



Fusobacterium-Stromal Cell Interactions in Colorectal Cancer

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Fusobacterium-Stromal Cell Interactions in Colorectal Cancer

A thesis presented

by

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to

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in fulfillment of the requirements

for the degree of

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Abstract

Colorectal cancer (CRC) is the second most common cause of cancer related deaths among adults within the United States. The complexity of the CRC tumor microenvironment (TME), comprising immune cells and stromal cells, such as cancer-associated fibroblasts (CAFs), and microbiota, further complicate the challenge in developing effective therapeutic targets. *Fusobacterium*, a Gram-negative, anaerobic oral microbe that has been shown to invade human gingival fibroblasts and promote a pro-inflammatory environment in the mouth, has also been associated with worsened CRC patient prognosis, specifically in CRC patients displaying a stromal cell-rich microenvironment. Additionally, substantial evidence has established a positive correlation between *Fusobacterium*, stromal cells, and immune dysregulation during CRC development. Thus, understanding the multifactorial components of the CRC TME may reveal potential therapeutic targets. We hypothesized, thus, that *Fusobacterium* within the CRC TME similarly play a pivotal role in inducing a pro-inflammatory environment through its interactions with CAFs which exacerbates CRC development.

We sought to investigate the interactions between *Fusobacterium species (spp.)* and fibroblasts by first developing optimized techniques for indirect immunofluorescence microscopy-based detection of *Fusobacterium* on Formalin-Fixed, Paraffin-Embedded (FFPE) sections. This was an important initial step, as *Fusobacterium spp.* have not been visually presented in FFPE of mouse colons within published works to date, however successful detection of *Fusobacterium spp.* within the colon allows us to further localize *Fusobacterium* in the gut and further evaluate its impact on normal tissue and in conditions of colonic pathology. In these experiments, bacterial cultures of *Fusobacterium spp.* (*Fn7-1*, *Fn7-3*, and *Fn25586*) were each

incubated with resected colons of Germ-Free (GF) mice, which are completely devoid of microorganisms, to assess and ensure the validation of our antibody detection. *Fusobacterium spp.* presence was assessed using primary antibodies against each species, and a fluorescent secondary antibody specific to the host of the primary antibody.

After successfully detecting and optimizing the detection of *Fusobacterium* on resected colons of GF mice, we used the optimized techniques to assess *Fusobacterium* localization in the colons of Altered-Schaedler Flora (ASF) mice following oral inoculation. These mice contain a well-defined, limited consortia of eight, non-pathogenic microorganisms, typically employed for the controlled study of physiologically relevant microorganisms and their impacts on a host. In assessment of the potential influence of *Fusobacterium* and colonic fibroblasts, we assessed the expression of CAF markers within the colons of *Fusobacterium*-inoculated ASF mice, which revealed a correlation between the presence of *Fusobacterium* and upregulated expression of two CAF markers, namely platelet-derived growth factor receptor alpha (PDGFR)- α and alpha smooth muscle actin (α SMA). This suggested that the presence of *Fusobacterium* may have influence on the transition between normal fibroblasts to a more CAF-like phenotype, validated by the upregulated expression of CAF-specific markers.

Next, we sought to investigate these findings in Specific Pathogen-Free (SPF) mice, which contain a conventional microbiota composition devoid of a specific pathogen. A time-point experiment tracking the abundance of orally inoculated *Fusobacterium* showed that *Fusobacterium* abundance falls below the threshold of detection after 24-hours, and therefore does not stably colonize the colons of these mice for further investigation. Due to this result, we sought to implement *in vitro* experiments consisting of isolated mouse colonic fibroblasts in coculture with *Fusobacterium* or microbially associated elements. The *in vitro* co-culture studies

revealed increases in interleukin (*Il*)-6, and *Pdgfr- α* . An increase in fibroblast secretion of IL-6 was also observed, further supporting our hypothesis that *Fusobacterium* induces a pro-inflammatory phenotype.

We next investigated whether *Fusobacterium* presence may further promote a pro-inflammatory microenvironment within a mouse tumor model. SPF mice were orthotopically-injected with organoids harboring mutations in KRAS, p53, and NOTCH, which mimic a stromal-cell rich TME and result in primary tumor formation and metastasis to the liver. Mice were then tail-vein intravenously inoculated with *Fusobacterium*, and *Fusobacterium* presence within tumors was validated by qPCR. Immunofluorescence imaging on mouse colon tumors of *Fusobacterium*-inoculated mice revealed upregulation of CAF marker expression when compared to non-inoculated mice. Through immunofluorescence imaging, RNA expression analysis, *in vitro* coculture, and *in vivo* inoculation studies, this thesis aims to provide a deeper analysis of *Fusobacterium* and its potential impact on inducing a pro-inflammatory environment through its interaction with colon and colonic fibroblasts.

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Chapter 1: Background

Colorectal cancer (CRC) is the second leading cause of cancer deaths worldwide, and the global burden of new CRC diagnoses is estimated to surge to 3.2 million by 2040 (compared to a reported 1.93 million in 2020)¹. CRC emerges from normal epithelium of the colon, where initial stages consist of increasing degrees of benign growth, known as early and late adenomas, before reaching its final stage as a malignant adenocarcinoma² (Figure 1).

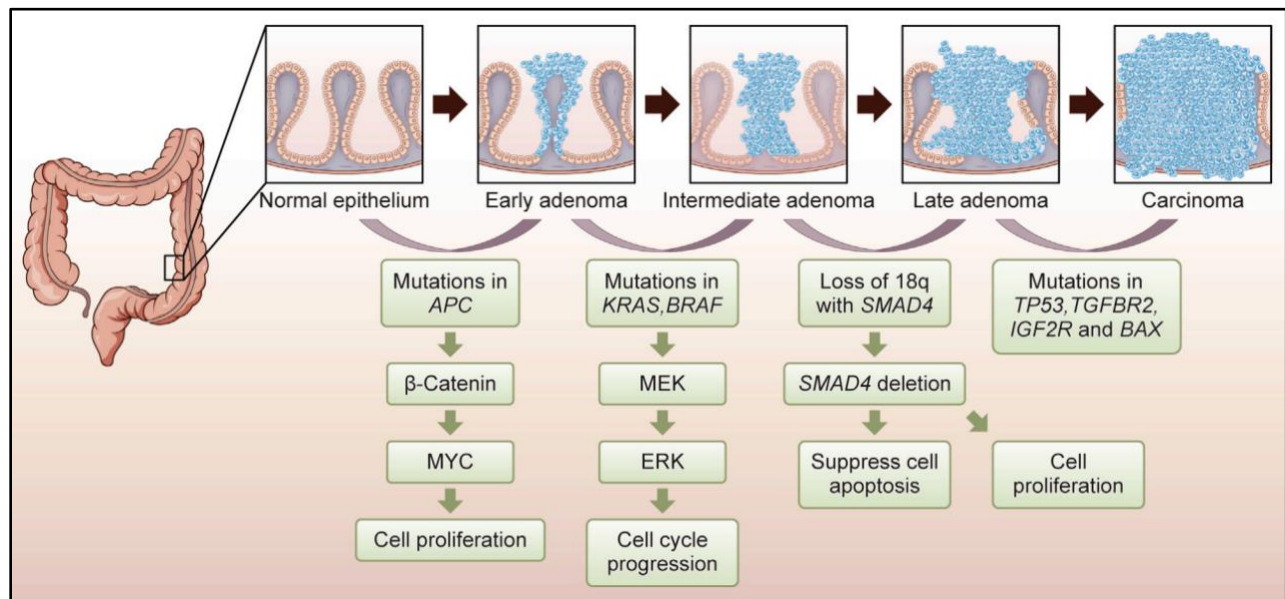


Figure 1: A simplified model of the adenoma-to-carcinoma sequence in CRC tumor progression. Tumor growth extends from normal epithelium of the colon and progresses into increasingly growing stages associated with acquired mutations that drive pathogenesis. From normal epithelium, acquired inactivating mutations in *APC* lead to the formation of an early adenoma. Mutations in *KRAS* and *BRAF* lead to enhanced cell cycle progression, resulting in the intermediate adenoma. The late adenoma is characterized by the deletion of *SMAD4*, leading to cell proliferation. Mutations acquired in *TP53*, *TGFBR2*, *IGF2R*, and *BAX* result in the formation of the final carcinoma. From: “Novel Genetic and Epigenetic Biomarkers of Prognostic and Predictive Significance in Stage II/III Colorectal Cancer”. Luo XJ (2021), Molecular Therapy, 9(2), 588.

A major driver of CRC is the occurrence of mutations in the adenomatous polyposis coli (*APC*) gene, leading to the production of truncated, inactivated APC gene products³. Under normal conditions, the *APC* gene acts as a tumor suppressor by negatively regulating the

wingless-related interrogation site (WNT)/ β -catenin signaling pathway. Under homeostasis, this pathway becomes downregulated through inactivation of WNT signaling, a result of the formation of APC association with destruction complexes which degrade β -catenin. Inactivation of the *APC* gene inhibits the formation of these degrading complexes, promoting the signaling of the WNT/ β -catenin pathway, leading to β -catenin accumulation and translocation to the nucleus. This translocation induces cell proliferation and subsequent tumor growth⁴, and acts as an early event in the onset of CRC progression⁵. Following the mutation of the *APC* gene during CRC development is typically a mutation in the oncogene, Kirsten Rat Sarcoma viral oncogene homolog (*KRAS*)⁶. Mutations in the *KRAS* gene, observed in 40% of sporadic CRC cases, leads to its constitutive activation which promotes cell proliferation and the formation of a “late adenoma”. Mutation in the *TP53* gene serves as a later stage critical point in CRC tumor progression, occurring between the transition from adenoma to carcinoma. Under homeostatic conditions, *TP53* serves as a tumor suppressor gene and tight regulator of cell cycle and apoptosis⁷. During CRC, loss-of-function mutations in *TP53* lead to aberrant cell proliferation and subsequent tumor growth⁸.

Patient-specific data has shown that patients diagnosed with CRC often display heterogeneous tumor microenvironments, exhibiting varying phenotypes within their CRC tumor microenvironment (TME)⁹. This observed patient-to-patient heterogeneity of the CRC TME translates to varying responses to therapeutic treatment⁹. This variability has prompted researchers to develop a method for characterizing individual patients' CRC TME based on common characteristics observed to be present among patients, with hopes to better understand the underlying drivers of CRC progression to develop effective treatments¹⁰. A method of CRC TME categorization based on gene expression profiles, known as the Consensus Molecular

Subtyping (CMS) system, encompasses four distinct subtypes of CRC TME characterization to better understand the local environmental and molecular contributors to CRC disease initiation, progression, recurrence, and response to treatment. These subtypes include **CMS1**, accounting for 14% of CRC patients and characterized as a proximal manifestation containing hypermutation, B-Raf proto-oncogene (*BRAF*) mutation, and high immune infiltration; **CMS2**, accounting for 37% of CRC patients and characterized as a distal manifestation containing increased activation in WNT signaling and myelocytomatosis oncogene (MYC) pathways; **CMS3**, accounting for 13% of CRC patients and characterized as right-sided manifestation containing overrepresentation of KRAS mutation; and **CMS4**, accounting for 24% of CRC patients and characterized to contain increased stromal cell infiltration, activation of transforming growth factor (TGF)- β , and the activation of genes related to epithelial-to-mesenchymal transition. For this thesis, I will mainly focus on the phenotypic characteristics of patients exhibiting the CMS4 subtype within their CRC TME.

Patients of the CMS4 subtype are often diagnosed at Stages III and IV, and this late diagnosis contributes to poor patient response to treatment, worsened overall, and worsened relapse-free survival¹⁰. Surgical resection remains a primary modality of treatment for resectable, early-stage CRC¹¹. For CRC deemed non-resectable due to its progression, size, location, or state of metastasis, available therapies include local radiation therapy, systemic chemotherapy, and for some patients immunotherapy¹². Avenues of immunotherapy for CRC that have been explored include adoptive cell transfer therapy, immune checkpoint inhibition, CRC vaccines against tumor neoantigens, and monoclonal antibodies against cytokines and growth factors. For some patients, combinatorial therapy containing multiple modalities of treatment, such as chemotherapy and radiation, may be utilized¹¹. Still, though the availability of these treatments is

widespread, patients may experience complications such as the development of resistance to treatment, as has been observed in over 90% of patients with metastatic CRC^{12,13}. A study modeling CRC cell clone responses to treatment showed that chronic treatment of FOLFOX, a highly effective and commonly administered antimetabolite treatment for CRC, resulted in acquired chemoresistance, which was an effect rescued after the administration of low dose synergistic drug combinations¹⁴. Resistance, however, hinders effective treatment, contributes to the onset of recurrent CRC, and may influence a patient's resistance to other drug treatments¹⁵. In efforts to combat this, emphasis has been placed on the development of novel, target-specific therapeutics including therapy containing monoclonal antibodies against cytokines and anti-angiogenic factors such as vascular endothelial growth factor (VEGF)^{12,16}. Despite these efforts, the potential for toxicity and cancer cell resistance to these treatments pose a risk that requires further investigation through preclinical studies¹¹.

Components of CRC TME:

The CRC TME is comprised of cancer cells, immune cells, epithelial cells, endothelial cells, and stromal cells, each of which can affect a patient's response to therapy and disease progression¹⁷. The tumor stroma consists of both normal *and* cancer-associated fibroblasts (CAFs), mesenchymal stromal cells (MSCs), immune cells, among other tissue-specific cells^{18,19}. These components of the tumor stroma provide cellular signals, from immune cells and fibroblasts, and noncellular immunomodulatory signals, such as IL-6²⁰, IL-8²¹, TGF- β ²², and GM-CSF²³. The composition of the tumor stroma may influence a patient's resistance to therapy in ways such as limiting the accessibility of treatments to tumor cells, degradation of therapeutic agents, and enabling resistance to treatment¹⁸.

