Gr-1 expression were analyzed in CD45.1$^+$ and CD45.2$^+$ cells independently. Representative plots of n=2 independent experiments with 3-4 mice/experiment.

and Tnfa (all IL-17 dependent genes) as compared to CD45.2^{+}IL-17R$^{-/-}$ MO-MDSC (Figure 2.5B). These results, together with our previous identification of many of the same transcripts in MO-MDSC cultured in vitro with exogenous rIL-17 (Figure 2.5A), demonstrate direct involvement of IL-17 in establishing protumoral MO-MDSC in the ETBF-induced TME. S100A9, a Stat3-dependent gene responsible for MDSC accumulation and activation , was not differentially regulated by IL-17, which signals predominantly through NF-B, emphasizing that several regulatory pathways act in tandem to fully activate MDSC.

Interestingly, although reduced in numbers compared to CD45.1^{+}IL-17R^{+}CD11b^{+} cells, CD45.2^{+}IL-17R-CD11b^{+} cells were still present in the colon TME (69% CD45.1^{+} cells versus 26% CD45.2^{+} cells, respectively; Figure 2.5C), again implying that signals in addition to the direct action of IL-17 on IMC coordinated MDSC recruitment to the ETBF colon TME. Nevertheless, the proportions of MO-MDSC and other myeloid cells subsets (CD45.2^{+}CD11b^{hi}Gr-1^{-}), including MΦ and DC, were drastically decreased in IL-17R-deficient tumor-infiltrating CD45.2+ cells compared to cells derived from CD45.1^{+}IL-17R^{+} hematopoietic progenitors. Notably, the proportion of PMN-MDSC in tumors decreased two fold in the IL-17R-deficient tumor-infiltrating CD45.2^{+} cells compared to CD45.1^{+}IL-17R^{+} cells (2.3% to 4.7%, respectively), indicating a similar dependency on IL-17 (Figure 2.5C). These results highlight the ability of IL-17 to preferentially activate MO-MDSC in vivo and drive their accumulation to the TME. However, other stimuli act in concert with IL-17 to mold the overall

myeloid TME in ETBF-colonized Min mice.

2.4 Recruitment of IL-17-driven, iNOS-expressing MO-MDSC constitutes an immune signature of ETBF colon tumorigenesis

Our final aim was to address whether the myeloid environment that is established upon colonization with ETBF and is associated with ETBF-triggered colon tumorigenesis is specific to ETBF or common among oncogenic bacteria. To test for specificity, we compared ETBF colonization of WT mice with *Fusobacterium nucleatum* and *pks+ E. coli*, two bacteria strongly associated with CRC in humans[10] and shown to induce colon tumorigenesis in experimental murine models.[20,22] As a control, we used the non-colitogenic *Bacteroides fragilis* 9349 pfd340 (NTBF) and *E. coli* lacking the genotoxic pks island (*E. coli* Δpks).

Although mice were successfully colonized with each bacterium (Figure 6.8), only ETBF and *Fusobacterium* induced a strong myeloid response upon colonization, which was characterized by an increased proportion of MO- and PMN-IMC in the distal colon by day 7 post-colonization (Figure 2.6A). This aligns with studies by Kostic et

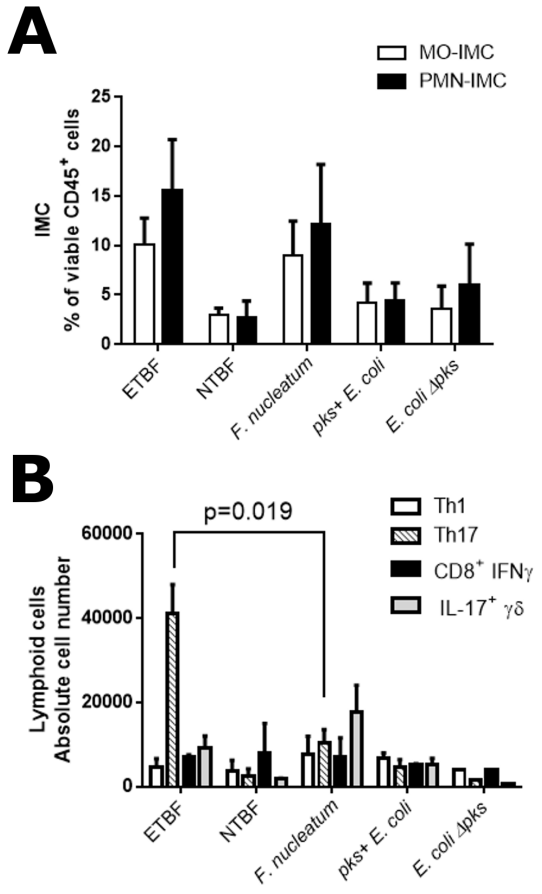

Figure 2.6: Mucosal immune response to *Fusobacterium nucleatum*, *pks+ E. coli*, ETBF and NTBF colonization in WT mice

A, Proportion of CD11b^{+}Ly6C^{hi}Ly6G^{-} MO-IMC (white bars) and CD11b^{+}Ly6C^{low}Ly6G^{+} PMN-IMC (black bars) as percent of viable CD45^{+} leukocytes isolated from distal colon of WT mice one week post-infection with oncogenic bacteria listed along the x-axis.

B, Absolute numbers of Th1 (CD3^{+}CD4^{+}IFN-γ^{+}, Th17 (CD3^{+}CD4^{+}IL-17^{+}), cytotoxic T cells (CD3^{+}CD8^{+}IFN-γ^{+}) and $\gamma\delta$ T cells (CD3+$\gamma\delta$+IL-17+) lymphoid populations isolated from distal colon as described in A.

Aggregate data of n=2 independent experiments with 3 mice per group.

al.[22] who showed an increase of myeloid subsets in small intestinal tumors following daily inoculation of Min mice with *Fusobacterium* for 8 weeks; colon tissues were not examined. It is worth pointing out that the authors used SI tumors as a surrogate for the TME in colon tumors. Per contra, in the context of ETBF infection, SI and colon tumors attract distinct myeloid populations (as shown in Figure 2.2D).

Infection with *pks+ E. coli* had little impact on myeloid recruitment, consistent with published findings comparing oncogenic *E. coli* to control *E. faecalis* in which neither lymphoid (CD3) nor myeloid (as measured by Ly6B, F480 immunohistochemistry) responses differed.[20] However, and in stark contrast to ETBF,[19] no other oncogenic bacterium triggered a predominant production of IL-17 (Figure 2.6B). Altogether, these results demonstrate that high IL-17 levels in combination with MO-MDSC accumulation may mark the pathogenic interaction of ETBF with colon CEC and be considered as the immune signature of tumorigenic ETBF infection.

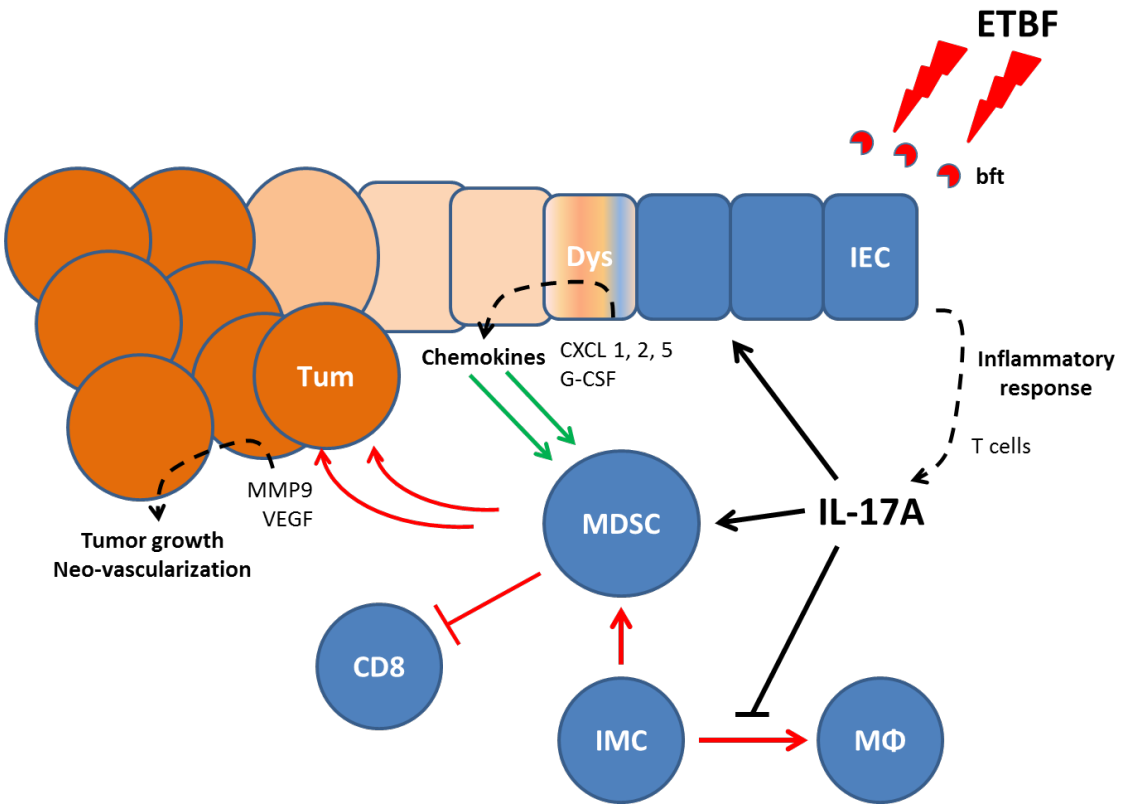

Figure 2.7: Proposed model for IL-17 recruitment of MDSC into colon TME of ETBF-colonized Min mice

ETBF induces submucosal IL-17 expression, which orchestrates the myeloid environment of ETBF-triggered colon tumors both directly by interacting with myeloid IL-17 receptors and indirectly by further inducing the ectopic production of chemokines and/or growth factors by dysplastic IL-17R+ colonic epithelial cells (CEC). The combined action of IL-17 and transformed CECs drives MDSC to promote tumor growth via the suppression of immune effector cells, the activation of proliferative pathways (Stat3) as well as the production of pro-angiogenic mediators (i.e. MMP-9 and VEGF).