The extracellular matrix (ECM) provides structural support to tissue by containing cells and signaling molecules, and becomes replenished by the collagenous secretions of fibroblasts. The basement membrane of the ECM provides an important platform of attachment for epithelial cells through integrin binding; a structure typically maintained during homeostatic conditions²⁴. This platform acts as a barrier between the epithelial cells of tissue from other components of the interstitial ECM, including fibroblasts, while allowing the passage of immune cells¹⁸. In this way, under homeostatic conditions, epithelial cells and fibroblasts typically reside within their respective histological compartments, where fibroblast activation, proliferation, and motility are negatively regulated, and fibroblasts are essentially present to maintain ECM homeostasis through their production of ECM and matrix metalloproteinases (MMPs)²⁵. During malignant tumor formation, however, this basement membrane is breached as the motility of fibroblasts becomes upregulated, allowing for malignant epithelial cell outgrowth and the transmigration of cells, including fibroblasts and MSCs¹⁸. The infiltration of these cells into the epithelial compartment provides immunomodulatory signals, such as secreted pro-inflammatory cytokines, growth factors, and ECM proteins that progressively modify the local microenvironment to promote tumorigenesis¹⁸.

Fibroblasts are a critical component of tissue and tumor stroma¹⁸. Under homeostatic conditions, these cells provide secretions of collagenous fibers needed to replenish the ECM such as collagen and fibronectin, and thus, are integral to the structural support maintained within a given tissue²⁶. In addition to its importance in maintaining structure, fibroblasts also secrete enzymes such as matrix metalloproteinases (MMPs), namely MMP-2 and MMP-9²⁷, which degrade components of the ECM. At homeostasis, the process of controlled tissue remodeling is essential to maintaining normal organ homeostasis by promoting tissue turnover, development,

and wound healing following injury²⁸. In excess and under pathogenic conditions, the elevated expression of MMP-2 and MMP-9 have been associated with poor CRC patient prognosis²⁹, and overexpression of MMP-2 within the tumor stroma has been associated with decreased CRC patient survival, rendering these MMPs as potential biomarkers for CRC^{30,31}. Fibroblasts may also release cytokines and growth factors, such as IL-1- β which can activate and amplify inflammatory immune responses³², IL-6 which can induce inflammation on multiple cells types and has been shown to be important for tumor angiogenesis in CRC^{33,20}, and GM-CSF³⁴, which play an immunomodulatory role in recruiting dendritic cells, macrophages and promoting inflammation.

During wound repair, fibroblasts, and a specific subset of fibroblasts called myofibroblasts³⁵, become activated where they increase proliferation, motility as evidenced by their upregulation of expression of motile/contractile markers, and increased secretion of pro-MMPs in efforts to rapidly clear a wound or site of injury²⁵. Cancer and malignant tumor growth, considered wounds that never heal, similarly result in fibroblast adoption of this myofibroblastic phenotypic signature³⁶⁻³⁸, as evidenced by their increased expression of α SMA and upregulation of motility programs, allowing for fibroblast invasion of the epithelial compartment. In turn, the invasion of fibroblasts promotes malignant tumor growth, progression, and metastasis such as through secretion of ECM proteins to increase tissue stiffness, secretion of IL-6, and by its binding to cancer cell-expressed Neural (N)-cadherin, providing a means for cancer cell motility³⁹. While present within the tumor microenvironment, these cells are termed cancer-associated fibroblasts (CAFs), due to their activation, presence within the tumor, upregulation of proliferation and myofibroblastic genes relating to motility (such as ACTA2), and increase in

their secretion of tumorigenic pro-inflammatory factors such as TGF- β ⁴⁰, VEGF⁴¹, and IL-6²⁰, among many others¹⁸.

Cancer-Associated Fibroblasts:

CAF characterization, presence, and functionality within the CRC TME are essential for research efforts toward developing therapeutic agents against CRC^{17,42}. The TME of patients of CMS4 subtype CRC patient is characterized as containing high stromal infiltration, elevated activation of tumor promoting factors such as TGF- β , and poor patient response to currently available immune checkpoint inhibitor treatments due to high intratumoral heterogeneity⁴³. Despite the contribution of the stroma of the CRC TME on conferring patient resistance/lack of response to treatments, currently available treatments are often developed with a focus on targeting cancer cells, such as immune checkpoint inhibitors which target immune checkpoint molecules that are highly expressed on cancer cells¹². Thus, further study into development of therapeutic agents that mitigate the influence of the tumor stroma on tumor progression may bring forth substantial progress in the development of effective treatments by circumventing issues such as resistance to treatment or lack of response^{17,42,43}, especially for patients who display high stromal infiltration as observed with patients of the CMS4 subtype.

CAFs originate from several different lineages such as tissue-resident normal fibroblasts, epithelial cells, and endothelial cells^{44,45}. Signals derived from within the TME may act on CAF precursors to drive differentiation, such as is the case with TGF- β ²², platelet-derived growth factor (PDGF)⁴⁶, and fibroblast growth factor 2 (FGF2) on tissue-resident normal fibroblasts. Other CAF precursors that may undergo transdifferentiation include epithelial and endothelial cells³⁷, bone marrow-derived mesenchymal stem cells, pericytes⁴⁷, and adipocytes⁴⁸. Recent

experimental characterization methods, including flow cytometric analysis, immunofluorescence staining, in situ hybridization, and single-cell RNA sequencing, have helped researchers to further classify CAFs into three categories based on their phenotypic characteristics: myofibroblastic, inflammatory/immunoregulatory, and antigen-presenting CAFs⁴⁵. These subtypes possess respective phenotypes and functions that are further influenced by the local tissue environment in which they may reside, which was demonstrated after spatial transcriptomics analysis revealed a spatial distribution of CAF subsets and correlation to their function⁴².

Myofibroblastic CAFs (myCAFs) are a subset which are mainly involved in ECM remodeling⁴⁹. Often expressing markers of contractility such as *ACTA2*⁵⁰ (encoding for alpha-smooth muscle actin, α SMA), myCAFs exhibit wound healing properties and remodeling of the ECM, while also promoting cancer growth and metastasis through the secretion of immune factors such as TGF- β ⁴⁹, which acts on myCAFs in a positive feedback loop to induce proliferation and differentiation resulting in the increase in stromal composition, and VEGF, which is expressed highly in precancerous CRC lesions and promotes tumor angiogenesis^{16,51}. Other markers expressed by myCAFs that are relevant to wound healing properties include the expression of periostin (POSTN)⁵², which has been shown to promote CRC tumorigenesis through the secretion of IL-6⁵³, secretion of collagen which provides structure and stiffness to the ECM, and expression of platelet-derived growth factor receptor α/β (PDGFR- α/β)⁵⁴, which plays an important role in the growth and spread of CRC by promoting angiogenesis, invasion, and metastasis⁵⁴. The expression of these markers promotes the differentiation of normal fibroblast to myofibroblast which are highly involved in epithelial cancers as they influence cancer cell invasion and metastasis^{39,49}. myCAF-mediated ECM remodeling within the tumor

stroma is strongly associated with tumor migration and metastatic potential²⁴. Located throughout the tumor stroma and near cancer cells, myCAFs exhibit immunosuppression within the TME through excess production of ECM, increasing the stiffness of the TME and inhibiting the immune cell infiltration within the stroma, as has been shown within lung adenocarcinoma⁵⁵.

Inflammatory/immunoregulatory CAFs (iCAFs), characterized by low α SMA expression and high expression of interleukin (IL)-6 and C-X-C motif chemokine 12 (CXCL12)⁵⁶, are involved in the modulation of inflammation either through the recruitment or suppression of immune cell trafficking into tissue. This modulation is mediated by their expression of pro-inflammatory cytokines such as interleukins (IL) IL-6, known for its pleiotropic activity including driving the differentiation of Th17 cells³³, expression of IL-8, which recruits neutrophils to the site of inflammation²¹, and expression of granulocyte-monocyte colony stimulating factor (GM-CSF), which recruits dendritic cells and macrophages, and promote their differentiation into wound-healing associated macrophages²³. iCAFs secrete chemokines, another important axis for immune cell recruitment, such as CXCL12, a chemoattractant, which binds its receptors CXCR4 on cells such as T cells and monocytes to promote leukocyte homing within tissue⁵⁷. Within the TME, this chemotactic axis can recruit immune cells, such as CD8+ T cells, away from tumor cells and sequestering them into stromal compartments, thereby interfering with their migration and cytotoxic function within the TME⁵⁸. iCAFs are distributed among several compartments of the TME seemingly based on their expression profiles, where CD34+ iCAFs tend to localize to blood and lymphatic structures⁵⁹, C-C motif chemokine ligand 21 (CCL21) expressing iCAFs localize to tertiary lymphoid structures, and iCAFs responsive to interferons localize around tumor cells⁴².

With regards to immunomodulation, there is an inverse relationship between the presence of CAFs and CD8+ T cell-mediated activity within the stroma⁵⁸. CD8+ T cells are an important cell type due to their antigen recognition, trafficking, and cytotoxic capabilities. Antigens that are expressed by cancer cells are taken up by dendritic cells, which then prime and activate T cells and lead to their trafficking (and infiltration) to tumors where they kill cancer cells⁶⁰. In the presence of CAFs, this process is disrupted where the secretion of IL-6 and TGF- β from CAFs results in a reduced cytotoxic T cell response⁵⁸. CAF-derived IL-6 has also been shown to generate immunosuppressive dendritic cells, which reduce the cytotoxicity of CD8+ T cells by reducing their production of interferon (IFN) - γ ⁶¹. CAFs inhibit the trafficking of CD8+ T cells chemotactically by recruiting them away from tumor cells through CAF-expressed CXCL12⁶², or physically by providing ECM proteins such as collagen and fibronectin, which stiffen the TME and prevent immune cell infiltration⁶³. This negative regulation of the T cell infiltration and response within the tumor stroma allows for tumor-promoting immunosuppression, where the tumor immune-evasion allows for tumor growth, progression, and metastasis.

Antigen-presenting CAFs (apCAFs) are the third major classification of CAFs, characterized by their major histocompatibility complex (MHC)-II expression. As is the case for canonical antigen-presenting cells such as dendritic cells, macrophages, and B cells, antigen-presenting CAFs present antigens to CD4+ T cells by their expression of antigen bound to MHCII⁶⁴. As such, antigen-presenting CAFs participate in CD4+ T cell activation, recruitment, and infiltration within tissue. Due to their canonical classification by MHC-II expression⁶⁵, it is difficult to determine the spatial localization of apCAFs within the TME as they are indistinguishable from other MHC-II-presenting cells⁴², however other markers used to identify apCAFs include CD74⁶⁶, which also aids in antigen presentation⁶⁷. These cells have recently

been shown to promote T cell-mediated antitumor immunity by promoting CD8⁺ T cell cytotoxicity⁶⁵. Further spatial analyses will provide deeper insights into apCAF preferential localization within the TME and may advance our understanding of their role within the TME. Understanding these TME dynamics will give insight to how best to develop and administer treatments, whether it be through understanding the spatial enrichment of myCAFs that increase tissue stiffness and prevent immune cell infiltration, localizing the release of cytokines and chemokines by iCAFs to polarize or repel cells from accessing tumor cells, or understanding how the distribution of apCAFs strengthens an antitumor T cell response. As such, the classification of CAFs, coupled with a deeper understanding of the contribution of CAFs to tumor maintenance and progression provide an important area for which therapeutic development may benefit much from to combat CRC progression and recurrence⁴⁵.

Oncomicrobial Influence on CRC:

A distinct and equally important feature of the CRC TME is the presence and contribution of microorganisms. These oncomicrobes play a role in inducing cancer initiation and progression^{68,69}. The presence and enrichment of certain microorganisms within CRC tissues are associated with worse clinical outcomes. One microorganism of note is *Fusobacterium* of the *Fusobacteriales* order, which include the species *Fusobacterium nucleatum* and *Fusobacterium animalis*⁷⁰. *Fusobacterium* is a Gram-negative, rod-shaped, non-spore forming, obligate anaerobic oral bacterium that is also enriched in CRC tumors⁷¹. This bacterium is associated with worse prognosis in colon cancer patients⁷² and in CRC patients who also display stromal cell-rich CRC tumors, such as those whose CRC TME is of the CMS4 subtype⁷³. Several

publications have established a positive correlation between *Fusobacterium*, stromal cells, and immune dysregulation in CRC^{35,71–73}.

Within its native niche of the oral cavity, *Fusobacterium* associates with other oral microorganisms within the oral cavity to promote the formation of oral biofilms⁷⁴. Through its elongated shape and surface adhesins, such as fatty-acid-binding protein 2 (Fap2)⁷⁵, binding to neighboring microorganisms is an important step in the initiation of the polymicrobial interactions, and the interactions between microbes and host cells. *Fusobacterium*, through the binding of these surface adhesins, coaggregates with other oral microorganisms, such as *Porphyromonas gingivalis*^{76,77}. This interaction allows the formation of a biological bridge between microorganisms, some of which may invade host cells by the binding its Fap2 outer membrane protein to D-galactose-B(1-3)-N-acetyl-D-galactosamine (Gal-GalNAc), a polysaccharide expressed on the surface of host and bacterial cells⁷⁸. This interaction within the oral cavity facilitates the development of oral pathologies, such as periodontitis and periodontal disease, and explicates *Fusobacterium*'s role as a facilitator of enhanced invasion of other oral microbes⁷¹. *Fusobacterium* also engages in this interaction with a myriad of mammalian host cells, including human buccal epithelial cells, gingival and periodontal ligament fibroblasts, as well as murine lymphocytes, and macrophages⁷⁹. Inhibition of this polymicrobial binding interaction through the administration of galactose, lactose^{80,81}, and monoclonal antibodies against adhesin binding proteins⁸¹ can prevent both the coaggregation of *Fusobacterium* with its microbial binding partner *P. gingivalis* and invasion into host cells. In the context of CRC, a strong correlation has been established between host cell expression of Gal-GalNAc within precancerous lesions and cancer, where its expression was observed throughout the colons of patients with precancer and cancer, and not seen within normal colons⁸². Abed *et al.* further the

connection between *Fusobacterium* and CRC by showing that Fap2-expressing *Fusobacterium* localizes to Gal-GalNAc-overexpressing CRC cells⁸³.