Chapter 3

Discussion

We show herein that ETBF-triggered *de novo* colon carcinogenesis in Min mice leads to the specific recruitment and activation of MO-MDSC. We conclude that ETBF affects the myeloid compartment in two ways. First, ETBF-induced intratumoral IL-17 together with additional factors, likely epithelial-derived, orchestrate the recruitment of myeloid cells to the TME. Second, BFT-driven oncogenesis polarizes the differentiation of IMC towards MDSC. We identified an 'immune signature' associated with ETBF-driven colon tumorigenesis, defined by high levels of IL-17 and an accumulation of iNOS^{high} MO-MDSC. This was radically different from the TME observed in ETBF-independent SI tumors, which were more infiltrated by MC and less by MDSC, or sporadic colon tumors, which presented with a higher proportion of M2 MΦ and few MO-MDSC (Figures 2.2 and 2.3). Finally, among bacteria linked to the etiology of CRC, the immune landscape associated with ETBF infection was

unique, affirming the distinct mechanisms via which these pathobionts can initiate and promote tumorigenesis.

Importantly, since ETBF accelerates the tumorigenesis process observed in the colons of Min mice, we cannot formally rule out that the differences in the proportions of MΦ and MO-MDSC between sham and ETBF-triggered tumors was not a consequence of rapid recruitment of IMC to the ETBF-associated TME rather than of skewed myeloid differentiation related to the inflammatory and metabolic conditions within the ETBF-associated TME. However, the adoptive transfer of BM-derived IMC demonstrated that they were:

1. recruited to the ETBF-associated TME and

2. upregulated Nos2 (Figure 2.4D) and Il17 gene expression (Figure 6.6).

Recruitment, together with *Nos2* and *Il17* expression were hallmarks of MO-MDSC activation in this study. This result supports a specific role of ETBF on altered myeloid differentiation in the TME rather than a kinetic effect. Namely, peripherally injected BM-derived IMC ($iNOS^{low}$) differentiated into intratumoral MO-MDSC ($iNOS^{hi}$) within one week in ETBF-colonized Min mice.

In this study, IL-17 acted as a key inflammatory mediator for myeloid recruitment and polarization. Genetic ablation of IL-17R in the hematopoietic compartment profoundly affected proportions of MO-MDSC in the TME of ETBF Min mice, but did not abrogate myeloid recruitment. Recent studies in human CRC as well as numerous

mouse models have proposed that IL-17 is critical for recruitment of MDSC to the TME.[73,152,158,159] However, sporadic colon tumors (that result from Apc deletion but not ETBF activity) are also infiltrated by Th17 cells (unpublished results), though without the accumulation of MO-MDSC, proof that additional ETBF-dependent epithelial signals, yet to be identified, shape local myelopoiesis.

The primary virulence determinant of ETBF is bft.[160] Bft is a metalloprotease that upon binding to a putative colonic epithelial cell receptor triggers cleavage of E-cadherin, thereby releasing E-cadherin-associated β-catenin and consequently activating Wnt (targeting c-Myc and resulting in epithelial proliferation)[61] and, separately, NF-κB (targeting proinflammatory mediators)[51] signaling pathways. BFT-induced production of IL-8 by human colonic epithelial cells, a NF-κB-dependent factor, and its murine homologue, CXCL1, can direct MDSC recruitment.[158,161] Herein, we demonstrated that ETBF has the capacity to shape the inflammatory environment differently in $CEC^{Apc/-}$ versus WT epithelia (no Apc deletion but intact BFT activity), resulting in a specific immune signature observable in Min colon tissue. In contrast, no sustained accumulation of MO-MDSC was evident in WT mice.

Select bacteria and their virulence factors can shape the colonic mucosal immune response, resulting in immune signatures defined by distinct proinflammatory gene expression profiles and the presence of specific immune cell subsets. Fusobacterium nucleatum which is enriched in a subset of colorectal carcinoma tissue samples was suggested to accelerate colon tumorigenesis in Min mice via the polarization of the

myeloid compartment in absence of histologic colitis and Th17 activation.[22] Similarly, *pks+ E. coli* strains were shown to promote colon tumorigenesis in the context of experimentally-induced colitis via colibactin, encoded in the pks island with the ability to trigger DNA-damage.[20] Our findings herein, together with recently published data, collectively propose that the TME associated with ETBF colon tumors likely results from the combined action of BFT, IL-17 and a permissive genetic background ($CEC^{Apc-/-}$).[19,65,71,162] It will be critical to investigate IL-17R-expressing tumor CEC under the influence of IL-17 and BFT, and identify mediators capable of polarizing the myeloid compartment towards protumoral MDSC (Figure 2.7).

How and whether MDSC, in particular MO-MDSC, contribute to ETBF-driven colon tumorigenesis remains unknown. We report here that colon tumor-infiltrating $iNOS^{high}$ MO-MDSC suppressed antigen-specific $CD8^+$ T cell responses in vitro, suggesting that the selective accumulation of immunosuppressive MO-MDSC in ETBF-colonized Min mice may account, at least in part, for the development of gross colon tumors. While in vitro inhibition of T cell proliferation is considered a poor surrogate of in vivo immunosuppression, the concomitant gene expression profiling of MO-MDSC, displaying a strong upregulation of inhibitory metabolic enzymes (Arg1, Nos2 and Ido), immune checkpoint ligands (PD-L1) and soluble mediators (Il10 and Tgfb1) among other genes supports our conclusion that tumor-infiltrating MO-MDSC are potent suppressors of anti-tumor immunity. Furthermore, in the context of intestinal chronic inflammation, by inhibiting Th1 responses and producing high

levels of IL-1, IL-6 (not shown) and IL-23 (Figure 2.4C), MDSC in the ETBF TME may contribute to the recruitment of Th17 and amplify IL-17-driven ETBF oncogenesis, while, simultaneously, contributing to the promotion of tumors by enabling an environment permissive for tumor growth and angiogenesis.[158] This agrees with recent findings that IMC are capable of promoting carcinogenesis via mechanisms other than T cell inhibition.[163] Furthermore, it supports our observation that ETBF-associated MO-MDSC were immunosuppressive in vitro even though they did so at fairly modest levels (approximately 40% inhibition of proliferation) compared to suppression levels of 80-90% typically reported for MDSC.[164]

Human and murine observations suggest that long term carriage of ETBF with subclinical colon inflammation may have oncogenic consequences and support the notion that ETBF is a novel candidate for initiation/promotion of human colon carcinogenesis. We show herein that a specific IL-17$^+$ MO-MDSC-dominant immune signature is associated with ETBF tumorigenesis. In humans, we postulate that identification of similar immune signatures in ETBF-infected individuals may be predictive of malignant transformation. Longitudinal studies are required to assess if the use of cellular and molecular immune signatures as a biomarker holds promise of identifying individuals at risk for developing cancer, and for whom early eradication of ETBF could possibly prevent oncogenesis.

Chapter 4

Outlook

4.1 Targeting critical signaling pathways in MDSC

The ability of MDSC to suppress anti-tumor immune responses and supply pro-tumoral factors is dependent on a two-step process:

1. Recruitment and

2. activation.

Stat3 and NF-κB are examples of signaling pathways which are critical to both processes and play overlapping roles.[154] Our findings showed that ETBF-driven tumorigenesis in Min mice is characterized by the accumulation of IMC to the tumor

microenvironment and their subsequent activation to MO-MDSC. Thus, we hypothesized that disruption of critical signaling pathways in MDSC may inhibit their function, and reduce their purported oncogenic activity. In the ETBF Min model, tumor numbers in the murine distal colon correlated with the severity of pro-tumoral inflammation.[19] Therefore, we generated BM chimera Min mice with conditional deficiency of either Stat3 or MyD88 myeloid compartment, and challenged the mice with ETBF to trigger colon tumors. MyD88 is a universal adapter protein used by toll-like receptors to activate NF-κB. Unexpectedly, Stat3 or MyD88 knockout BM chimera mice were not protected from tumorigenesis: tumors developed in numbers comparable to WT mice (see Figure 4.1A, B).

Similar experiments were conducted in BM chimera Min mice lacking CSFR3 (encoding the G-CSF receptor). Chung et al. showed that an IL-17-mediated paracrine network induced G-CSF through NF-κB and ERK signaling, leading to IMC mobilization and recruitment to the murine colon tumor micronenvironment.[73] CSFR3$^{-/-}$ mice had fewer circulating and tumor-associated MDSC and exhibited delayed growth of transplanted EL-4 tumors following α-VEGF antibody treatment. Welte et al. reported that mTOR signaling in cancer cells stimulates MDSC accumulation through the regulation of G-CSF.[165] However, we observed no differences in tumor counts between CSFR3$^{-/-}$ and WT BM Min chimera after 12 weeks of ETBF colonization (data not shown).

Hif-1α is another critical regulator of MDSC function: it controls suppressive activity and drives their differentiation to tumor-associated macrophages.[98] We crossed conditional Hif-1α knockout mice to Min mice and subsequently infected them with ETBF. At 12 weeks of colonization, tumor counts revealed no significant differences between knockout and WT groups (see Figure 4.1C). Notably, LysM^{cre} mediated deletion of Hif-1α in the myeloid compartment was more than 80% efficient, suggesting that our observations were not due to insufficient penetrance of the knockout genotype (data not shown).

We performed additional experiments aimed at reducing tumorigenesis in the ETBF Min model, employing established pharmacological tools such as α-Gr-1 antibody treatment or phosphodiesterase-5 (PDE-5) inhibitors to target MDSC. In previous studies, α-Gr-1-mediated depletion of MDSC enhanced the activity of antigen-presenting cells and increased the frequency and activity of NK and T cell effectors in a murine lung cancer model.[166] Sildenafil, a PDE-5 inhibitor, has been reported to down-regulate Arg1 and Nos2 expression, thereby reducing the suppressive machinery of MDSC.[167] In our hands, neither treatment was successful in reducing ETBF Min tumorigenesis (data not shown).

In sum, our experiments showed that single pathway inhibition in MDSC was not sufficient to impact ETBF tumorigenesis. Likely, a combination of robust myelopoiesis and redundant pathways may have counteracted all genetic or pharmacological targeting strategies. With only partial therapeutic efficacy, MDSC were recruited to

the tumor microenvironment and subsequently activated in large enough numbers to allow progression.