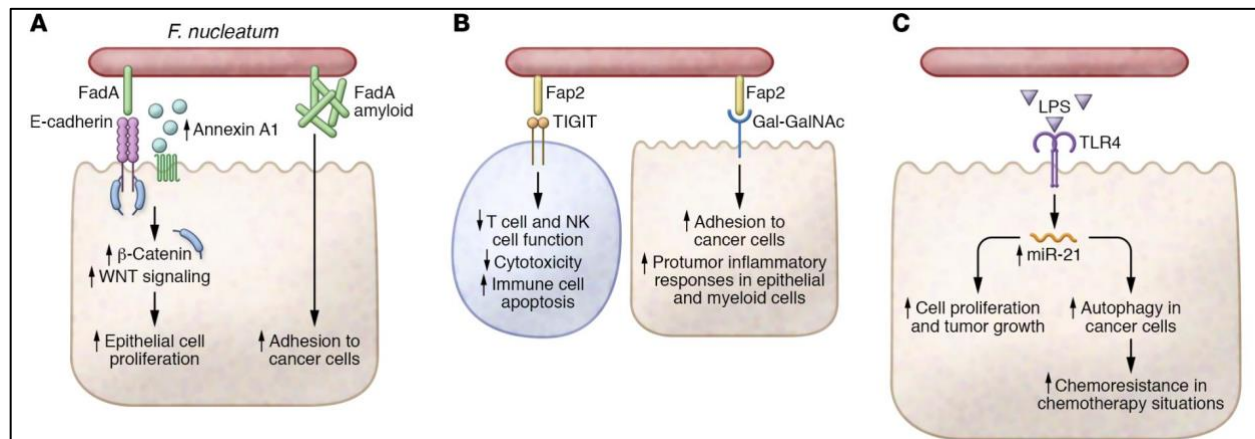


Figure 2: A summary of known oncogenic mechanisms of *Fusobacterium*. *Fusobacterium* can invade tumor cells through attachment of its surface proteins FadA (**A**) and Fap2 (**B**) to host cell surface proteins E-Cadherin and Gal-Gal-NAc (respectively). *Fusobacterium* can also be recognized by host cell surface receptor, toll-like receptor (TLR) 4, through detection of bacterial membrane component lipopolysaccharide (LPS) (**C**). The result of each of these mechanisms is the translocation of transcription factors that induce cell proliferation as well as the transcription and subsequent release of pro-inflammatory cytokines, thereby shaping the tumor microenvironment. From: “Colorectal cancer: the facts in the case of the microbiota”. Clay, S.L., Garrett, W.S. J Clin Invest. 2022; 132(4):e155101.

The *Fusobacterium* surface molecule *Fusobacterium* adhesin-A (FadA) also plays an important role in *Fusobacterium* binding and subsequent invasion of host epithelial and endothelial cells^{84,85}. By binding to epithelial/vascular endothelial (VE)-cadherin, *Fusobacterium* promotes the internalization of E-cadherin and formation of the E-cadherin/β-catenin complex. β-catenin translocates to the nucleus and promotes the upregulation of oncogenes, such as MYC, and the production and release of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α⁸⁶. This process downregulates the surface expression of E-cadherin, resulting in the activation of the epithelial-to-mesenchymal transition, that drive the invasiveness of epithelial cancers, including CRC.

In addition to its activity in biofilm formation within its native niche, *Fusobacterium* possesses additional virulence features that promote disease. There have been numerous studies on *Fusobacterium*'s LPS and its roles in eliciting pro-inflammatory cytokine production from host cells, including both IL-6 and IL-8^{87,88}. There have been many recent studies investigating the roles of *Fusobacterium* metabolites and their roles in CRC, these include: butyrate⁸⁹, propionate⁹⁰, acetate⁹⁰, and formate⁹¹. The secretion of these metabolites can recruit suppressive cells such as myeloid-derived suppressor cells⁹²⁻⁹⁴, and conventional and regulatory dendritic cells⁹². These cells act to suppress T effector cell activities, such as dendritic cell-mediated dampening of CD8+ T cell cytotoxicity, which increases immunosuppression and promotes tumor growth⁹⁵.

Although its native niche is the oral cavity, *Fusobacterium species (spp.)* are enriched in CRC tumor tissues and in the stool of patients with CRC⁹⁶. There are at least two ways that *Fusobacterium* may reach the gut. *Fusobacterium* may reach the colon through the oral cavity to the intestinal via swallowing dental plaques containing biofilms or via a hematogenous route by entering the oral vasculature³⁵. Recent data support that *Fusobacterium* presence and abundance within the TME of CRC patients positively correlate with poor patient prognosis⁹⁷. Enrichment of *Fusobacterium* and *Fusobacteriales* within CRC patients of the CMS4 subtype were at a two-fold higher risk of worse overall survival when compared to patients with non-CMS4 subtype tumors. This identifies CRC patients of the CMS4 subtype as a high-risk population. Accordingly, targeting the unique components of their tumor stroma and microenvironment, such as *Fusobacterium*-derived metabolites, or CAFs, or inhibiting the cytokines that they produce, are of great importance for the development of effective therapeutics.

It has become increasingly appreciated that alterations associated with cancer are influenced by factors and signals from throughout the tumor microenvironment, and recent technologies, such as spatial transcriptomics, has allowed for the localization and characterization of many solid tumors of patients. A recent profiling focused in *Fusobacterium* in the TME examined oral squamous cell carcinoma (OSCC) and CRC⁹⁸. In this investigation, *Fusobacterium* are found in specific locations within the TME and in areas with lower densities of CD4 and CD8+ T cells and higher densities of CD11b+ myeloid cells⁹⁸. Additionally, the host cells within these bacteria-positive regions often express cytotoxic T-lymphocyte-associated protein (CTLA)-4, an inhibitory molecule which suppresses the antitumor activity of vital T cells within the TME⁹⁹. These data suggest a strong association between intratumoral bacteria distribution and immunosuppressive regions of the CRC TME⁹⁸.

Fusobacterium presence influences the immune landscape in the gastrointestinal tract in both human and mouse tissues. M2 macrophages, associated with wound healing, tissue repair, and tumor promotion, are affected by *Fusobacterium* in a TLR4-dependent manner in preclinical CRC mouse models¹⁰⁰. These macrophages become the dominant population of macrophages within the tumor, and are exploited by tumor cells for their matrix-remodeling capabilities to promote tumor progression¹⁰¹. Another important cell type, T-helper 17 (Th17) cells, which are induced by IL-6 and TGF- β in mice and known for their involvement in clearing extracellular pathogens, are important mediators of inflammation at mucosal barrier sites where they are most abundant¹⁰². Th17 cells contribute to intestinal inflammation by secreting IL-17, a pro-inflammatory cytokine highly implicated in human pathologies¹⁰³, and IL-22, which has been reported to possess both pro- and anti-inflammatory roles during inflammation¹⁰⁴.

Fusobacterium-derived SCFAs engage the free fatty acid receptor-2 (FFAR2) and increase Th17

cells in the mouse colon¹⁰⁵. In this study, detection of *Fusobacterium*-derived SCFAs leads to increased frequency of intestinal Th17 cells, and increased expression of IL-17, which has been shown to promote colonic tumorigenesis¹⁰⁶.

T-regulatory cells (Tregs), known for their importance in suppression of immune responses are also affected by *Fusobacterium*¹⁰⁷. Important in dampening the effector functions of cells through mechanisms such as secretion of inhibitory cytokine IL-10, Tregs are increased in the presence of *Fusobacterium* in human esophageal squamous cell carcinoma (ESCC)¹⁰⁸, as well as in human intestinal tissue and mouse models¹⁰⁷. In these models, *Fusobacterium* stimulation of TLR2 and TLR4 induces Tregs, and leads to a reduction of the secretion of pro-inflammatory cytokines such as IL-8, IL-1 β , and TNF- α ¹⁰⁷. In this way, Tregs contribute to the suppression of intestinal inflammation following *Fusobacterium* infection. In CRC cell lines, the presence of *Fusobacterium* leads to an increase in the expression of programmed cell death ligand 1 (PD-L1)¹⁰⁹, a protein upregulated on the surface of tumor cells which promotes immune escape by suppressing T cell signaling¹¹⁰.

Notably, exposure of tumor cells to *Fusobacterium* may increase the success of anti-programmed death 1 (PD-1) therapy¹¹¹. PD-1, a protein upregulated on T cells upon activation, provides an inhibitory signal when bound to its cognate ligands, programmed death ligand 1 (PD-L1) and PD-L2, which are expressed on both hematopoietic and non-hematopoietic cells. During homeostatic conditions, this interaction helps maintain immune tolerance by inhibiting T cell signaling. Under pathogenic conditions such as cancer and chronic antigen stimulation, however, this interaction prevents the mounting of an efficient immune response and reinforces poor anti-tumor and anti-viral immunity. A study focusing on the TME of microsatellite stable (MSS) CRC within *Fusobacterium*-monocolonized mice has shown, however, that

Fusobacterium-derived butyric acid downregulates tumor-infiltrating CD8 T cell expression of PD-1 and enhances their effector functions after exhaustion¹¹¹. In this way, *Fusobacterium* may support the anti-tumor activity of CD8⁺ T cells, and its abundance in MSS CRC supports a successful response to immunotherapy. Together, these findings demonstrate the complexity of the immunomodulation meted out by *Fusobacterium* and the need for further investigation to understand what influences these different outcomes.

Given the growing appreciation of the importance of microbiota in the prognosis of CRC (and other diseases), as well as the strong association between *Fusobacterium* abundance and poor patient CRC clinical outcomes, the goal of my thesis is to further characterize the interactions between *Fusobacterium* and fibroblasts as they contribute to colorectal pathology. My central hypothesis is that *Fusobacterium* plays a pivotal role in inducing a pro-inflammatory environment that exacerbates CRC development. To investigate the interaction between fibroblasts and *Fusobacterium* spp., I will employ tissue-bacteria coculture techniques utilizing colons of Germ-Free mice cocultured with different species of *Fusobacterium*. Following incubation, the tissues will be processed as formalin-fixed, paraffin-embedded (FFPE) samples. Indirect immunofluorescence microscopy staining will then be performed to confirm the presence and spatial distribution of *Fusobacterium* spp. within the colonic tissue microenvironment. Immunofluorescence techniques will include markers specific for *Fusobacterium* and CAF markers. The CAF markers of focus include PDGFR- α , overexpression of which has been associated with increased angiogenesis, invasion, and metastasis¹¹⁴, and α -SMA, shown to increase the invasive potential of CAFs and reduce immune cell invasion¹¹³.

To investigate the effect of *Fusobacterium* on murine fibroblasts, I will perform *in vitro* coculture experiments where RNA transcript levels will be assessed by RT-qPCR. Additionally,

I will conduct *in vivo* oral gavage and intravenous inoculation studies to assess the direct influence of *Fusobacterium* on the stroma and immune compartments in healthy and CRC tumor mouse models. These experiments will be performed in mice with varying microbiota complexities (such as germ-free, specific pathogen free) to evaluate how *Fusobacterium* influences the tumor microenvironment under different microbial conditions.

I will employ immunofluorescence microscopy techniques on FFPE tissue sections to visualize *Fusobacterium* in colon tissue and CAF markers in healthy colonic tissue and primary colonic tumor in the presence and absence of *Fusobacterium* inoculation. By investigating the interactions between *Fusobacterium* and the stroma of the CRC TME through multiple analytic approaches, this thesis aims to provide further insight into the dynamics influencing CRC progression and to unveil potentially applicable translational targets for CRC treatment.

Chapter 2: Materials, Methods, and Results

MATERIALS AND METHODS:

Animal experiments:

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) and Harvard Medical School's Standing Committee on Animals. WT C57BL/6 were bred under specific-pathogen-free (SPF) conditions with a 12-hour light cycle and *ad libitum* food and water in our vivarium. For gnotobiotic experiments, C57BL/6 germ-free (GF) mice were housed at the Harvard T.H. Chan Gnotobiotics Center for Mechanistic Microbiome Studies in semi-rigid isolators (Plastic Concepts Inc.) and fed with sterile food and water. Experiments were conducted in individual ventilated IsocageP system (Tecniplast). Mixed gender and age-matched mice between 6 and 8 weeks were used for all experiments.

Bacterial Strains and Culture Preparation:

The bacterial strains used in our experiments are as follows:

<i>Fusobacterium</i> isolate	Taxonomy	Origin/Patient status	Source
<i>Fn7-1</i> (EAVG_0002)	<i>Fusobacterium animalis</i>	Crohn's Disease ¹¹⁵	Provided by Dr. Emma Allen-Vercoe Lab
<i>Fn7-3</i> (CC 7/3 JVN3C1)	<i>Fusobacterium animalis</i>	CRC tumor ¹¹⁶	Provided by Dr. Emma Allen-Vercoe Lab
<i>Fn25586</i> (ATCC 25586)	<i>Fusobacterium nucleatum</i>	Cervical/urogenital cancer ¹¹⁷	Purchased from ATCC

To prepare a culture of *Fusobacterium* for bacterial inoculation, *Fusobacterium spp.* were streaked from a frozen stock onto Fastidious Anaerobe Agar (FAA) plates under anaerobic conditions at 37°C using a flexbile vinyl chamber (Coy Lab Products). *Fusobacterium spp.* used

in these experiments consisted of either: *Fn7-1*, isolated from a Crohn's Disease Patient; *Fn7-3*, isolated from the tumor of a patient with CRC as described by Cochrane, et al.; or *Fn25586*, isolated from a patient diagnosed with cervical/urogenital cancer. 48-hours after streaking onto FAA plates, one colony was selected and inoculated into 3-5mL supplemented Trypticase Soy Broth (sTSB), (TSB supplemented with 5mg/mL of hemin, 1mg/mL of menadione) as previously described¹¹⁸. After 24h, the overnight (ON) culture was diluted 100-fold in 30 mL of sTSB to prepare a subculture, which yielded approximately 1×10^{10} colony forming units (CFU)/mL. ON *Fusobacterium* culture was centrifuged at 4000xg for 5 minutes, then resuspended in 1 mL of sTSB media (for *in vivo* inoculation), or resuspended in 1 mL of fibroblast culture media (for *in vitro* inoculation coculture).

Bacterial Inoculation:

Oral:

For oral inoculation of *Fusobacterium*, 10^9 CFUs/100 μ l was administered per mouse using a sterilized 22-gauge gavage needle. This procedure was performed within a laminar flow hood in the animal facility.

Intravenous:

For intravenous inoculation of *Fusobacterium*, 10^7 CFUs/100 μ l, was administered per mouse through a tail vein injection using a sterilized injection needle. This procedure was performed in a laminar flow hood within the animal facility.