4.2 The CXCR2 inflammatory chemokine receptor

Numerous treatment options are being explored for the pharmacological regulation of MDSC (summarized in[154]). A novel approach involves the inhibition of the CXCR2 inflammatory chemokine receptor, a heptahelical G protein-coupled receptor that mediates the biological effect of inflammatory chemokines. The differential expression of inflammatory chemokine receptors can be utilized to delineate MO- and PMN-MDSC (see Figure 6.11). CXCR2 is a key regulator of recruitment and effector responses of Ly6G-expressing neutrophils. Mei et al. proposed a model in which neutrophil-expressed CXCR2 is required for migration into tissues, especially mucosal sites, to downregulate the IL-17/G-CSF axis. Colon epithelial cell express the CXCR2-ligand and chemoattract CXCL5 to promote neutrophil transmigration to the gut, where they regulate granulopoiesis and neutrophil homeostasis through feedback inhibition of IL-17/G-CSF.[168] Importantly, CXCR2 is also expressed by MDSC in tumor-bearing mice.[114,169]

Research by the group of Raymond DuBois showed that COX-2 and the COX-2 derived prostaglandin E_2 (PGE_2), which is potently induced by pathogenic bacte-

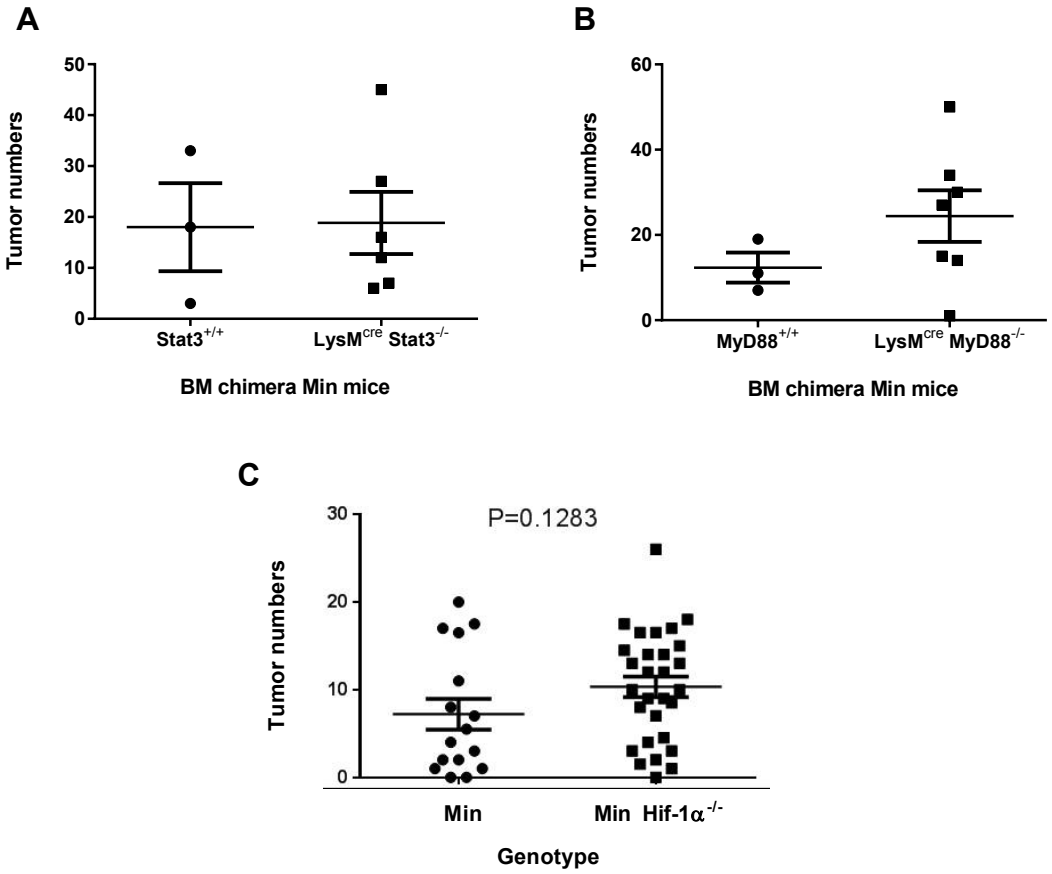

Figure 4.1: Disruption of critical MDSC signaling pathways does not impact tumorigenesis in ETBF Min mice
A, BM from WT or LysM^{cre} Stat3$^{fl/fl}$ donors was transferred to Min recipients to generate BM chimera Min. 6 weeks after transfer, mice were infected with ETBF. 12 weeks later, the colon was harvested for tumor scoring.
B, As above, except with LysM^{cre} MyD88$^{fl/fl}$ donor mice.
C, Tumor numbers in colon of Min or Hif-1α-deficient Min mice 12 weeks after ETBF infection.

ria from the gut flora, can stimulate CRC cells to produce another CXCR2 ligand, CXCL1.[170] Using the AOM/DSS model of colitis-associated cancer, they show that loss of CXCR2 suppresses chronic colonic inflammation and tumorigenesis in a MDSC-dependent manner.[171]

In light of this data we hypothesized that high levels of IL-17, a hallmark of ETBF inflammation, may trigger recruitment of PMN-MDSC to the Min colon via the CXCL chemokine/CXCR2 axis. We confirmed that CXCR2 is expressed in ETBF tumor-associated PMN-MDSC (see Figure 6.10A). We found that the CXCR2-ligands CXCL1, CXCL2 and CXCL5 are all upregulated in distal colon epithelium segments of ETBF versus sham-inoculated mice (Figure 4.2A). Importantly, ETBF-induced CXCL chemokine upregulation was dependent on IL-17 signaling, since ETBF-infected IL-17$^{-/-}$ and IL-17-R$^{-/-}$ mice did not express CXCL1, CXCL2 or CXCL5 (Figure 4.2B).

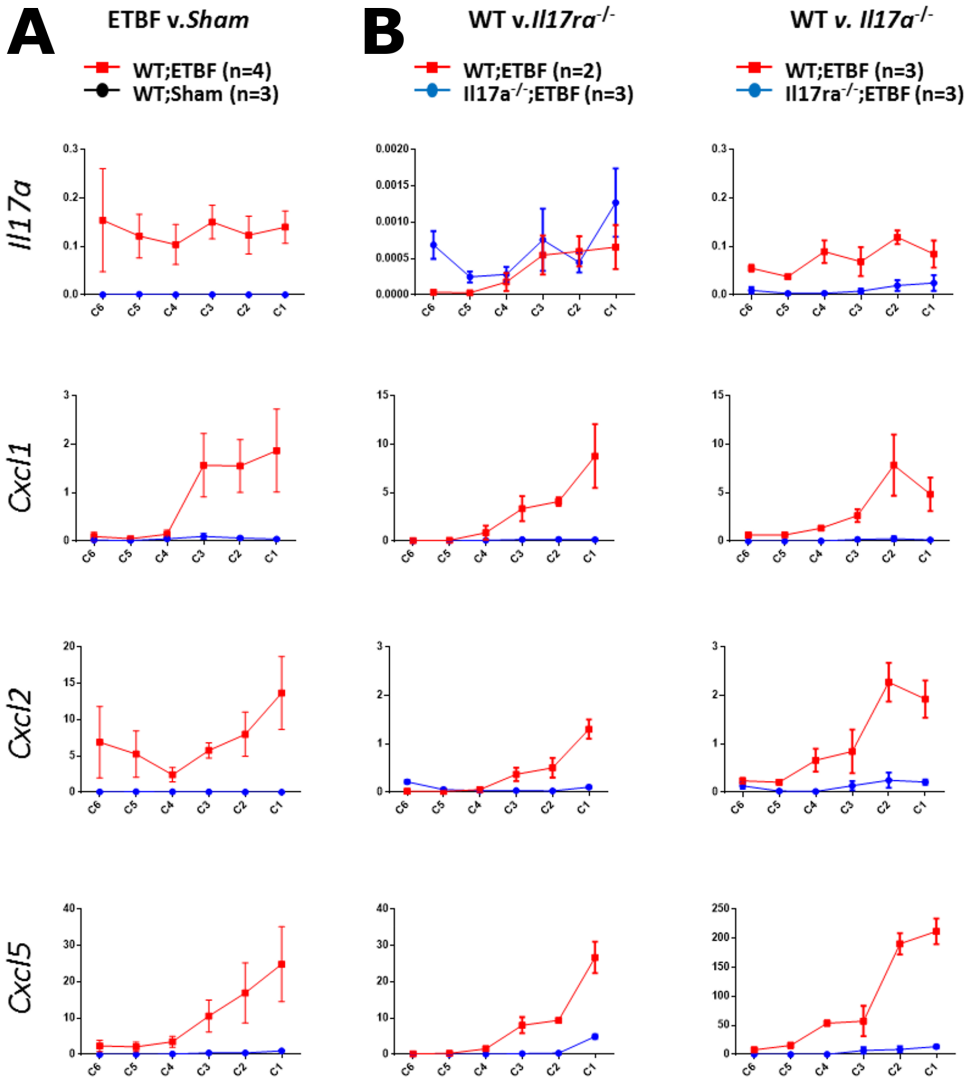

Figure 4.2: ETBF induces expression of the CXCR2 ligands CXCL1, CXCL2 and CXCL6 in distal colon epithelium segments in an IL-17-dependent manner
A, Expression of *Il17a*, *Cxcl1*, *Cxcl2* and *Cxcl5* relative to *Gapdh* in 1 week ETBF-infected WT or sham-inoculated mice. Expression was quantified in individual colon segments (C1 through C6, representing segments from the distal to proximal colon, respectively).
B, As above, except in ETBF-inoculated WT versus IL-17-R$^{-/-}$ (left) and IL-17$^{-/-}$ (right) mice.
Courtesy of Abby Geis and Liam Chung.

4.3 Inhibition of CXCR2 suppresses recruitment of MDSC to the ETBF-inflamed colon and reduces tumorigenesis in Min mice

Next, we sought to characterize ETBF-inflammation in CXCR2$^{-/-}$ mice. As previously reported, knockout of CXCR2 results in potent suppression of neutrophil transmigration to inflammatory sites, i.e. the inflammatory tumor microenvironment, as evident by the reduction of Ly6G^{+} MPO^{+} neutrophils in DMBA/TPA-induced skin papillomas[172] and PMN-MDSC in AOM/DSS-induced colon tumors.[171] We confirmed by flow cytometry that expression of CXCR2 was absent at the surface of distal colon-infiltrating PMN cells from ETBF-infected CXCR2$^{-/-}$ mice (see Figure 6.10B).