Tissue Harvesting (fecal and colon samples):

Colons were resected and trimmed longitudinally to expose the mouse intestinal lumen. Forceps and scissors were used to remove any remaining hair or adipose tissue. Feces were collected and stored immediately at -20°C. Colons were then washed in 1X phosphate-buffered saline (PBS) to remove residual fecal matter and trimmed transversally to fit the width of the histology sponge and placed into labeled tissue cassettes. Colons were then placed flattened and extended onto histology sponges, into tissue cassettes, and immersion fixed in 10% Neutral Buffered Formalin for 24 hours at room temperature (RT). After 24 hours of formalin fixation, colons were stored in 70% ethanol where they remained stored until submission to the Rodent Histopathology Core at Harvard Medical School for embedding, sectioning, and slide generation.

Germ-Free (GF) mouse colon Incubation Experiment Protocol:

GF mouse colons were excised and harvested as described, then incubated in 10-cm petri dishes containing either sTSB or sTSB containing bacterial culture of *Fn7-1*, *Fn7-3*, or *Fn25586*, in an anaerobic hood at 37°C. After 30 minutes of incubation, colons were washed in 1X PBS and prepared for histology and subsequent immunofluorescence detection as described below.

Fusobacterium* detection:*DNA Extraction Protocol:**

Frozen fecal pellets were added to tubes containing 300 µl extraction beads, with 300µl of sodium dodecyl sulfate (SDS) and 500 µl of ultra-pure phenol ([#J64011.36](#)). Samples were then subjected to bead-beat for 2 minutes and centrifuged at 15,000 rpm for 5 minutes at 4°C. After centrifugation, the top layer (aqueous phase) was transferred to a new tube, minimizing as much

contamination between separated phases as possible. According to aqueous phase volume, an equal volume of phenol-chloroform-isoamyl (#15593031) was added to the tube containing the aqueous phase. Sample was vigorously homogenized, centrifuged at 15,000 rpm for 5 minutes at 4°C, and the top layer (aqueous phase) was added to a new tube. A 1:10 volume of 3M sodium acetate and 1:1 volume of 100% isopropanol was added. Samples were mixed by inversion and incubated at -20°C for 10-60 minutes. After incubation, samples were centrifuged at 15,000 rpm for 5 minutes at 4°C. The supernatant after centrifugation was discarded, and 500 µl of cold 70% Ethanol was added to the pellet. Pellet was then mixed by inversion, vortexed, and centrifuged at 15,000 rpm for 5 minutes at 4°C. Supernatant after centrifugation was discarded a second time, and another 500 µl of cold 70% ethanol was added to the pellet. Pellet was again mixed by inversion, vortexed, and centrifuged a final time at 15,000 rpm for 5 minutes at 4°C. The supernatant was again discarded, and the pellet was air-dried in a sterilized tissue culture (TC) hood for 20-30 minutes. 50-100 µl of room-temperature molecular grade H₂O was added to each sample and incubated at 37°C for 30 minutes. Once a sample was fully dissolved, the sample was lightly vortexed and quickly spun using a microcentrifuge. Total DNA concentration was determined using an ND-1000 spectrophotometer (Denville Scientific Inclusive).

qPCR Analysis:

Fusobacterium presence was assessed by detection via qPCR analysis. Primers were obtained from Thermo Fisher Scientific and primers directed against the *Fusobacterium* spp. 16S rRNA gene was ordered as described by Boutaga et al. (2005)¹¹⁹. Gene expression was evaluated using KAPAPROBE FAST qPCR Kit (KK4718). The qPCR thermocycling protocol consisted of an initial denaturation cycle for 3.5 minutes at 95°C, then 40 successive cycles of denaturation for 3

seconds at 95°C, annealing for 20 seconds at 60°C, followed by elongation for 20 seconds at 72°C. Relative expression was evaluated using the $2^{-\Delta Ct}$ method, where $\Delta Ct = (Ct \text{ of gene of interest}) - (Ct \text{ of housekeeping gene})$.

Primers listed below were used:

Marker (16S rDNA)	Forward Sequence 5'-3'	Reverse Sequence 3'-5'
<i>Fusobacterium spp.</i>	GGATTATTGGGCGTAAAGC	GGCATTCTACAAATATCTACGAA
<i>Eubacteria</i>	AGAGTTTGATCMTGGCTCAG	GGTTACCTTGTTACGACTT

The following master mix protocol was used to perform qPCR analysis:

Reagent	Volume (μl, per reaction)
Forward Primer (10μM)	0.4
Reverse Primer (10μM)	0.4
SYBR	10
Molecular Grade H₂O	5.2
DNA template mix (20 ng)	4

***Fusobacterium* spp. Detection by Immunofluorescence on Formalin-Fixed Paraffin-**

Embedded Sections:

Reagents:

Tris-Tween (sterile filtered with 0.22 µm filter): 20X Tris-Buffered Saline-Tween (TBS-T) in Milli-Q H₂O

Blocking solution (sterile filtered with 0.22 µm filter): 0.1% Triton-X100, 0.1% saponin, 3% bovine serum albumin (BSA), 5% normal serum (Donkey serum) in 1X TBS-T

Antibody diluent (sterile filtered with 0.22 µm filter): 0.1% Triton-X100, 1% BSA, in 1X TBS-T

Antibodies	Concentration	Source	Product Identification
Rabbit-α-Fn7-1/7-3	1:800	Dr. Emma Allen-Vercoe	<u>Allen-Vercoe Lab</u>
Rabbit-α-Fn25586	1:800	Dr. Emma Allen-Vercoe	<u>Allen-Vercoe Lab</u>
Rat-α-PDGFR-α	1:200	Invitrogen	<u>14-1401-82</u>
Mouse-α-αSMA	1:1000	Millipore Sigma	<u>A2547</u>
Donkey-α-Rabbit AF555	1:1000	Invitrogen	<u>A31572</u>
Donkey-α-Rat AF647	1:1000	Invitrogen	<u>A78947</u>
Donkey-α-Mouse AF488	1:1000	Invitrogen	<u>A21202</u>
Donkey-α-Rabbit AF488	1:1000	Invitrogen	<u>A21206</u>
4',6-diamidino-2-phenylindole (DAPI nuclear stain)	1:1000	Invitrogen	<u>D3571</u>

Day 01: Deparaffinization, Antigen Retrieval, Blocking, and Primary Antibody Application

Deparaffinization:

After tissue harvesting and fixation, samples were sent to the Rodent Histopathology Core at Harvard Medical School where they were paraffinized, embedded, and sectioned to 5 μm thickness according to the standards of their facility. For immunofluorescence staining, slides were baked at 60°C for 1 hour and immediately placed into a xylene wash. Xylene washes were repeated, totaling 3 xylene washes at 5 minutes each. Slides were then immediately placed into 100% ethanol (EtOH) washes, totaling 2 washes at 5 minutes each. After 100% EtOH washes, slides were placed in 95% EtOH, totaling 2 washes at 3 minutes each. After 95% EtOH washes, slides were incubated in 3% hydrogen peroxide (H_2O_2) in methanol (MeOH) solution (25 mL of 30% H_2O_2 in 225 mL MeOH) in a chemical hood at room temperature for 20 minutes. Finally, samples were washed in DD H_2O for 2 minutes, totaling 2 washes.

Heat-Induced Antigen Retrieval:

In preparation for antigen retrieval, 1 L of double-distilled (DD) H_2O was added to a pressure cooker, 250 mL of Borg Decloaker antigen retrieval in one histology slide chamber, and 250 mL of DD H_2O into another histology slide chamber containing a thermometer for temperature tracking. Antigen retrieval solution and DD H_2O were boiled in the pressure cooker to 80°C. Once 80°C temperature was achieved, slides were inserted into Borg Decloaker antigen retrieval and boiled for 25 minutes, being sure that temperature did not exceed 100°C. Slides were cooled on the benchtop until temperature reached below 50°C, then rinsed in DD H_2O twice.

Blocking:

Slides were dried using kimwipes to allow PAP Pen encirclement of tissue, creating a hydrophobic barrier for containment of subsequent solution additions. Slides were briefly washed with Tris-Tween, then blocking solution was applied for 1 hour, in dark humidity chambers, at room temperature. After blocking, primary antibodies were added to each slide and maintained within dark humidity chambers at 4°C ON. All antibodies (primary and secondary) were prepared in antibody diluent, centrifuged at 15,000 rpm for 5 minutes, then sterile filtered in a sterilized TC hood using a 0.22 µm sterile filter.

Day 02: Secondary Antibody Application, Counterstain, Coverslip

After incubation ON with primary antibodies, slides were washed with Tris-Tween, totaling 3 washes, 5 minutes each, at RT in humidity chambers. Secondary antibodies were applied for 1 hour at RT in humidity chambers. Slides were again washed with Tris-Tween, totaling 3 washes, 5 minutes each, at RT in humidity chambers. Slides were counterstained with DAPI nuclear stain for 1.5-2 minutes at room temperature, then immediately mounted using Avantor cover glasses (#48393-230) and Aquapoly/Mount solution (#87001-902).

Mouse Colonic Fibroblast Isolation:

To investigate the effects of *Fusobacterium* on fibroblasts of the mouse colon under normal, homeostatic conditions, we performed *in vitro* coculture experiments using isolated normal mouse colonic fibroblasts. For coculture experiments, we first isolated and expanded mouse colonic fibroblasts. We used the selective adhesion method, in which colons were excised and

trimmed to expose the mouse intestinal lumen. Fecal matter was discarded, and samples were washed 3 times in 1X PBS. Samples were transferred to an ethylenediaminetetraacetic acid (EDTA) buffer (containing HEPES (5mM), EDTA (10mM), 1% Penicillin-Streptomycin (P/S), and 2% non-heat-inactivated fetal bovine serum (FBS) in 1X PBS). Samples were incubated on a rotator at 37°C for 20 minutes, then passed through 100 µm filters and washed 3 times in 1X PBS to remove EDTA residues. Samples were transferred to a 1.5 mL Eppendorf tube containing 200 µl of digest buffer **without** enzymes (RPMI 1640, 1% P/S), where they were chopped very finely (100 chops per colon) using sharp scissors. Samples were then transferred to digestion buffer containing enzymes (RPMI 1640, 1% P/S, 100 µg/mL LiberaseTM, 100 µg/mL DNase I), and incubated on a rotator at 37°C for 20 minutes. Samples were resuspended using a 5 mL pipette, then re-incubated on a rotator at 37°C for 10 minutes. Samples were resuspended using a 1mL pipette, then placed on ice where 1 mL of Non-Heat-Inactivated FBS was added. Samples were filtered twice using a 70 µm filter, topped up to 45mL with 1X PBS, and centrifuged at 400 G for 10 minutes. In a sterile TC hood, the supernatant was removed, and the pellet was resuspended in 1 mL culture media (RPMI 1640, 20% non-heat inactivated FBS, 1% sodium pyruvate, 1% P/S, 0.1% 2-mercaptoethanol). Cells were then transferred to a 6-well plate containing 2 mL culture media and incubated at 37°C. Since fibroblasts are expected to attach to the well-plate surface after 20 minutes, the supernatant after 20 minutes was replated into new wells, and fresh culture media was added to original cells. Culture media was refreshed among all wells every 2-3 days. Cells were grown to full confluency and expanded. Expansion of cells consisted of a brief wash in 1X PBS, followed by the addition and incubation of cells with 1 mL of Trypl-E recombinant enzyme at 37°C for 3-5 minutes.

Coculture Experiments:

Prior to Infection:

Fibroblasts passaged 3 to 6 times were used for coculture experiments. Cells were collected into a 50 mL conical, centrifuged at 300xg for 5 minutes, resuspended in 1 mL fibroblast culture media, and counted using a hemocytometer. Cells were then plated at 5×10^5 per 10-cm petri dish per condition. Approximately 5×10^5 of cell suspension was retained for flow cytometric immunophenotyping.

Day of Infection:

After centrifugation of bacterial culture and before resuspension in 1 mL of culture media, *Fusobacterium* supernatant was collected and sterile filtered using a 0.22 μ m sterile filter to be used for coculture condition containing 10% conditioned media (CM). After resuspension in culture media, *Fusobacterium* infection was performed at an MOI of 1:10. Coculture with lipopolysaccharide (LPS) was performed at a concentration of 1 μ g/mL. *Fusobacterium* cells were resuspended in culture media to the desired CFU.

Collection:

After 6 hours of infection/coculture, the supernatant from each condition was sterile-filtered using a 0.22 μ m sterile filter, collected in a 1.5 mL tube, and stored immediately at -20°C for enzyme-linked immunosorbent assay (ELISA) analysis. After removing the supernatant, plates were washed with 1X PBS, replenished with 1.5 mL of 1X PBS for which adherent cells were scraped from the surface of the petri dishes and collected into 1.5 mL tubes. Scraped cells were collected for RNA analysis by reverse-transcriptase qPCR (RT-qPCR). All collected samples

were centrifuged at 400xg for 10 minutes at 4°C, after which time, the supernatant was removed, and cell pellet stored at -20°C.

Flow Cytometric Immunophenotyping:

Cells were collected as previously mentioned during initial plating for coculture experiments, centrifuged at 400xg for 5 minutes, and resuspended in 250 µl of fluorescent activated cell sorting (FACS) buffer (containing 1 mM EDTA, 2% FBS in 1X PBS). 50 µl of cell suspension from each sample was used for live/dead stain, and 50 µl of cell suspension from each sample was used for unstained control. Fc block and live/dead stain were added to cells, then incubated in the dark at 4°C for 10 minutes. 50 µl of surface antibody cocktail was added to cells, then incubated in the dark at 4°C for 30 minutes. Cells were then topped up with 100 µl of FACS buffer, centrifuged at 400xg for 5 minutes, and resuspended in 200 µl of FACS buffer before being filtered and prepared into FACS tubes.

Antibodies used for immunophenotyping were utilized as follows:

Antibody/Target	Fluorochrome	Concentration	Purpose	Product Identification
CD45	FITC	1:200	All immune cells	<u>103114</u>
CD31	PE	1:200	Endothelial cells	<u>102507</u>
CD326 (EpCAM)	PerCPCy5.5	1:200	Epithelial cells	<u>118220</u>
PDGFR-α	APC	1:100	Fibroblast marker	<u>17-1401-81</u>
Fc Block	N/A	1:100	Blocks Fc receptor binding	<u>101339</u>
Live/dead stain	Aqua	1:1000	Cell viability	<u>423101</u>

ELISA analysis:

To detect the secretion of IL-6 into the supernatant of the coculture experiments, we performed ELISA analysis according to the protocol provided with Legend Max Mouse IL-6 ELISA kit (#431307).