As shown in Figure 4.3A, PMN-IMC were significantly reduced in CXCR2$^{-/-}$ mice during acute infection with ETBF (7 days). MO-IMC and macrophages were not affected. In particular, the inflammatory IL-17 response — mediated chiefly by Th17 and $\gamma\delta$ T cells[65] — was not negatively impacted, contrariwise IL-17^{+} trended to be increased in CXCR2$^{-/-}$. This is consistent with reports of Mei et al.,[168] where IL-1β and IL-23-responsive IL-17producing cells were significantly increased in tissues of CXCR2-deficient mice relative to WT.

To extend these findings to the Min mouse model, we chose to use a synthetic peptide inhibitor of CXCR2, pepducin. Pepducins were developed by Kaneider et al. to specifically target the CXCR chemokine receptors, and originally devised to block sepsis in mice.[173] They were also used by Jamieson et al. to illustrate the requirement of CXCR2 for spontaneous tumorigenesis: daily pepducin treatment of Min mice suppressed the spontaneous development of benign intestinal adenomas.[172]

In agreement with our findings in ETBF-infected CXCR2$^{-/-}$ mice, pepducin-treated, ETBF-infected Min mice exhibited significantly reduced PMN-IMC numbers (Figure 4.3B). M1 macrophages, characterized by high surface expression of MHC-II and F4/80, were increased in pepducin-treated mice, although not statistically significant. As in CXCR2$^{-/-}$ mice, IL-17-secreting lymphocytes trended to be increased.

In an attempt to establish whether inhibition of CXCR2 can reduce tumorigenesis in the ETBF Min model, we generated bone marrow chimeras with Min recipients and CXCR2-sufficient or deficient donors. As expected, their characterization revealed that PMN-MDSC were drastically reduced in ETBF-induced colon tumors of CXCR2$^{-/-}$ Min chimera mice (Figure 4.3C). Reaffirming the specificity of the CXCLX:CXCR2 interaction (which occurs primarily at mucosal sites), PMN-MDSC levels in the spleen were comparable between knockout and WT chimera mice. Strikingly, M1 macrophages were again increased in knockout versus WT chimeras (Figure 4.3D). Tumor counts revealed that CXCR2$^{-/-}$ mice had significantly fewer colon tumors after longterm ETBF colonization (12 weeks, Figure 4.3E), despite equivalent

levels of *Il17* mRNA in distal colon tissue samples (data not shown).

Thus, in contrast to the disruption of signaling pathways in MDSC, which remained largely unsuccessful in abrogating tumorigenesis, inhibition of MDSC recruitment to the distal colon succeeded. This is a significant observation for the following reasons:

- Of numerous transgenic strains on a Min background, only IL-17A, IL-17R and Stat3 knockouts have been protected from ETBF-induced tumorigenesis. The CXCR2$^{-/-}$ mouse now joins these strains, demonstrating that it is a strong modifier of pro-tumoral MDSC function.

- As we described in Chapter 2, MO-MDSC emerged as the *distinct* myeloid population associated with ETBF-triggered colon tumors. In contrast, both sporadic and ETBF-induced Min colon tumors were highly infiltrated by PMN-MDSC (Figure 2.3), which upregulated S100A8/9 and Mmp9 as compared to MO-MDSC (Figure 6.4). However, MO-MDSC do not express CXCR2 and are not numerically reduced in CXCR2$^{-/-}$ mice. Thus, our findings that reduction of PMN-MDSC correlates with tumor suppression must be reconciled therewith. Notably, the mechanism via which inhibition of PMN-MDSC is anti-tumoral is poorly understood. Perhaps recruitment of PMN is a common tumorigenic mechanism in both inflammation-induced and sporadic tumors, while MO may act as a specific pro-tumoral modifier (tumorigenesis is markedly accelerated by ETBF). Further studies are necessary to explore whether inhibition of CCR2, a

chemokine receptor expressed predominantly by MO-MDSC (see Figure 6.11), can achieve tumor reduction.

- M1 macrophages were increased across all models of CXCR2 inhibition. Phenotypically, they resembled inflammatory macrophages, which also occurred in high numbers in sporadic Min colon tumors. We may speculate that the $CXCR2^{-/-}$ anti-tumor effect is achieved in part through a modified myeloid immune infiltrate, characterized by mature macrophages exhibiting an M1 phenotype.

- We observed that CXCR2 ligands are upregulated in distal colon segments, with increasing expression from proximal to distal (Figure 4.2). By comparison, IL-17 levels are consistently elevated throughout the colon, mirroring the uniform colonization of ETBF in the entire colon mucosa.[64] However, tumorigenesis occurs predominantly in distal segments. Future studies may provide insight into whether a CXCL chemokine gradient precludes tumor localization.

- Experiments to address whether pepducin administered to longterm ETBF-colonized Min mice may reduce tumorigenesis are ongoing.

Our data supports the notion that CXCR2 is a leading mechanism for the recruitment of MDSC to tumor sites, and its inhibition may prove to be a powerful tool to study MDSC biology. The ability to selectively target specific myeloid populations by excluding their migration to the tumor microenvironment will enable deeper under-

standing of their contribution to tumorigenesis. Lastly, pharmacological development of MDSC-specific chemokine receptor inhibitors for clinical use may pave the way for a novel modality of immunotherapy.

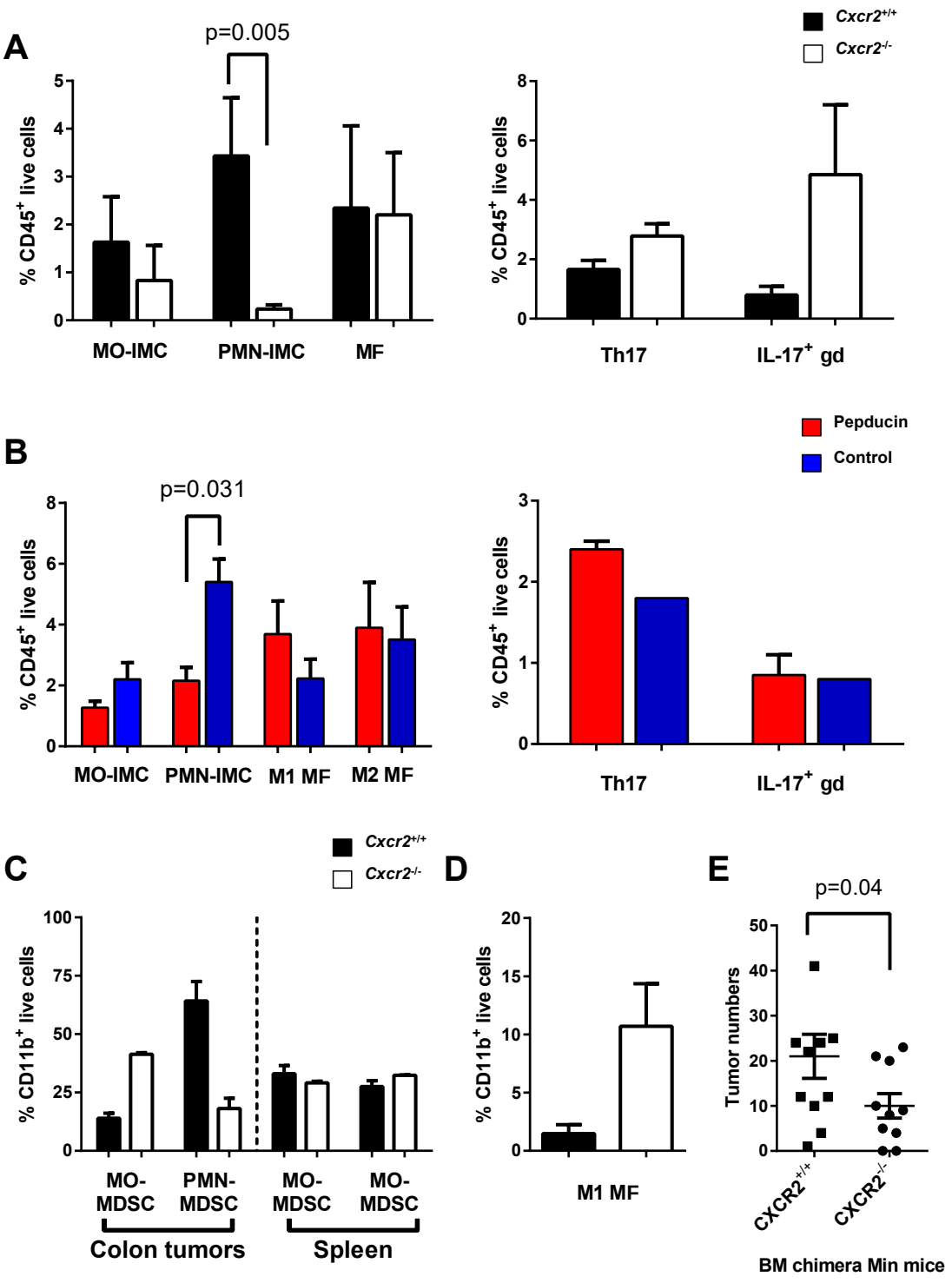

Figure 4.3: Inhibition of CXCR2 suppresses recruitment of MDSC to the ETBF-inflamed colon and reduces tumorigenesis in Min mice

Continued on next page.

Figure 4.3: Inhibition of CXCR2 suppresses recruitment of MDSC to the ETBF-inflamed colon and reduces tumorigenesis in Min mice
A, Relative abundance of myeloid and lymphoid populations in distal colon of 1 week ETBF-colonized WT or CXCR2-deficient mice. MO-IMC, PMN-IMC, MΦ, Th17 and $\gamma\delta$ T cells are shown in percentage relative to $CD45^+$ leukocytes.
B, As above, except in ETBF-inoculated Min mice that received pepducin, a CXCR2 antagonist, or control peptide. M1 and M2 MΦ are characterized by high or low F4/80 and MHC-II expression, respectively.
C, Recovery of MDSC populations in colon tumors or spleen of longterm ETBF-colonized $CXCR2^{+/+}$ or $CXCR2^{-/-}$ BM chimera Min mice, shown as percent $CD11b^+$ myeloid cells.
D, Proportion of M1 MΦ in colon tumors of $CXCR2^{+/+}$ or $CXCR2^{-/-}$ BM chimera mice as percent $CD11b^+$ myeloid cells.
E, Tumor numbers in longterm ETBF-colonized $CXCR2^{+/+}$ or $CXCR2^{-/-}$ BM chimera Min mice.

Chapter 5

Materials and methods

5.1 Mice and bacteria

C57BL/6 (wildtype, WT), CD45.1 C57BL/6 and Min ($Apc^{\Delta 716/+}$) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) or obtained from Dr. David Huso (Johns Hopkins University) and bred in the vivarium. OT-1 T cell receptor transgenic $RAG^{-/-}$ mice were bred in-house. Mixed bone marrow (BM) chimeric mice were established by intravenous injection of 10^7 BM cells from donor mice into lethally irradiated (900 cGy) recipient mice. Unless otherwise stated, n=2-4 mice were used per experimental group, the colon of each mouse was processed independently and, thus, data reflects measurements in individual mice. Mice were maintained according to protocols approved by the Johns Hopkins University Animal Care and Use Committee. ETBF strain 86-5443-2-2, NTBF strain 9343 pfd340, *Fusobacterium nu-*

cleatum strain 2432 (provided by Brandon Ellis, Johns Hopkins Medical Microbiology Laboratory), *pks+ E. coli* strain NC101 and *E. coli* Δpks strain NC101 (provided by Dr. Christian Jobin, University of Florida) were used in this study. Mice were challenged at 4-6 weeks of age following per os antibiotic pre-treatment (clindamycin and streptomycin) and harvested at the time points described.[19] BM chimeric mice were inoculated 6 weeks after BM engraftment. Fecal samples were cultured periodically post-inoculation to quantify and assure consistent ETBF colonization.

5.2 Leukocyte isolation from gut lamina propria, tumors and spleen

Small intestine and distal colon (Figure 6.1) were cut, washed and enzymatically digested (400 U/ml Liberase and 0.1 mg/ml DNAse1; Roche Diagnostics, Indianapolis, IN). Colon and blood leukocytes were isolated using Percoll gradient separation (GE Healthcare Life Science, Pittsburgh, PA). Splenocytes were isolated from Liberase-treated spleen samples using Lymphoprep density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY).

5.3 Flow cytometry and cell sorting

Single cell suspensions were stained with Live/Dead Yellow (Life Technologies, Grand Island, NY), CD11b-PerCp/Cy5.5 (M1/70, Biolegend, San Diego, CA), I-A/I-E-AF488 (M5/114.15.2, Biolegend), Gr-1-Pacific Orange (RB6-8C5, Life Technologies), F4/80-PE-Cy7 (BM8, Biolegend), CD11c-APC-Cy7 (HL3, BD Biosciences, San Jose, CA), CD117-PE-Cy5 (2B8, Biolegend) and FcϵRI (Mar-1, Biolegend). Ly6-C-Pacific Blue (HK1.4, Biolegend) and Ly6-G-AF647 (1A8, Biolegend) were used in adoptive transfer experiments. For intracellular cytokine staining, cells were stimulated for 4.5 h with 30 nM phorbol 12-myristate 13-acetate and 1 M ionomycin (both eBioscience) in presence of Golgistop (BD). Cells were subsequently stained against cell surface markers (CD4-PerCp/Cy5.5, GK1.5; CD3-AF700, 17A2; CD8-PE/CF594, 53-6.7; all Biolegend) followed by fixation/permeabilization (Cytofix/Cytoperm, BD) and staining using IL-17A-Pacific Blue (TC11-18H10.1, Biolegend). Flow cytometry was performed using a LSRII cytometer (BD) and data was analyzed with FACSDiVa 6.1.3 software (BD). For cell sorting (FACS) experiments, myeloid populations were sorted using an AriaII cytometer (BD).

5.4 Suppression of antigen-specific proliferation

FACS-sorted IMC were co-incubated with CFSE-labelled OT-1 $CD8^+$ T cells at T cell:IMC ratios of 10:1 or 1:1 in the presence of 1 μg/ml $OVA_{SIINFEKL}$ peptide. Irradiated splenocytes were used as source of antigen-presenting cells. Proliferation was measured as dilution of CFSE and assessed by flow cytometry. Inhibition of proliferation was measured as 100 x [1-(% $CFSE^{low}$ OT-1 in co-culture with MDSC / % $CFSE^{low}$ OT-1 alone)].

5.5 Nitric oxide assay

FACS-sorted MO-MDSC were cultured for 16 hours in MEM medium alone or in the presence of recombinant mouse IL-17A (Biolegend) and LPS-B5 (Invivogen, San Diego, CA). Culture supernatants were screened for nitric oxide production using a NO detection kit (Enzo, Farmingdale, NY) as per the manufacturers instructions.

5.6 Adoptive transfer of BM-derived IMC

CD45.1+ BM cells were cultured for 5 days with G-CSF (100 ng/ml), GM-CSF (250 U/ml) and IL-13 (80 ng/ml) (Preprotech, Rocky Hill, NJ). MO-IMC were cell-

sorted as described above and injected into sham Min or ETBF-colonized tumor-bearing Min mice. Cells were recovered from enzymatically digested colon tumors using FACS according to CD45.1, CD11b, Ly6C and Ly6G expression (Figure 6.9).

5.7 Taqman-based PCR array

Expression of 48 target genes by FACS-sorted myeloid populations were measured using Taqman technology-based custom-designed PCR array plates or individual assays following the manufacturers instructions (Life Technologies). Expression of the genes of interest was normalized to Gapdh expression. Results were expressed as ΔCt or $\Delta\Delta$Ct (RQ, relative expression).

5.8 Immunohistochemistry

Serial FFPE tissue sections were deparaffinized and antigens retrieved by incubation in citrate buffer. Slides were stained with anti-iNOS (N-20, sc-651, Santa Cruz Biotechnology, Dallas, TX) or anti-arginase 1 (LS-B4789, LifeSpan BioSciences, Seattle, WA). Poly-HRP conjugated anti-rabbit IgG (PV6119, Leica) was used as secondary. Sections were analyzed on an EcliPSE E800 microscope (Nikon Corporation).

5.9 Cytospin

MO- and PMN-IMC were cell-sorted as described above, fixed in 4% paraformaldehyde and annealed to slides via cytospin (800 rpm for 3 minutes). Slides were subsequently stained with Wright-Giemsa dye to visualize cells. Images were captured as described above.

5.10 Statistical analysis

Data were analyzed using the Student t, ratio-paired t, Mann-Whitney U or chi square tests. Data is presented as mean ± SEM. p values < 0.05 were considered statistically significant.

Chapter 6

Supplemental Figures

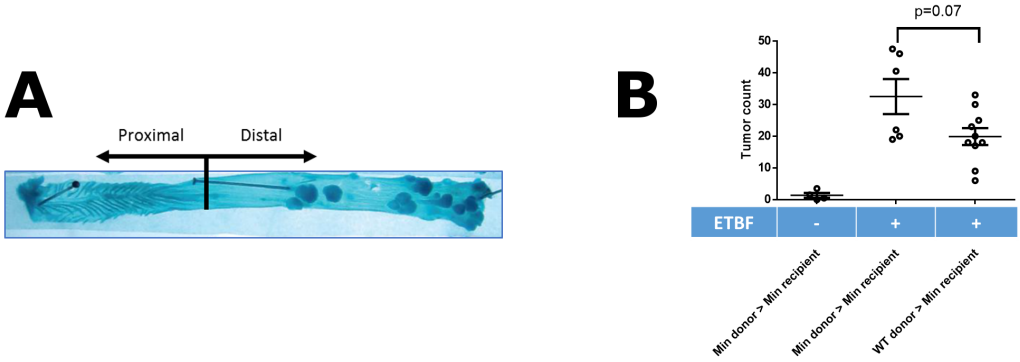

Figure 6.1: ETBF Min mouse colons
A, ETBF-triggered tumorigenesis is prominent in distal colons of Min mice.
B, Colon tumor counts from chimeric Min mice reconstituted with bone marrow from wildtype (C57BL/6) or Min donors. Tumor numbers were assessed at 12 weeks after inoculation with ETBF. Representative image and graph of n=2 or more independent experiments.

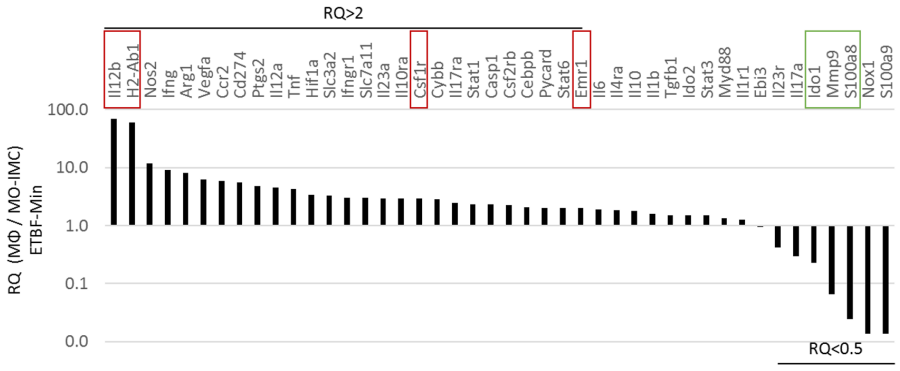

Figure 6.2: Gene expression analysis in ETBF tumor-infiltrating myeloid cells
MΦ and MO-IMC were cell-sorted from 3 month ETBF Min colon tumors as $CD11b^{hi}GR1^{-}MHC^{+}F4/80^{+}$ and $CD11b^{hi}GR1^{lo}MHC^{lo}F4/80^{-}$, respectively. Bars represent fold increase of gene expression (RQ) in MΦ compared to MO-MDSC. RQ greater than 1, genes are overexpressed in tumor-associated macrophages; RQ less than 1, genes are overexpressed in MO-IMC. Genes characterized by RQ greater than 2 and RQ less than 0.5 are highlighted above and below the graph. Red boxes indicate genes characteristic of differentiated MΦ; green boxes indicate genes characteristic of MO-MDSC. Representative graph of n=2 independent experiments.