RNA Extraction:

1 mL of Trizol was added to tubes containing samples, then homogenized by resuspension. Sample was left to stand for 5 minutes at room temperature, during which time, phase-lock gel tubes were pre-centrifuged at 10,000 G for 30 seconds. The Trizol homogenate was added on top of the gel of the phase-lock tubes, then incubated for 5 minutes at room temperature. 0.2 mL of chloroform was added to each tube, then vigorously mixed by inversion until homogenized for 15 seconds. Samples were centrifuged at 12,000xg for 10 minutes at 4°C. The top layer (aqueous phase) was transferred to a new tube, precipitated by adding 0.5 mL ice cold isopropanol, mixed well by inversion, and incubated ON at -20°C. After 24h, samples were centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed, and 1 mL of ice cold 75% ethanol was added. Samples were vortexed, then centrifuged at 10,000xg for 5 minutes at 4°C. The supernatant was removed, and the pellet was permitted to air dry in a sterilized TC hood for 10 minutes. 25 µl of molecular grade H₂O was added to each sample, and samples were transferred to 55°C water bath to dissolve pellet. Once sample was fully dissolved, sample was kept on ice, lightly vortexed and quickly spun using a microcentrifuge. Total RNA concentration was determined with an ND-1000 spectrophotometer (Denville Scientific Inclusive). Sample were then frozen at -20°C until further use for RT-qPCR.

RT-qPCR analysis:

RNA expression was evaluated using KAPA SYBR® FAST One-Step RT-qPCR Kit ([KK4651](#)) following the manufacturer's protocol. RT-qPCR thermocycling protocol consisted of an initial enzyme activation step for 5 minutes at 42°C followed by incubation at 95°C for 3.5 minutes, then 40 successive cycles of denaturation at 95°C for 3 minutes, 95°C for 3 seconds, 20 seconds of annealing at 60°C, and 20 seconds of elongation at 72°C. A final cycle consisted of incubation at 95°C for 1 minute, 60°C for 20 seconds. Relative expression was evaluated using the $2^{-\Delta\Delta C_t}$ method¹²⁰, where $\Delta C_t = (C_t \text{ of gene of interest}) - (C_t \text{ of housekeeping gene})$ and $\Delta\Delta C_t = (\Delta C_t \text{ of treated sample}) - (\Delta C_t \text{ of untreated sample})$.

The primers used to evaluate mRNA expression were as follows:

Marker	Forward Sequence 5'-3'	Reverse Sequence 3'-5'
<i>Il-6</i>	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
<i>Pdgfr-α</i>	AGAGTTACACGTTTGAGCTGTC	GTCCCTCCACGGTACTCCT
<i>Acta2</i> (α SMA)	ATGCAGAAGGAGATCACAGC	GTATTCCTGTTTGCTGATCCAC
<i>Fap</i>	GTCACCTGATCGGCAATTTGT	CCCCATTCTGAAGGTCGTAGAT
<i>Actb</i>	TACCACCATGTACCCAG	CTCAGGAGGAGCAATGATCTTGAT

The following master mix protocol was used to perform RT-qPCR analysis:

Reagent	Volume (μ l, per reaction)
Forward Primer (10 μ M)	0.4
Reverse Primer (10 μ M)	0.4
dUTP	0.4

kRT	0.4
SYBR	10
Molecular Grade H₂O	3.4
RNA template mix (20 ng)	5

CRC preclinical mouse model:

Mouse tumor organoids, harboring mutations in KRAS (Kirsten rat sarcoma viral oncogene homolog), p53 (tumor protein p53 gene), and NOTCH (neurogenic locus notch homolog protein 1), were orthotopically injected into distal colons of mice as previously described¹²¹. Four weeks post-injection, mice were randomized and intravenously injected via the tail vein with either PBS control or *Fn25586*. Tumor pieces were resected to validate of *Fn25586* translocation by qPCR. Tumor samples were submitted for microscope slide generation at the Rodent Histopathology Core as previously described. Microscope slides were then used for immunofluorescence detection of *Fn25586* and CAF marker PDGFR- α using optimized techniques as previously described.

Statistical Analysis:

Statistical analyses and graph generation were conducted using Prism 9 (GraphPad Software, San Diego, CA).

RESULTS:

***Fusobacterium* detection on Germ-Free (GF) colon samples:**

To assess the detection of *Fusobacterium* on FFPE mouse colon samples, we first performed an incubation experiment. In GF colon samples incubated sTSB media alone, there is no detection of *Fusobacterium* (Figure 3). GF colon samples that were incubated with an inoculum of either *Fn7-1* or *Fn7-3* displayed positive detection of *Fusobacterium* (Figure 4). This result is consistent among all strains of *Fusobacterium* tested and supports the binding affinity of the antibodies tested on tissues, as they had previously been tested only in pure cultures on slides.

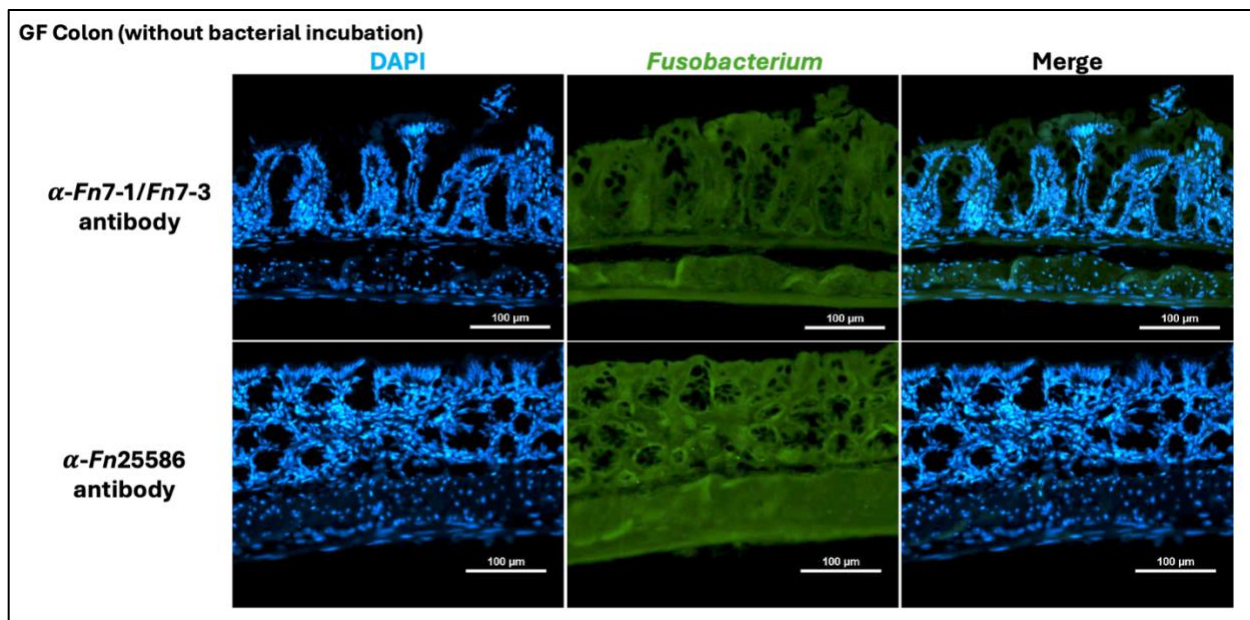


Figure 3: Immunofluorescence staining performed on colons of GF mice incubated with sterile media only. Germ-free (GF) mouse colon samples were treated with antibodies against *Fusobacterium* spp. for assessment of antibody binding specificity. **DAPI**: nucleus stain, 1:1000 concentration; **Fn7-1/7-3** and **Fn25586**: conjugated with **AF488**, 1:200 concentration. **Antigen Retrieval**: Borg Decloaker. **Magnification**: 20X.

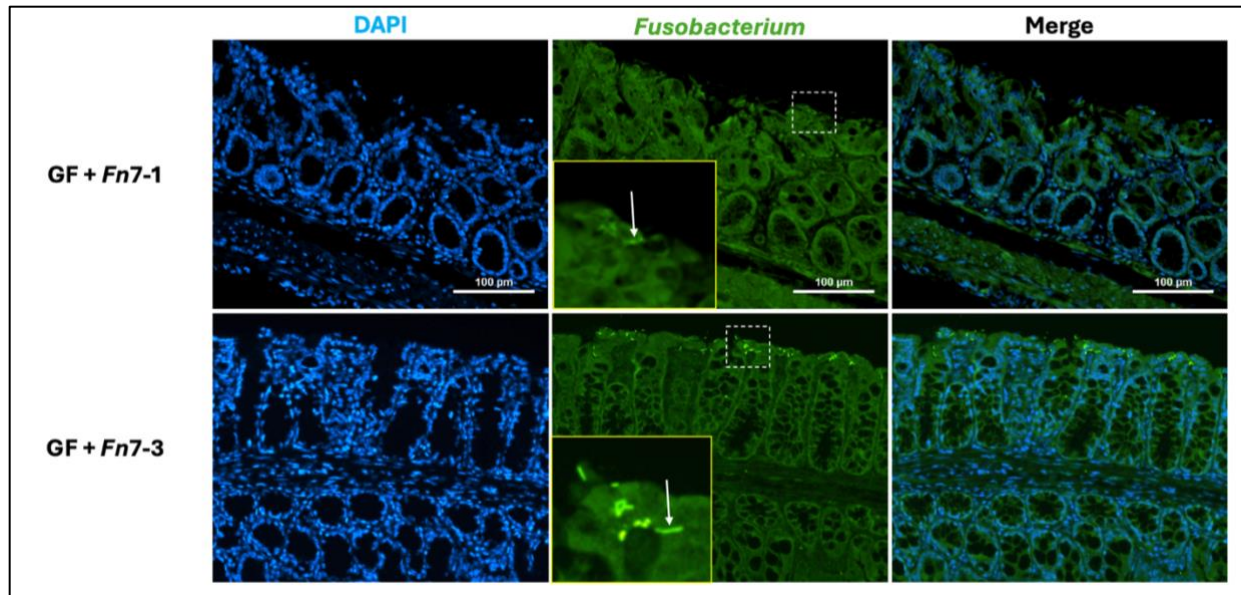


Figure 4: Immunofluorescence staining on colons of GF mice after incubation with differing strains of *Fusobacterium*. Germ-free (GF) samples were incubated with respective strains of *Fusobacterium* (*Fn7-1*, *Fn7-3*), then stained with antibodies against each *Fusobacterium* species for assessment of antibody binding specificity. **DAPI:** nucleus stain, 1:1000 concentration; ***Fusobacterium* species:** conjugated with **AF488**, 1:200 concentration. **Antigen Retrieval:** Borg Decloaker. **Magnification:** 20X. White arrows indicate positive *Fusobacterium* spp. detection.

Optimization of Immunofluorescence Techniques:

Optimization of immunofluorescence techniques was performed to further validate the immunofluorescence for *Fusobacterium* spp.-specific antibodies and detection conditions.

Parameters of assessment for optimal immunofluorescence detection consisted of an assessment of decreasing concentrations of *Fusobacterium* antibodies (*Fn7-1/Fn7-3*, and *Fn25586*), in search of a concentration that minimized background signaling while still allowing visualization of the bacteria. As such, we tested the detection of *Fusobacterium* using primary antibodies at concentrations of 1:200, 1:400, 1:600, and 1:800. It was found that the 1:800 antibody concentration yielded visible *Fusobacterium* detection (Figure 5), and thus was selected as an optimal working concentration for our antibody for detection of *Fusobacterium* in subsequent experiments. Antigen retrieval methods were also tested for optimization. Antigen retrievals

differing in their optimization for antigens at high pH (Borg Decloaker) or lower pH (Citrate Buffer) were assessed. Differing compositions of blocking solutions and of antibody diluents were also tested. Final optimal condition determinations are displayed in Table 1.

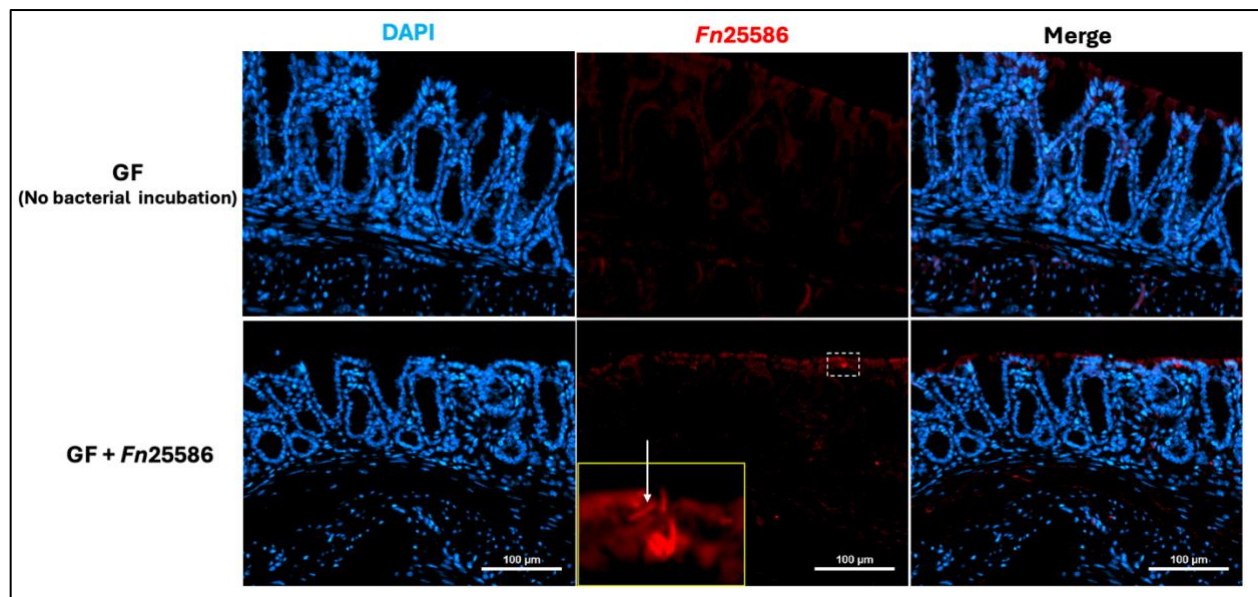


Figure 5: Optimized immunofluorescence staining of *Fn25586*. Germ-free (GF) colons were incubated with *Fn25586* or sterile media as control, then stained with antibodies against *Fn25586* for assessment of antibody binding specificity. **DAPI:** nucleus stain, 1:1000 concentration; ***Fusobacterium*:** conjugated with AF555, 1:800 concentration. **Antigen Retrieval:** Borg Decloaker. **Magnification:** 20X. White arrow indicates positive *Fn25586* detection.