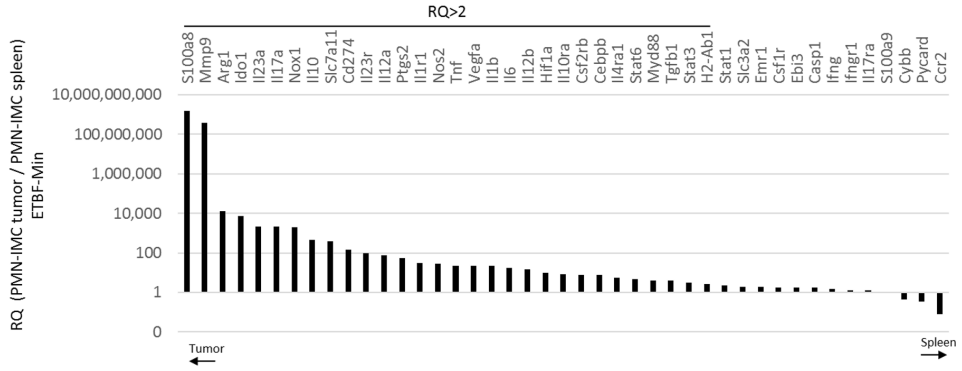

Figure 6.3: Gene expression array in PMN-IMC sorted from colon tumors or spleen of 3 month ETBF Min mice
Bars represent fold increase of gene expression (RQ) in IMC sorted from tumors compared to those sorted from spleen. RQ greater than 1 when genes are overexpressed in tumor IMC; RQ less than 1 when genes are overexpressed in spleen. Genes characterized by RQ greater than 2 are highlighted. Representative graph of n=2 independent experiments.

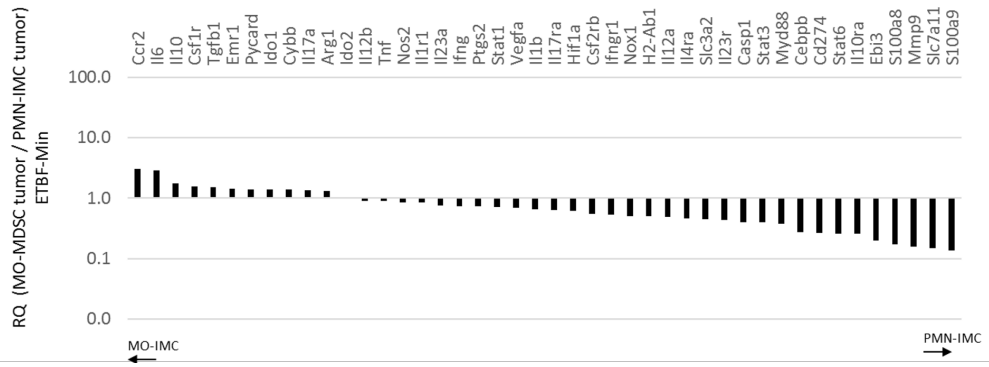

Figure 6.4: Gene expression array intratumoral MO-MDSC to PMN-IMC sorted from colon tumors or spleen of 3 month ETBF Min mice
Same as in Figure 6.3. Representative graph of n=2 independent experiments.

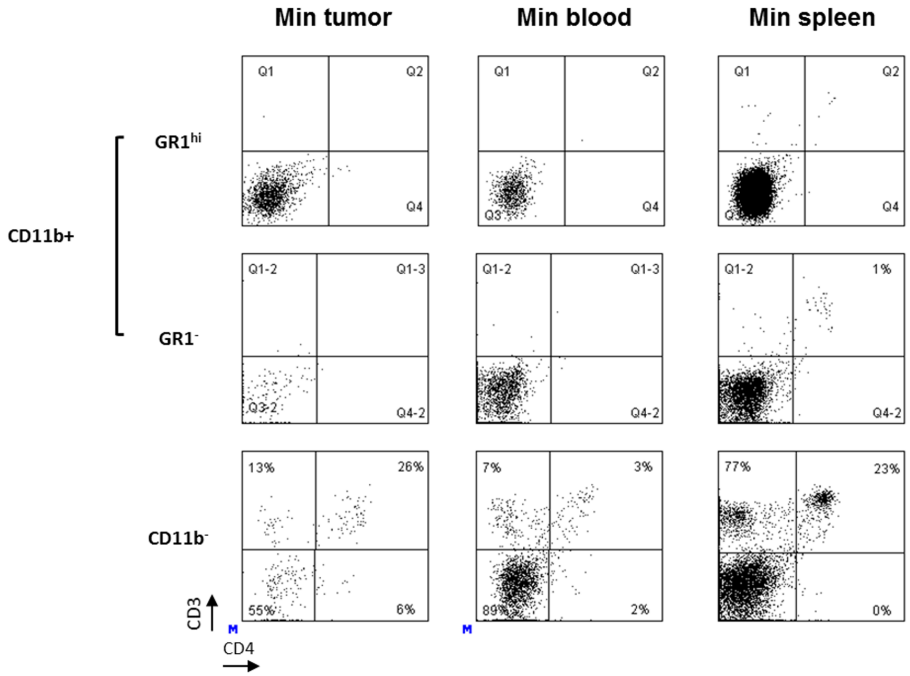

Figure 6.5: Myeloid cell populations ($CD11b^{+}GR1^{hi}$ and $CD11b^{+}GR1^{neg}$) cell-sorted from colon tumors for IL-17 qPCR were not contaminated by T cells

Plots represent pre-sort CD3 and CD4 staining in $CD11b^{+}GR1^{hi}$, $CD11b^{+}GR1^{neg}$ and $CD11b^{-}$ gates used for cell sorting and subsequent *Il17a* mRNA detection in myeloid cells associated with colon tumors, blood or spleen of 3 month ETBF-colonized Min mice. Representative staining of n=2 independent samples from one cell sorting experiment.

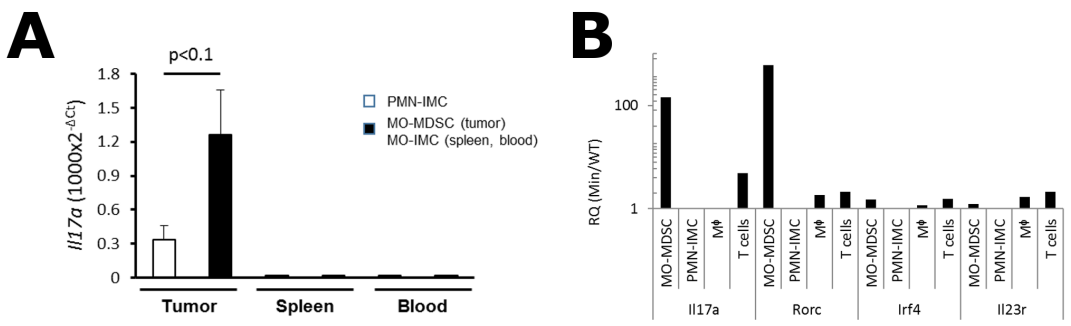

Figure 6.6: Tumor-infiltrating MDSC express *Il17a* gene in colon tumors A, PMN-IMC and MO-MDSC or MO-IMC were sorted from tumors, spleen or blood of 3 month ETBF-colonized Min mice. RNA extracted from each cell subset was assessed by qPCR for Il17a gene expression. Mean SEM is shown. Ct values were normalized with Ct_{Gapdh} ($\Delta Ct = Ct - Ct_{Gapdh}$) and bars represent 2-$\Delta$Ct. Aggregate data of n=2 independent experiments.
B, Myeloid and lymphoid populations were cell-sorted from wildtype or Min distal colon lamina propria at day 7 post-ETBF colonization and assessed for *Il17a*, *Rorc*, *Irf4* and *Il23r* expression by qPCR. Bars represent fold increased (RQ) between Min and wild type cell populations, RQ=2-$\Delta\Delta$Ct. Representative staining of n=2 independent samples from one cell sorting experiment.

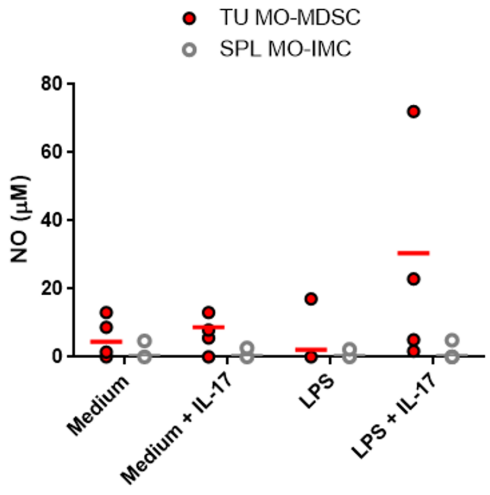

Figure 6.7: Confirmation of Nos2 gene expression by detection of nitric oxide in culture supernatant of rIL-17-conditioned purified MO-MDSC
MO-MDSC cell-sorted from colon tumors or MO-IMC sorted from spleen in ETBF Min mice were incubated overnight with IL-17 (10 ng/ml) in presence or absence of LPS (100ng/ml). Nitric oxide (NO) was measured in culture supernatants using a colorimetric assay. Lines represent geometric mean. Aggregate data from n=3-4 independent experiments.

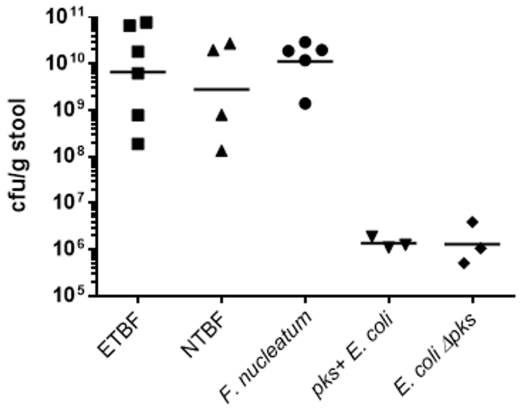

Figure 6.8: Stool culture of oncogenic bacteria to confirm colonization
Fresh stool samples were collected 7 days after inoculation with *F. nucleatum*, *pks+ E. coli*, *E. coli* Δ*pks*, ETBF or NTBF. Samples were homogenized in PBS, serially diluted and cultured on Brucella (*F. nucleatum*), BHI (ETBF, NTBF) or MacConkey (*E. coli*) agar under optimal anaerobic or aerobic conditions. Colony forming units were manually counted within 24-48h of culturing.