Table 1: Optimal immunofluorescence staining conditions for detection of *Fusobacterium*.

Parameter of Assessment	Optimal Condition
<i>Fusobacterium</i> Antibody Concentration	1:800
Antigen Retrieval	<u>Borg Decloaker</u>
Blocking Solution	0.1% Triton-X100, 0.1% Saponin, 3% Bovine Serum Albumin (BSA), 5% normal serum (Donkey serum) in 1X TBS-T

Antibody Diluent	0.1% Triton-X100, 1% BSA, in 1X TBS-T
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Detection of *Fusobacterium* abundance in ASF mice *in vivo* at steady state:

To validate the detection of *Fusobacterium* in mice *in vivo*, we performed oral inoculation experiments on Altered Schaedler Flora (ASF) mice. ASF mice were administered an oral inoculation of *Fn25586* once a week for two weeks. Fecal samples were collected once a week for 2 weeks to assess concurrent fecal detection of *Fusobacterium*. In stably colonized mice, *Fusobacterium* was detected by qPCR analysis in ASF mice that received oral inoculation (Figure 6).

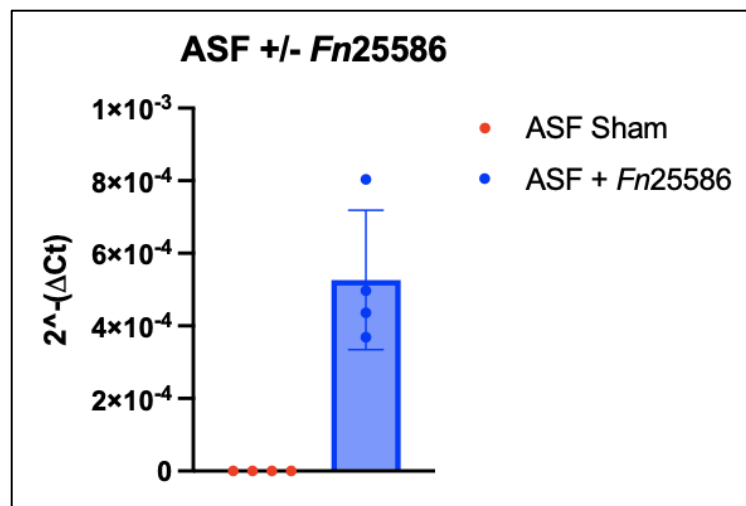


Figure 6: *Fusobacterium nucleatum* detection validated in fecal samples of ASF mice. qPCR analysis was performed on DNA extracted from fecal samples of ASF mice after receiving oral inoculation of *Fn25586* or H₂O as control. Red data points indicate results from ASF Sham-gavaged mice (n=4); Blue data points indicate results from mice inoculated with *Fn25586* (n=4). *Fusobacterium* abundance for each sample was assessed by calculating 2^{-ΔCt}, where ΔCt is the difference between cycle threshold of detection of *Fusobacterium* and the cycle threshold of detection of total bacteria (Eubacteria).

After detection of *Fusobacterium* was confirmed within fecal samples of ASF mice, optimized immunofluorescence staining was employed to assess *in vivo* *Fusobacterium* detection on FFPE sections (Figure 7). Based on our immunofluorescence staining results on FFPE

sections of ASF mouse colons, we found background or possible positive signal within the ASF sham samples with the *Fusobacterium* antibody we employed. This finding suggests that our antibody may recognize species within the limited ASF community beyond specificity for *Fusobacterium nucleatum* and *animalis*. We did observe a difference in expression of our CAF markers, PDGFR- α and α SMA, in our experimental mice that received *Fusobacterium* gavage (Figures 8 and 9). These data suggest that the presence of *Fusobacterium* may play a role in the induction of these markers' expression. These increases in CAF marker expression were examined using a semi-quantitative method for immunofluorescence quantification.

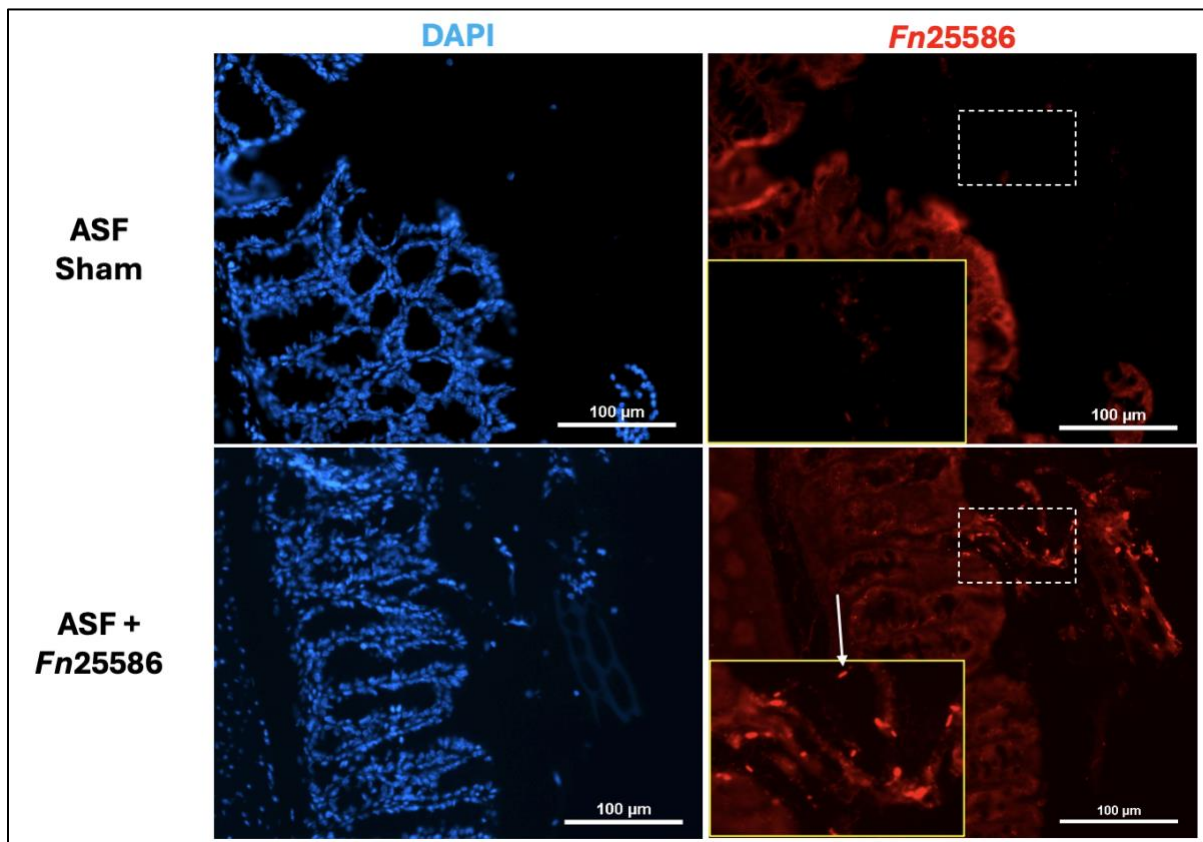


Figure 7: Immunofluorescence staining positively detects *Fn25586* within FFPE ASF mouse colon samples. After ASF mice were inoculated with *Fn25586* or Sham control, colons were harvested and used for FFPE immunofluorescence detection of *Fusobacterium*. **DAPI:** nucleus stain, 1:1000 concentration; ***Fn25586*:** conjugated with AF555, 1:800 concentration. **Antigen Retrieval:** Borg Decloaker. **Magnification:** 20X. White arrows indicate positive *Fn25586* detection.

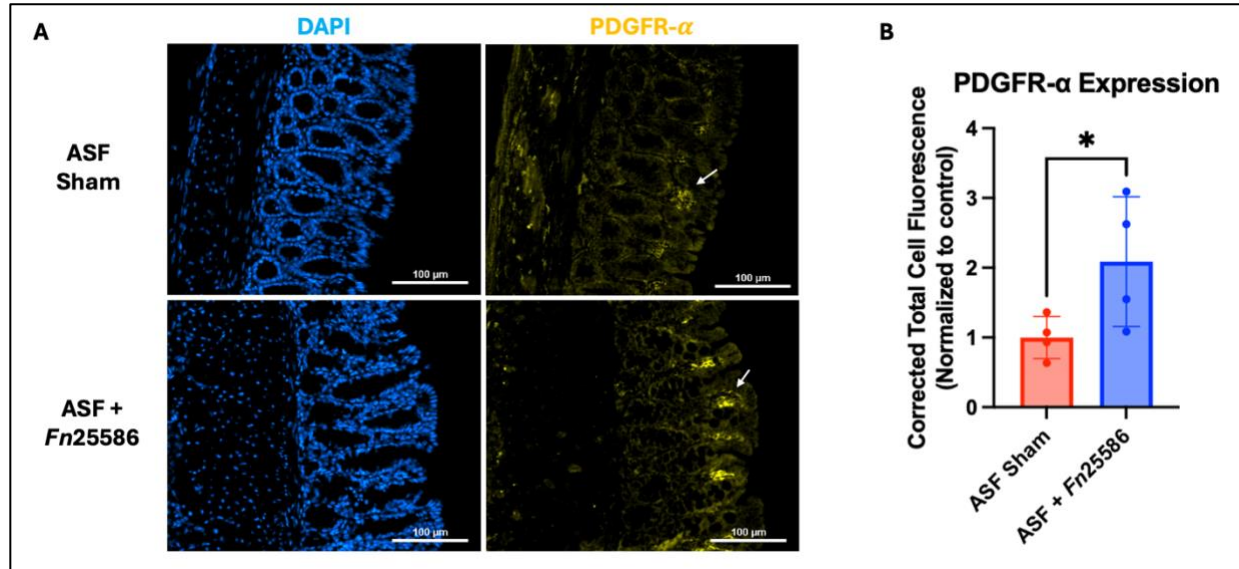


Figure 8: Immunofluorescence staining and quantification of PDGFR- α detection on FFPE ASF colon samples after *Fusobacterium* inoculation or Sham control treatment. After ASF mice were inoculated with *Fn25586* or Sham control treatment, colons were harvested and used for **(A)** FFPE immunofluorescence detection of platelet-derived growth factor receptor alpha (PDGFR- α). **DAPI:** nucleus stain, 1:1000 concentration. **PDGFR- α :** conjugated with **AF647**, 1:200 concentration. **Antigen Retrieval:** Borg Decloaker. **Magnification:** 20X. White arrows indicate positive **PDGFR- α** detection. **(B)** Immunofluorescence quantification of PDGFR- α staining. Red data points indicate results from ASF Sham mice (n=4); Blue data points indicate results from ASF mice inoculated with *Fn25586* (n=4). Immunofluorescence intensity was assessed using ImageJ Software. Quantification was calculated as the corrected total cell fluorescence (CTCF). $CTCF = [Integrated\ density - (Area \times Mean\ fluorescence\ of\ Background)]$. Data are represented as results from the same staining batch conditions.

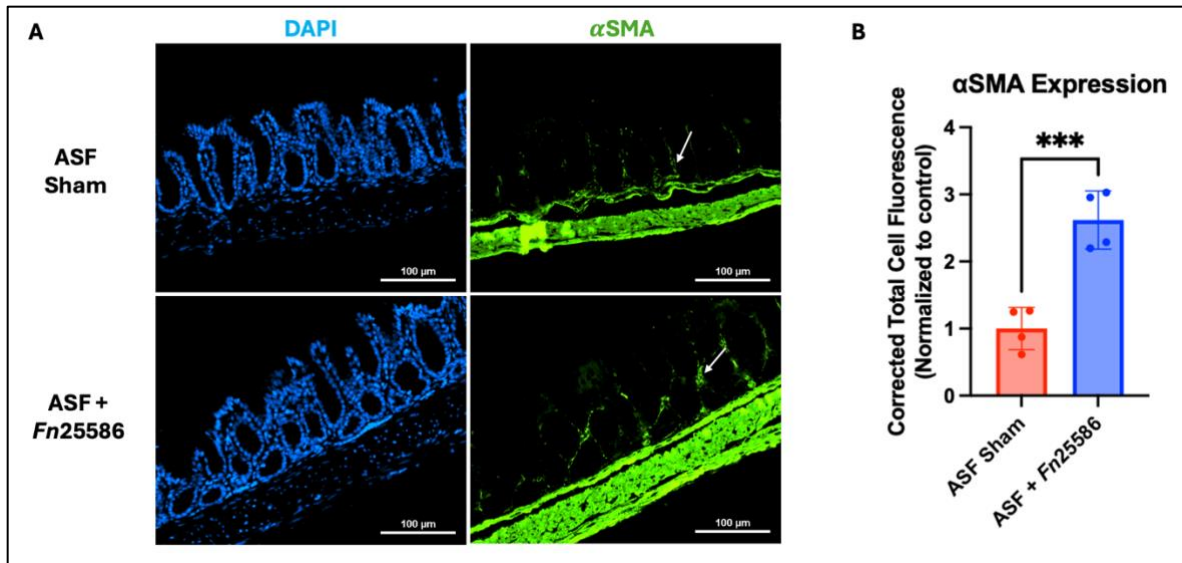


Figure 9: Immunofluorescence staining and quantification of α SMA detection on FFPE ASF colon samples after *Fusobacterium* inoculation or Sham control treatment. After ASF mice were inoculated with *Fn25586* or Sham control treatment, colons were harvested and used for (A) FFPE immunofluorescence detection of alpha smooth muscle actin (α SMA). **DAPI:** nucleus stain, 1:1000 concentration; **α SMA:** conjugated with **AF488**, 1:1000 concentration. **Antigen Retrieval:** Borg Decloaker. **Magnification:** 20X. White arrows indicate positive α SMA detection. (B) Immunofluorescence quantification of α SMA staining. Red data points indicate results from ASF Sham mice (n=4); Blue data points indicate results from ASF mice inoculated with *Fn25586* (n=4). Immunofluorescence intensity was assessed using ImageJ Software. Quantification was calculated as the corrected total cell fluorescence (CTCF). $CTCF = [\text{Integrated density} - (\text{Area} \times \text{Mean fluorescence of Background})]$. Data are represented as results from the same staining batch conditions.