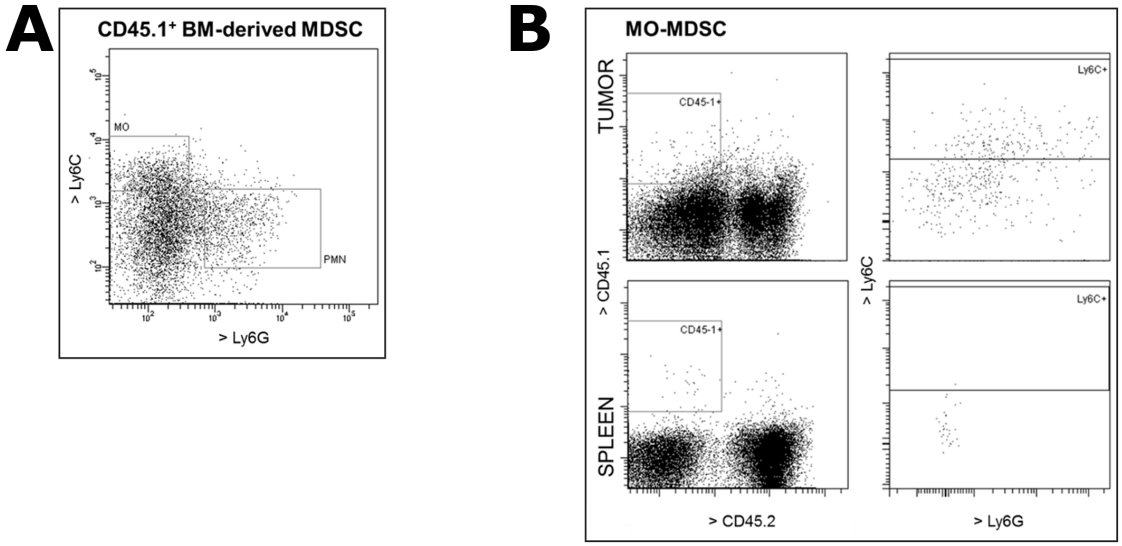

Figure 6.9: Gating strategy for the recovery of adoptively-transferred in vitro derived BM-MDSC

A, CD45.1+ bone marrow cells were harvested and MDSC were derived in vitro by culture with G-CSF, GM-CSF and IL-13 for 5 days. MO-MDSC were cell-sorted and adoptively transferred to Min recipients previously infected with ETBF (ETBF 11 weeks) via tail-vein injection.

B, Colon tumors were harvested 1 week later (ETBF 12 weeks) and CD45.1$^+$ were recovered and sorted by FACS for RNA extraction and Arg1/Nos2 qPCR analysis.

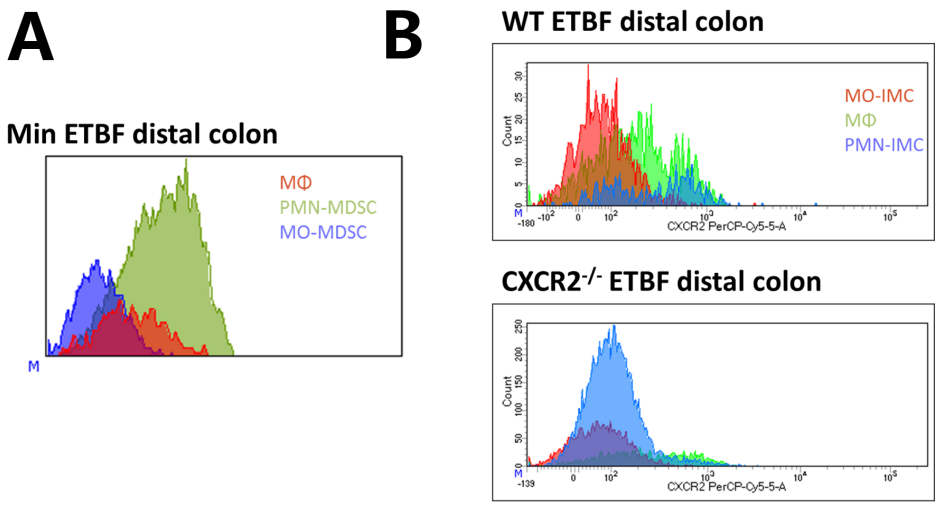

Figure 6.10: CXCR2 is strongly expressed on ETBF tumor-associated PMN-MDSC, while in the knockout staining is nullified
A, CXCR2 expression in $CD11^{+}$ myeloid subsets harvested from the distal colon of 12-week ETBF colonized Min mice.
B, Comparison of CXCR2 expression by flow cytometry on myeloid subsets in 4-week ETBF colonized WT versus $CXCR2^{-/-}$ mice.

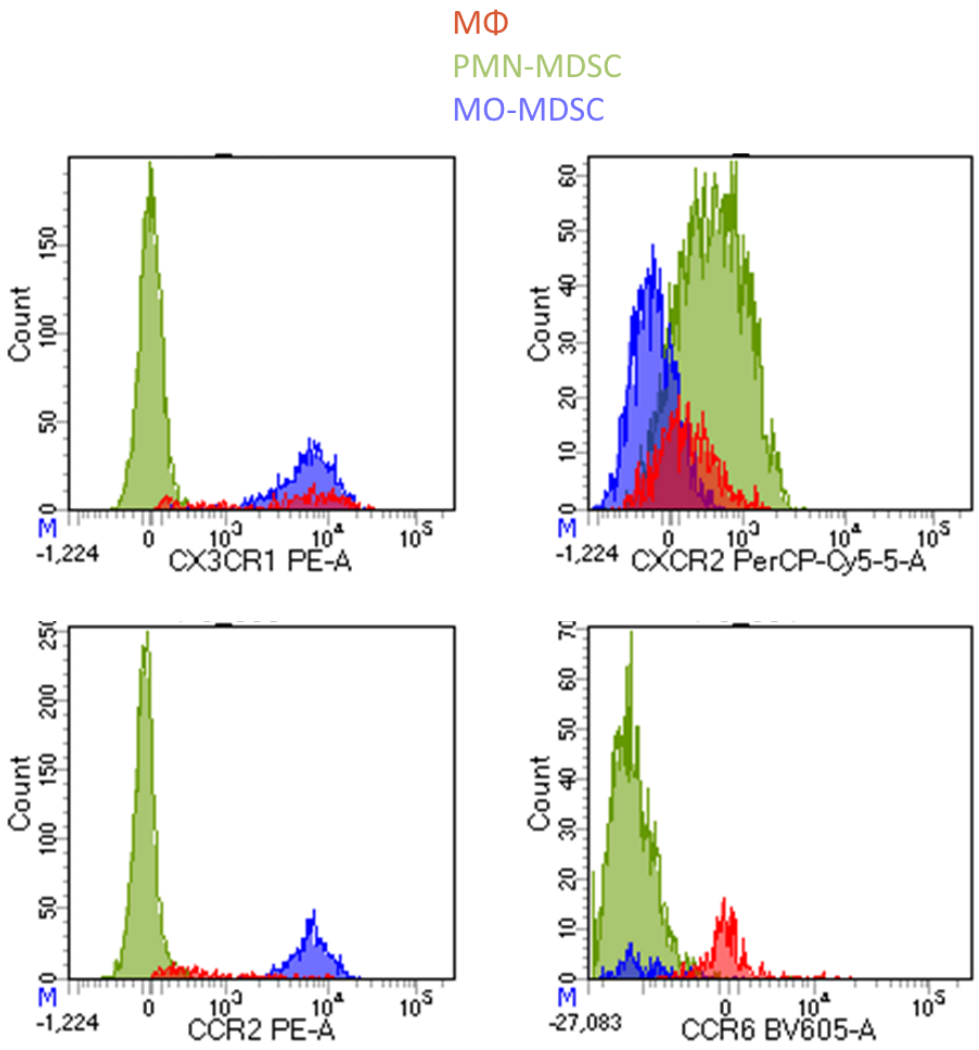

Figure 6.11: Expression of inflammatory cytokine receptors by colonic MDSC

Expression of CX3CR1, CXCR2, CCR2 and CCR6 by $CD11^+$ myeloid subsets in the distal colon of 12-week ETBF-colonized Min mice.

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Vita

Dr. med. univ. Erik Thiele Orberg

6.1 Personal

6.1.1 Address

3538 Greenmount Avenue

Baltimore, Maryland, USA

VITA

Phone: +1 (216) 367-2374

Email: eorberg@jhmi.edu

6.1.2 Personal Details

Gender: Male

Date of birth: 26th August 1985

Place of birth: Boston, USA

6.2 Education

2010–2016 Immunology Graduate Program, *Ph.D.*

Johns Hopkins University, Baltimore, USA

2004–2010 Medical School, *Dr. med. univ.*

Medical University of Vienna, Vienna, Austria

2003 *Reifeprüfung* with distinction

'Vienna Bilingual School' Draschestraße, Vienna, Austria

6.3 PhD Thesis

Title *The Myeloid Immune Signature of Enterotoxigenic Bacteroides fragilis-Induced Colon Tumorigenesis*

VITA

Mentors Dr. Franck Housseau, Dr. Cynthia Sears, Dr. Drew Pardoll

Institute The Sidney Kimmel Comprehensive Cancer Research Center at Johns Hopkins

Description Results Identified a myeloid immune signature characterized by the specific accumulation of protumoral monocytic myeloid-derived suppressor cells (MDSC) in a murine model of spontaneous carcinogenesis induced by Enterotoxigenic *Bacteroides fragilis* (ETBF).

Significance First, inhibition of MDSC trafficking to the colon can abrogate tumorigenesis and may represent a novel immunotherapy. Second, evidence of a malignant myeloid immune signature in patients with ETBF infection may allow for identification of those at risk of developing colorectal cancer.