***Fusobacterium* does not colonize conventional mice with complex microbiota:**

To track the detection of *Fusobacterium* in SPF mice, we performed a 72-hour time point experiment (Figure 10). SPF mice were either administered a single dose of *Fn7-3* or H₂O as control. Fecal samples were collected from all mice every 3-, 6-, 9-, 12-, 24-, 48-, and 72-hours post-inoculation. Mouse colon and fecal samples were then harvested as described above for further analysis by immunofluorescence detection or bacterial abundance via quantitative polymerase chain reaction (qPCR). After oral inoculation with *Fn7-3*, qPCR results show that *Fusobacterium* abundance is detected within the first 3-, 6-, 9-, and even 12-hours post-gavage, then reaches below the threshold of detection after 24 hours. Similar to prior results with *Fn7-1*,

Fn7-3 does not stably colonizes the colon of SPF mice harboring a complex microbiota, and therefore WT C57BL/6 under the SPF condition are not an idea model to study *Fusobacterium* effects as the strain does not stably colonize. As a result, we opted to use *in vitro* coculture experiments, utilizing normal fibroblasts harvested from the colon of SPF mice.

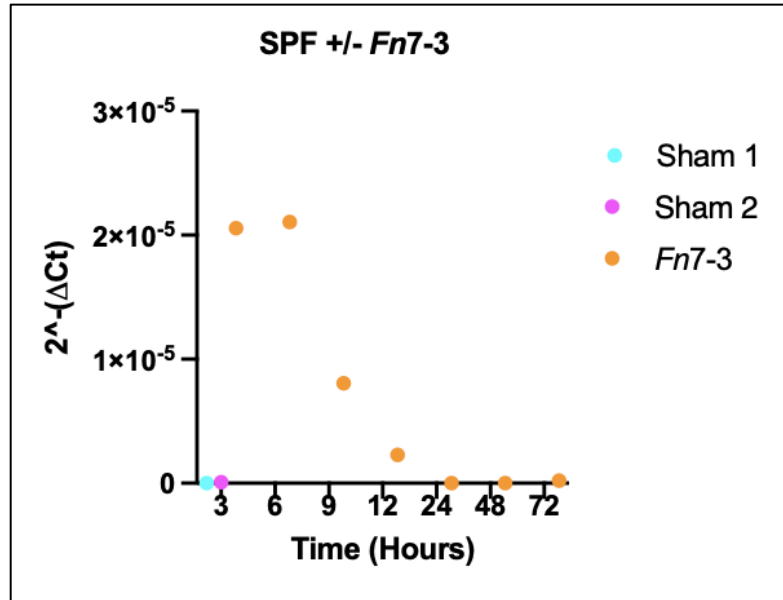


Figure 10: Detection of orally-inoculated *Fn7-3* in fecal samples of SPF mice 72-hours post-gavage. qPCR analysis was performed on DNA extracted from fecal samples after receiving oral inoculation of *Fn7-3*. Blue and magenta data points indicate results from SPF Sham control mice (n=2); Orange data points indicate results from SPF mouse inoculated with *Fn7-3* (n=1). *Fusobacterium* abundance for each sample was assessed by calculating $2^{-\Delta\text{CT}}$, where ΔCt is the difference between cycle threshold of detection of *Fusobacterium* and the cycle threshold of detection of total bacteria (Eubacteria).

***Fusobacterium nucleatum* coculture increases RNA expression of target genes on normal SPF colonic fibroblasts *in vitro*:**

To investigate the impact of *Fusobacterium* on fibroblasts of the mouse colon under normal, homeostatic conditions, we performed *in vitro* coculture experiments using colonic fibroblasts isolated from normal SPF mice. Cells were immunophenotyped after fibroblast isolation and prior to coculture to validate the purity of the resulting fibroblast population (Figure 11). Cells

negative for CD45 (a marker of immune cells), negative for CD31 (a marker for endothelial cells), negative for EpCAM (a marker for epithelial cells), and positive for PDGFR- α (a marker for fibroblasts) were considered the true fibroblast population.

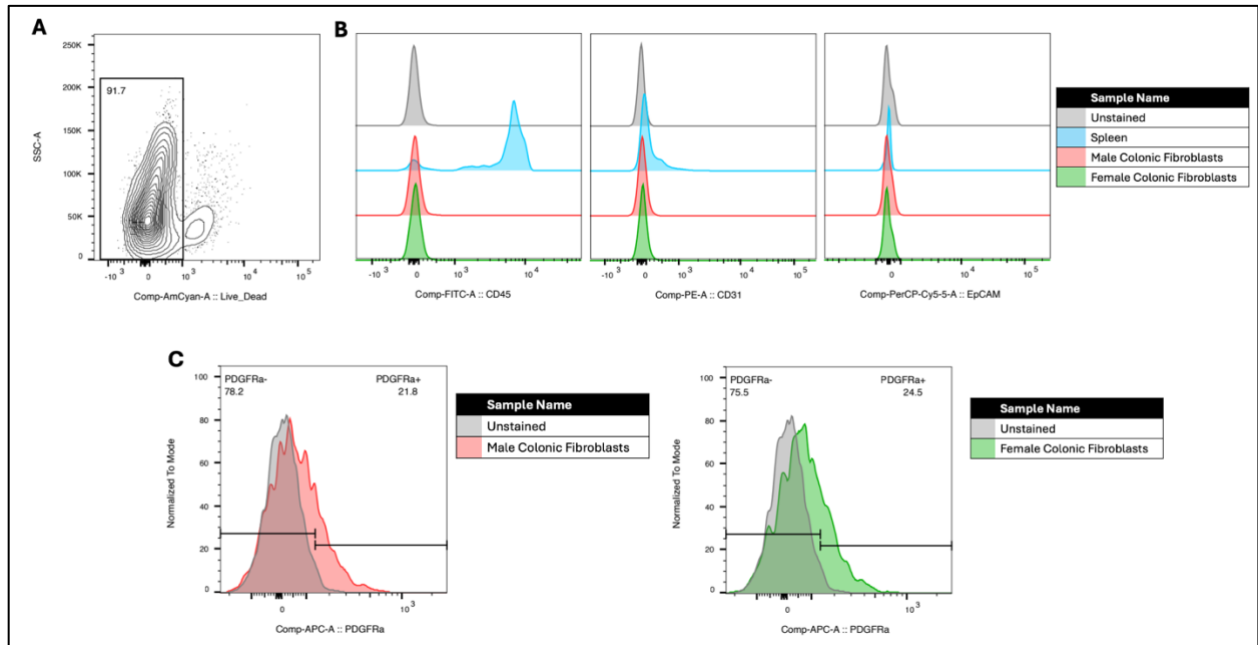


Figure 11: Phenotyping of isolated colonic fibroblasts from WT SPF mice. Representative flow cytometry and histogram plots showing frequency of candidate markers among live cells (A) after fibroblast isolation from colon of WT C57BL/6 mice under SPF conditions. Total live cells were gated as AmCyan- (live). (B) Frequency of candidate cell populations within the indicated live cell subset from isolated fibroblasts. **CD45**: conjugated with FITC for immune cell detection; **CD31**: conjugated with PE for endothelial cell detection; **EpCAM**: conjugated with PerCP Cy5.5 for epithelial cell detection. Grey plots represent frequency of unstained cells. Blue plots represent splenic cells as immune cell positive control. Red plots represent frequency of isolated male colonic fibroblasts. Green plots represent frequency of isolated female colonic fibroblasts. (C) Frequencies of PDGFR- α expression among representative male (Red plot) and female (Green plots) colonic fibroblasts compared to unstained control. Data are representative of one experiment.

Fibroblasts were cocultured with *Fusobacterium*, lipopolysaccharide (LPS), and 10% conditioned media (CM). After harvesting the cells, we investigated the effect of *Fusobacterium* in our coculture experiments by assessing mRNA expression levels of candidate genes. ELISA analysis was also performed on the supernatant of coculture experiments to assess for the secretion of IL-6 into the supernatant.

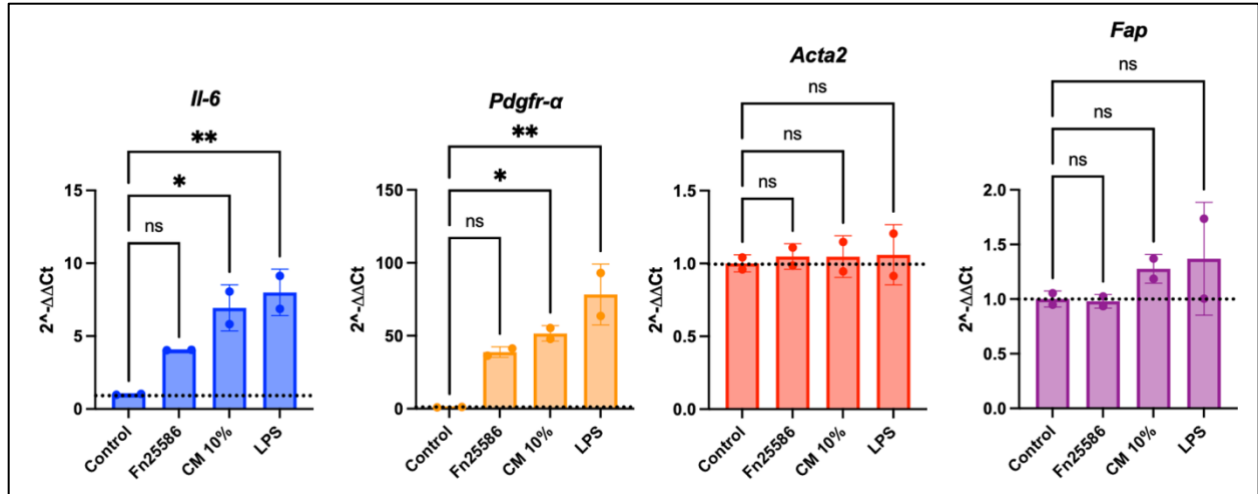


Figure 12: Assessment of RNA expression levels of candidate genes within normal SPF colonic fibroblasts after coculture experiments. After coculture experiments, RT-qPCR analysis of mRNA expression was performed on RNA extracted from normal SPF mouse colonic fibroblasts. Blue data points indicate mRNA expression of *Il-6* [encoding for interleukin-6 (**IL-6**)]. Orange data points indicate mRNA expression of *Pdgfr-α* [encoding for platelet-derived growth factor receptor (**PDGFR-α**)]. Red data points indicate mRNA expression of *Acta2* [encoding for alpha smooth muscle actin (**αSMA**)]. Purple data points indicate mRNA expression of *Fap* [encoding for fibroblast activation protein (**FAP**)]. All data points were performed in technical duplicate and represent detection of transcripts based on the cycle threshold (CT) of Actin beta. P values of <0.05 were considered significant.

Coculture with *Fusobacterium* or microbial associated elements (10% conditioned media, and LPS) resulted in a significant increase in mRNA expression levels of pro-inflammatory cytokine IL-6, and candidate CAF markers PDGFR-α (Figure 12). These results suggest that the presence of *Fusobacterium* and microbial associated elements induce a pro-inflammatory, CAF-like phenotype. These findings are consistent with our understanding of CAFs in the promotion of inflammation and tumor progression. Coculture with *Fusobacterium* (or microbial-associated elements) minimally altered αSMA and FAP mRNA expression levels. This finding suggests that *Fusobacterium* and microbially-associated elements selectively induce the expression of CAF markers, and the expression of αSMA and FAP may require further elements for their activation. ELISA analysis on the supernatant of coculture experiments revealed an increase in

IL-6 among all coculture conditions (Figure 13). Additional analysis through repeat experiments must be performed to establish a more robust phenotype across all experiments and conditions.

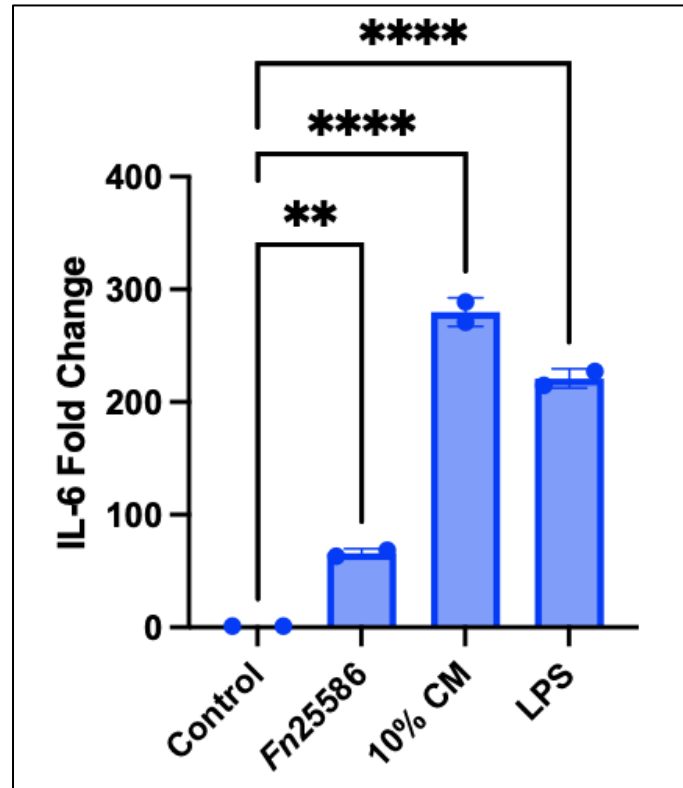


Figure 13: IL-6 detection in the supernatant of cocultured cells. After coculture with either *Fn25586*, 10% conditioned media (CM), or lipopolysaccharide (LPS), the supernatant of cells was collected, sterile filtered, and analyzed by enzyme-linked immunosorbent assay. All data points were performed in technical duplicate and represent detection of IL-6 according to the protocol provided with Legend Max Mouse IL-6 ELISA kit.

Visualization of *Fusobacterium* and PDGFR- α within the CRC tumor microenvironment

To assess the localization and effect of *Fusobacterium* on the protein expression within the colorectal cancer (CRC) tumor microenvironment (TME) of mice, organoids harboring mutations in **KRAS**, **p53**, and **NOTCH** (KPN organoids) were orthotopically injected into the colons of WT C57BL/6 mice¹²². Mice that develop tumors after injection display a tumor microenvironment similar to CRC patients with a CMS4 subtype, a subtype characterized by

high fibroblast and innate immune cell infiltration¹²³. In patients with CRC TME of the CMS4 subtype, the abundance of *Fusobacterium* within the TME is associated with worse prognosis, overall survival, and relapse-free survival^{71,73,124}. For this reason, we also performed intravenous injection of *Fusobacterium* and assessed resulting phenotypes. We hypothesized that the presence of *Fusobacterium* in the TME could induce the expression of our candidate CAF markers, potentially contributing to the worsened phenotypes observed in this model.

After confirming the presence of *Fusobacterium* in the tumors of mice via qPCR analysis, immunofluorescence staining was performed on FFPE tumor sections (Figure 14). Our immunofluorescence staining shows increased expression of PDGFR- α in mice that received *Fusobacterium* intravenous injection. Additionally, detection of *Fn25586* within the KPN TME colocalized with areas of increased expression PDGFR- α within certain areas, suggesting a potential relationship between the detection of *Fn25586* and expression of PDGFR- α . In comparison, the expression of PDGFR- α within the TME of Sham controls showed markedly reduced detection. Immunofluorescence quantification of PDGFR- α expression further validated this finding (Figure 14B). This result supports our hypothesis of the induction of expression of our candidate CAF marker in relation to *Fusobacterium* presence.

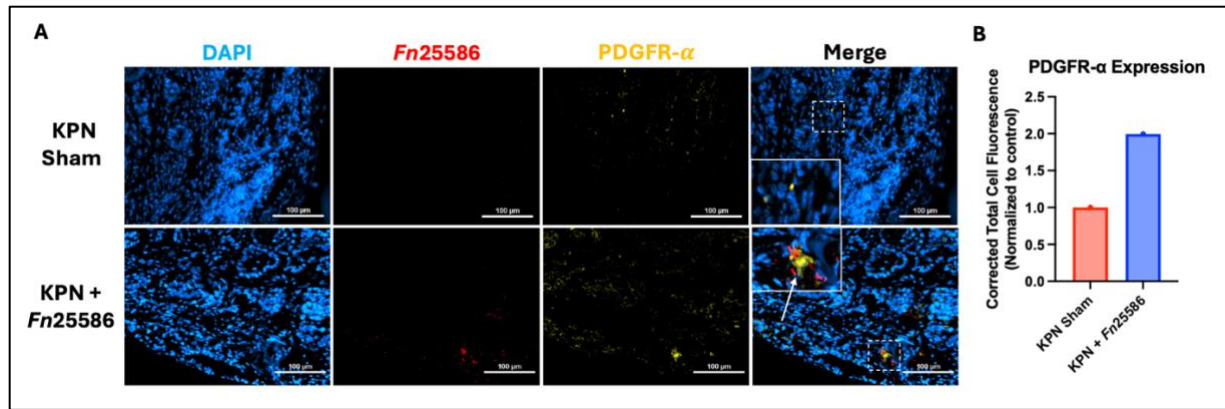


Figure 14: *Fusobacterium nucleatum* and PDGFR-α presence detected in FFPE SPF tumor samples by immunofluorescence staining. Organoids harboring mutations in **KRAS**, **p53**, and **NOTCH1** (KPN organoids) were orthotopically injected into the colons of WT C57BL/6 mice under SPF conditions. Mice were then intravenously inoculated with *Fn25586*, or with PBS as Sham control. After confirming the presence of *Fusobacterium* by qPCR analysis, **(A)** FFPE sections of tumor samples were stained with immunofluorescent antibodies to assess the presence of *Fn25586* and expression of platelet-derived growth factor receptor-alpha (**PDGFR-α**). **DAPI**: nucleus stain, 1:1000 concentration; **Fn25586**: conjugated with **AF555**, 1:800 concentration; **PDGFR-α**: conjugated with **AF647**, 1:200 concentration. **Antigen Retrieval**: Borg Decloaker. **Magnification**: 20X. **(B)** Immunofluorescence quantification of PDGFR-α staining on orthotopically-injected KPN organoids. Red data points indicate results from KPN Sham mice (n=1); Blue data points indicate results from KPN mice intravenously inoculated with *Fn25586* (n=1). Immunofluorescence intensity was assessed using ImageJ Software. Quantification was calculated as the corrected total cell fluorescence (CTCF). CTCF= [Integrated density – (Area x Mean fluorescence of Background)]. Data are represented as results from the same staining batch conditions.

Chapter 3: Discussion and Perspectives

The results of our experiments aided in answering several pertinent, yet previously unanswered questions. Prior to our experiments on Formalin-Fixed, Paraffin-Embedded (FFPE) sections, previous studies had not demonstrated the visualization of *Fusobacterium nucleatum* by indirect immunofluorescence microscopy in the colon of ASF mice using FFPE samples. A lack of antibodies for tissue-based *Fusobacterium* visualization has been a challenge that has hindered efforts to accurately assess the localization of *Fusobacterium* within the colonic oncogenic niche, a crucial environment for understanding its role in the onset of CRC. Microscopic visualization of *Fusobacterium* within the colon is therefore essential for elucidating its interactions with the host at the cellular level, providing critical insights into its role in CRC pathogenesis. To address this challenge, we optimized the use of a polyclonal antibody generated in the Allen-Vercoe lab using standard indirect immunofluorescence mic techniques to tackle this gap in current colorectal cancer (CRC) research.

Our optimization techniques demonstrated that *Fusobacterium* can be reliably detected in on the colon of mice. In experiments where we added *Fusobacterium* to germ-free resected mouse colons, we confirmed that *Fusobacterium* adheres to mouse colon tissue, such that we can detect it using our methods. The success of this experiment is critical for subsequent studies, as it establishes that *Fusobacterium* can (1) be detected by immunofluorescence on FFPE sections, and (2) be visualized in mouse colonic tissue.

Building on the success of these optimization techniques, we showed that *Fusobacterium* can be visualized in stably colonized ASF mice. However, our results revealed unexpected positive staining in negative control samples, raising concerns about the specificity of the

Fusobacterium detection antibody, which we use for detection of *Fn25586*. It is possible that the positive signal reflects detection of other species within the microbiota of our ASF mice, which contain a well-defined consortia of 8 microorganisms that include two Lactobacilli, one Bacteroides, one Flexistipes, and four extremely oxygen-sensitive (EOS) fusiform species¹²⁵. This finding underscores the need for further validation to ensure accurate and reliable detection. To address this issue, we will employ fluorescence *in situ* hybridization (FISH) techniques to further validate the presence of *Fusobacterium* on our FFPE mouse colon tissues. As FISH utilizes fluorescently-labeled DNA probes for detection¹²⁶, it is a highly specific method for identifying target organisms. Implementing this technique will enhance the specificity of our detection approach, providing greater confidence in the identification of *Fusobacterium*.

Nonetheless, in our ASF mouse experiments, we identified a positive association between the presence of *Fusobacterium* and our markers of interest. Specifically, in ASF mouse colon samples stained with immunofluorescence antibodies against the CAF markers PDGFR- α and α SMA, we observed increases in immunofluorescence signal in mice inoculated with *Fusobacterium*, compared to non-inoculated control mice (Sham). These results suggest an upregulation of CAF markers in normal colonic tissues in response to *Fusobacterium*, indicating its influence on protein expression. The potential for *Fusobacterium* to induce changes in protein expression, especially in the expression of CAF markers, may further implicate *Fusobacterium* as an important driver of tumorigenesis.

We successfully integrated an orthotopically-injected organoid model into our studies of *Fusobacterium* localization within the CRC tumor microenvironment. Our KPN organoid model, which contains organoids harboring mutations in KRAS, p53, and NOTCH1 signaling, induces a mouse CRC phenotype characterized by high stromal and fibroblast infiltration¹²². This

phenotype closely resembles the phenotype of patients with a CRC TME of the CMS4 subtype¹²³, which is similarly characterized by high stromal/fibroblast infiltration, poor therapeutic response, and reduced overall and relapse-free survival. Using our optimized immunofluorescence techniques, we observed a corresponding increase in the CAF marker, PDGFR- α , in the presence of *Fusobacterium*. Interestingly, *Fusobacterium* detection was observed to colocalize with certain areas of increased PDGFR- α expression. This may suggest that *Fusobacterium* presence within the tumor positively influences the expression of PDGFR- α , a marker of cancer-associated fibroblasts. These findings support our hypothesis that *Fusobacterium* induces pro-tumorigenic conditions that contribute to CRC, reinforcing its influence on host cells. To validate the effect of *Fusobacterium* on the induction of CAF markers within mouse colonic/CRC local environments, we employed quantification of our immunofluorescence staining and observed a corresponding result in quantified increases in our CAF markers. We will also perform additional immunofluorescence staining targeting other CAF markers including fibroblast specific protein-1 (FSP-1)¹²⁸, podoplanin¹²⁹, and periostin^{52,53}, which have been shown to be upregulated in the CRC TME.

In evaluating the influence of *Fusobacterium* on conventional mice, our coculture experiments with normal, SPF mouse colonic fibroblasts further validate our hypothesis. These experiments were conducted *in vitro*, using isolated mouse colonic fibroblasts, as *Fusobacterium animalis* and *nucleatum* are unable to stably colonize SPF mice. Notably, after coculture with *Fn25586*, we observed substantial increases in the mRNA expression of *Il-6* and *Pdgfr- α* , as well as increased secretion of IL-6 within the coculture supernatant. These results were consistent among all conditions either containing *Fn25586*, or microbially associated elements such as bacterial membrane component, LPS, or conditioned media.

These findings are also consistent with the known features of *Fusobacterium*, as *Fusobacterium* has been shown to induce pro-tumorigenic conditions through the promotion of pro-inflammatory signaling pathways within host cells⁷⁹. One mechanism by which this is accomplished is through host cell recognition of *Fusobacterium* bacterial membrane component, LPS, by toll-like receptor (TLR) 4. The recognition of LPS by TLR4 stimulates the NFkB pathway, which involves NFkB translocation to the nucleus, resulting in the induction of the secretion of pro-inflammatory cytokines, such as IL-6, by host cells⁸⁶. Another mechanism by which *Fusobacterium* promotes a pro-tumorigenic environment is through physical engagement with host cells. The bacterial adhesin protein Fap2 binds to host cell surface protein Gal-GalNAc, leading to *Fusobacterium* invasion and the subsequent induction and release of pro-inflammatory cytokines, such as IL-6¹²⁷. The secretion and presence of these cytokines likely promote a local environment that results in colonic fibroblasts adopting a more activated, CAF-like phenotype.

My preliminary data support that *Fusobacterium* may induce the mRNA expression of each of these targets. Upregulation of these transcripts in coculture with both *Fusobacterium* and LPS likely indicate that the detection of bacterial membrane components upon host-cell contact induces a pro-inflammatory response after recognition by host cell-expressed TLR4. This effect may be broadly characteristic of Gram-negative bacteria, which contain LPS within their bacterial membrane. Upregulation of mRNA transcripts in coculture with conditioned media may indicate that secreted factors of *Fusobacterium*, such as short-chain fatty acids (SCFAs) butyrate, propionate, acetate, and formate, induce a pro-tumorigenic environment that promotes cancer progression, consistent with findings by Ternes et al⁹¹ showing that microbiome-derived formate promotes the progression of CRC.

These findings are consistent with our immunofluorescence microscopy data on the cellular expression of PDGFR- α , further supporting our hypothesis. However, we did not observe a significant increase in the mRNA expression of *FAP*, a marker of fibroblast activation that is implicated in over 90% of carcinomas including CRC¹³⁰. This result is slightly confounding given that we might expect an increase in the mRNA expression of this marker, similarly to the resultant expression observed for PDGFR- α . One possible reason for this is because FAP is almost exclusively not expressed in normal tissues and is induced during conditions of wound healing, tissue repair, fibrosis, and cancer. Its induction is dependent on the transcriptional activity of early growth response factor 1 (EGR1)¹³¹, which has been implicated in cancer, and influenced by the presence of pro-inflammatory cytokines TGF- β , TNF α , and IL-1- β ¹³². Previously published experiments have shown that FAP induction in human samples can occur as early as 24 hours after stimulation with TGF- β , reaching its peak expression at 48 hours, with induction of α SMA following¹³³. Our coculture experiments consisted of a 6-hour infection of *Fusobacterium* cultured with normal mouse colonic fibroblasts; perhaps an extended infection time will reveal a more significant phenotype. It is also possible that our model simply does not induce FAP expression alone. As FAP is almost exclusively not expressed in normal tissues, and since the fibroblasts used in these experiments were isolated from under homeostatic conditions, our model may require a synergy of FAP inducing elements beyond coculture with *Fusobacterium* alone to induce a significant observed change in FAP expression. To further validate these results by establishing a more robust phenotype, we will conduct repeat of these experiments.

Future repeat coculture experiments will include the analysis of the expression of target proteins through western blotting and additional ELISA assessment of cytokine secretions. We

hope that these data will provide additional support for the hypothesis that *Fusobacterium* promotes a pro-tumorigenic environment that influences CRC progression. Western blotting results will provide insight into potential changes in protein expression in normal mouse colonic fibroblasts following coculture with *Fusobacterium*. Additionally, ELISA analysis, performed on filtered supernatants from our coculture experiments, will provide further data on the influence of *Fusobacterium* on the secretion of IL-6 by normal mouse colonic fibroblasts. The results of these analyses will provide insight into whether and how *Fusobacterium* spp. activate CAFs.

In conclusion, this study highlights the role of the microbial compartment on the stromal environment of colorectal tissue which may contribute to CRC progression. By utilizing *in vivo* models with varying gut microbiota complexities, we demonstrate that we can visually detect *Fusobacterium* in the colon of GF and ASF mice. Additionally, we can detect *Fusobacterium* in the local CRC TME and measure its impact on normal fibroblasts across multiple modalities. The results of this study improve our understanding of *Fusobacterium* as an important mediator of CRC progression by identifying other targets which may be influenced by *Fusobacterium*. Given that *Fusobacterium* abundance correlates with poorer treatment response and survival outcome^{73,124,134}, this study uncovers novel potential therapeutic targets to be investigated, with the hope that these targets may have translational relevance in developing effective clinical interventions for CRC.

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