Skills

- Broad knowledge of cancer immunotherapy
- Management of collaborative research projects
- Experimental design and statistical data analysis
- Characterization of suppressive tumor microenvironments
- Colony management of conditional transgenic mice with complex breeding schemes
- Primary culture of tumor infiltrating lymphocytes and myeloid cells
- Multiparameter flow cytometry and cell sorting

- Extraction, isolation and quantification of DNA & RNA

6.4 Publications

6.4.1 Scientific Articles

Oct 2015 Thiele Orberg E, Fan H, Tam A, Dejea C, Fathi P, Destefano-Shields C, Wu S, Wu X, Chung L, Finard, B, Ganguly S, Fu J, Pardoll DM, Sears CL, F Housseau. "The myeloid immune signature of Enterotoxigenic *Bacteroides fragilis*-induced murine colon tumorigenesis." *Mucosal Immunology*, in press.

Dec 2013 Llosa NJ, Geis AL, Thiele Orberg E, Housseau F. "Interleukin-17 and type 17 helper T cells in cancer management and research." *ImmunoTargets and Therapy*, 2014(3):39-54.

Sep 2009 Thiele Orberg E, Asklin J, Wizel B, von Gabain A. "Impact of Immunization Routes on Adjuvant-Induced T-Cell Immunity to Model Pathogen-Derived Antigens." *Med. Univ. Vienna Dipl.-Arb.*, 2009.

6.4.2 Presentations and Posters

Jan 2016 Thiele Orberg E, Chung L, Fan H, Tam A, Wu S, Wu X, Ganguly S, Fathi P, Dejea C, Pardoll DM, Sears CL, Housseau F. "The myeloid immune signature of Enterotoxigenic *Bacteroides fragilis*-induced colon tumorigenesis."

Keystone Symposium on Cancer Immunotherapy: Immunity and Immunosuppression Meet Targeted Therapies, Vancouver, Canada. Oral short talk and poster.

Sep 2015 Thiele Orberg E, Fan H, Tam A, Wu S, Wu X, Ganguly S, Pardoll DM, Sears CL, Housseau F. "The myeloid immune signature of Enterotoxigenic *Bacteroides fragilis*-induced colon tumorigenesis." *18th ECCO – 40th ESMO European Cancer Congress*, Vienna, Austria. Oral short talk.

Mar 2014 Thiele Orberg E, Fan H, Wick E, Wu S, Pardoll DM, Sears CL, Housseau F. "Immature myeloid cells as a link between inflammation and tumorigenesis in bacterial colitis-associated carcinogenesis." *Keystone Symposium on Immune Evolution in Cancer*, Whistler, Canada. Oral short talk and poster.

Feb 2013 Thiele Orberg E, Sears CL, Wick E, Pardoll DM, Housseau F. "Hif-1α and the regulation of pro-tumoral function of MDSC in colitis-induced colon cancer." *Keystone Symposium on Myeloid Cells: Regulation and Inflammation*, Keystone, USA. Oral short talk and poster.

6.5 Professional experience

6.5.1 Academic Experience

Jan–Mar 2010 *Visiting student*

Karolinska Institutet, Stockholm, Sweden

Mentorship Dr. Birgitta Henriques Normark, Dr. Staffan Normark

Institute Swedish Institute for Infectious Disease Control

Description Involved in a project analyzing *Pneumoccocus* patient isolates matched with clinical data to identify novel virulence factors.

Skills Culture of *Streptococcus pneumoniae* from clinical samples, DNA isolation, amplification and multilocus sequence typing.

Feb–Nov 2008 *Diploma thesis student*

Intercell (Valneva), Vienna, Austria

Title *Impact of Immunization Routes on Adjuvant-Induced T-Cell Immunity to Model Pathogen-Derived Antigens*

Mentorship Dr. Johanna Asklin, Dr. Benjamin Wizel, Dr. Alexander von Gabain

Description Evaluation of the impact of different routes of immunization on protective T-cell immunity. Immunized mice (intranasal, intraperitoneal,

oral or subcutaneous) with antigens from a mucosal pathogen, *Helicobacter pylori*, and from a systemic pathogen, *Borrelia burgdorferi*, together with Intercell's type 1 immunity-stimulating adjuvant IC31. Immunization route and administration of IC31 were major determinants for the induction of $CD4^+$ T-cells and protection.

Skills Independent experimental work employing ELISPOT and ELISA to quantify T-cell responses. Murine immunization and bacterial challenge studies. Expertise in microbiology of *Borrelia burgdorferi* and *Helicobacter pylori*. Extensively involved in initial lab setup.

6.5.2 Clinical Experience

Oct–Dec 2009 *Erasmus exchange student*

Université de Strasbourg, Faculté de Médicine, Straßburg, France

Rotations in gynacology, neurology and pediatrics.

Aug 2009 *Elective rotation student*

University of Illinois at Chicago, Division of Transplant Surgery, Chicago, USA

Elective rotation in surgery (transplantation).

July 2009 *Medical student clerkship*

Allgemeines Krankenhaus der Stadt Vienna, Vienna, Austria

Rotation in internal medicine (emergency medicine).

VITA

Feb 2009 *Medical student clerkship*

Landeskrankenhaus Klagenfurt, Klagenfurt, Austria

Clerkship in pathology.

Aug 2007 *Medical student clerkship*

Allgemeines Krankenhaus der Stadt Vienna, Vienna, Austria

Rotation in internal medicine (bone marrow transplantation).

July 2007 *Medical student clerkship*

Unfallkrankenhaus Linz, Linz, Austria

Clerkship in primary care.

July 2006 *Medical student clerkship*

Sozialmedizinisches Zentrum Ost der Stadt Vienna, Vienna, Austria

Rotation in internal medicine (oncology).

Feb 2006 *Medical student clerkship*

Sozialmedizinisches Zentrum Ost der Stadt Vienna, Vienna, Austria

Elective clerkship in trauma surgery.

6.6 Teaching

6.6.1 Graduate Teaching

Jan 2015 *Teaching assistant*

Johns Hopkins School of Medicine, Baltimore, USA

Course Genes to Society: Immunology

Course director Dr. Jonathan Schneck

Description Intensive immunology course designed for medical students. In afternoon small groups, students presented and discussed problem sets.

Skills Small group leader.

Feb–May 2012–2015 *Teaching assistant*

Johns Hopkins School of Medicine, Baltimore, USA

Course Graduate Immunology

Course director Dr. Scheherazade Sadegh-Nasseri (2012–2014), Dr. Mark Soloski (2015)

Description Semester-long advanced immunology course consisting of lectures and seminars. The seminars were designed to promote learning of class topics by discussing original articles relevant to the week's lectures.

Skills Small group leader, preparation and presentation of class materials, conducting journal club discussions as well as writing and grading of midterm and final exams.

Oct 2013–2014 *Teaching assistant*

Johns Hopkins School of Medicine, Baltimore, USA

Course Scientific Foundations of Medicine: Cell Physiology

Course director Dr. Erika Matunis

Description Intensive physiology course designed for medical students. Course objectives included fundamentals in physiology as well as experimental methods that have enabled the advancement of medical knowledge.

Skills Small group leader.

6.6.2 Undergraduate Teaching

Aug–Nov 2015 *Instructor*

Johns Hopkins University, Baltimore, USA

Course Hopkins Engineering Applications & Research Teaching Tutorials: Immunoengineering

Course director Course was led by myself with mentoring assistance from Dr. Jonathan Schneck

Description Development of a course on 'Immunoengineering', a nascent field marrying medicine, immunology and engineering. In this three part course, basics in immunology were introduced first, followed by applications in vaccine technology as well as cancer immunology. This course was well received by the student, as evident by a overwhelmingly positive online evaluation.

Skills

- Syllabus design
- Drafting accurate learning objectives
- Preparation of lectures, both traditional format and online ('flipped classroom')
- Rational choice of teaching methods and assessment modalities
- Moderation of 'active learning' activities

Jan 2009 *Teaching assistant*

Medical University of Vienna, Vienna, Austria

Course Krankheit, Manifestation und Wahrnehmung, Allgemeine Arzneimitteltherapie

Course director Dr. Alexander Hirschl

Description The laboratory practice in microbiology teaches medical students about laboratory practices for handling and diagnostics of pathogens.

Skills Responsible for introducing students to the concept of handling, culturing, staining and identifying bacteria, as well as microscopy. Lecturer on virological assays.

6.7 Merits

6.7.1 Fellowships

July 2015 *Teaching as Research Fellows Program*

Johns Hopkins University, Baltimore, USA

Fellowship awarded to instructors to promote scientific methods in instruction and evaluate in-class research projects regarding learning success.

2013–2015 *Preparing Future Faculty Certificate Program*

Johns Hopkins University, Baltimore, USA

The Preparing Future Faculty Program (PFF) recruits doctoral students pursuing academic careers. PFF fellows are trained in the basics of pedagogics and effective instruction methods, with the aim of meeting the future demands of modern university teaching.

6.7.2 Academic Awards

2013 & 2015 *Graduate Student Association Travel Award*

The Johns Hopkins School of Medicine

2013 *Best Poster Award*

The 12th Annual Immunology Training Program Retreat

2009 *Diploma thesis defense with distinction*

Medical University of Vienna

2005-2008 *Baxter Scholarship*

The Baxter International Foundation

6.8 Leadership Skills

2012–2014 *Student group president*

The Hopkins Marathon TeamBaltimore, USA

Description President and captain of the Hopkins Marathon Team, a university-wide student organization dedicated to promoting health and fitness on campus through long distance running.

Skills

- Homepage design
- Author of a weekly newsletter reaching over 400 campus subscribers

- Liasing with various student governing bodies on an all Johns Hopkins campus
- Writing of grant applications and lobbying for funds
- Editor of various recruitment and advertising campaigns (Highlight: Ads on Johns Hopkins shuttle busses)
- Coaching of marathon training groups

2012, 2015 *Student coordinator*

Johns Hopkins Immunology ForumBaltimore, USA

Responsible for inviting and hosting prominent reseachers to present at the Immunology Forum, a weekly seminar on recent advances in immunology.

2012–2014 *Student representative*

Johns Hopkins School of Medicine Graduate Student AssociationBaltimore, USA

Representative of the of the Immunology Graduate Program student body to the Graduate Student Association

2012 *Recruitment coordinator*

Johns Hopkins Immunology Graduate ProgramBaltimore, USA

Reponsible for organizing recruitment activities for students applying to the Immunology Graduate Program.

Updated on May 26, 2016.

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