

**Characterization of the Pro-Inflammatory Cytokine
IL-1[beta] on Butyrate Metabolism in Colorectal Cancer Cells**

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ABSTRACT

Genetic and environmental factors impact colorectal cancer incidence [2, 3].

Environmental factors include fiber intake and exercise, which are both postulated to reduce colorectal cancer risk [4-6]. While much is understood in regards to how fiber and its derived nutrients influence inflammation; little is known about how inflammation influences how nutrient impact cell metabolism and physiology.

The short chain fatty acid butyrate, is derived from the fermentation of fiber in the colon and is the preferred energy substrate for colonic epithelial cells (colonocytes) [7, 8]. Butyrate also slows cell proliferation in cancerous colonocytes through its ability to inhibit histone deacetylases (HDACs) [20]. HDACs are enzymes that remove acetyl groups from histones [9]. Inhibition of HDACs results in hyperacetylation, which leads to changes in gene expression [10]. In cancerous colonocytes, inhibition of HDACs by butyrate induces cell cycle arrest, in part, via increased p21 expression [10]. Cancer cells deficient in p21 have been reported to be resistant to butyrate's anti-cancer effects [11]. Thus, blocking p21 induction by butyrate would promote tumor development and may exclude the beneficial effect derived from a high fiber diet toward prevention of the disease.

Patients with colorectal cancer have elevated serum levels of pro-inflammatory cytokines [12-14]. Pro-inflammatory cytokines have been shown to suppress butyrate metabolism in cancerous colonocytes [18]. In addition, colorectal cancer patients have a significant reduction in butyrate producing bacteria [15-17]. The significance of this reduction is unclear, however, taken in conjunction with decreased butyrate metabolism it is likely to impact of butyrate's anti-proliferation activity in the colon.

Identification of mechanisms that mediate decreased butyrate metabolism may aid in the understanding of fiber's role in colorectal cancer. Here, we find that IL-1[beta] dampens butyrate metabolism in colorectal cancer cells. Furthermore, IL-1[beta] decreases butyrate's ability to slow cell proliferation. We identify two mechanisms by which IL-1[beta] induces these effects. First, we determine that IL-1[beta] is operating through its receptor, IL-1RI, and second through a p38-mediated mechanism. These findings are consistent with previous reports that pro-inflammatory cytokines promote cancer cell survival and proliferation [19-22].

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NOMENCLATURE AND ABBREVIATIONS

ABBREVIATIONS

HDAC	Histone deacetylase
HAT	Histone acetylase enzyme
SCFA	Short chain fatty acid
FAP	Familial adenomatous polyposis
IBD	Irritable bowel disease
CD	Crohn's disease
IL-1 β	Interleukin-1 β
IL-1R1	Interleukin-1 receptor, type I
IL-1RA	Interleukin receptor antagonist A
MAPK	Mitogen activated protein kinase
ATP	Adenosine triphosphate
NADH	nicotinamide adenine dinucleotide
TNF- α	Tumor necrosis factor
ECAR	extracellular acidification rate
OCAR	oxygen consumption rate
CAPE	caffeic acid phenethyl ester, NF κ B inhibitor
SB203580	p38 selective inhibitor
2DG	2-deoxyglucose
FCCP	mitochondrial ionophore

CHAPTER I

Literature Review and Specific Aims

Epidemiology

Approximately 1 out of 20 Americans will be diagnosed with colorectal cancer in their lifetime [1]. The majority of people diagnosed are over fifty years of age [2]. More than half of the cases will occur in “Westernized” regions of the world [2]. Incidence rates are not proportional to gender, as men are more likely to develop colorectal cancer than women [3]. Chemotherapeutic treatments, surveillance and lifestyle interventions have led to a decline in colorectal cancer incidence in individuals over the age of 65 [4]. Survival is highly dependent upon disease stage at the time of diagnosis [3, 5]. The majority of colorectal cancers occur sporadically and it is largely thought to be caused by “environmental factors,” [3, 5, 6]. It is estimated that more than half of all cancers and deaths are preventable through lifestyle interventions such as diet and exercise [3].

Risk Factors

Genetic and environmental factors influence colorectal cancer incidence. Factors such as age, gender, personal history with adenomatous polyps, family history of colorectal cancer, race, and inflammatory bowels disease are non-modifiable [1, 3, 6]. Physical activity, obesity, cigarette smoking, alcohol consumption and diet are factors that can be modified reduce colorectal cancer risk [3, 6]. Data suggests that up to 35% of cancer related deaths can be attributed to diet and related factors [7]. For example, evidence based research shows that a diet with a high intake of dietary fiber is associated with decreased colorectal cancer incidence [7-11].

Dietary Fiber

Dietary fiber is composed of indigestible polysaccharides derived from plants [12]. It includes soluble and insoluble fiber [12], where both can be fermented by colonic microbes to provide short chain fatty acids as energetic substrates used by colonocytes [13, 14]. Associations between dietary fiber and reduced risk of colorectal cancer have been around since the 1970's [15]. Epidemiological and experimental studies have provided evidence that dietary fiber has a protective effect against colorectal cancer incidence [4, 7, 8, 16, 17]. The relative risk derived from these experimental data is shown in Fig. 1.1. Relative risk is the rate or risk of the outcome of the intervention group divided by the rate of outcome in the control group [18]. A relative risk of one denotes that the intervention has no effect [18]. If the risk is below one, then the outcome is determined to have a positive or good outcome, whereas those that have a relative risk of above one, are seen to denote harm or poor outcome as compared to control group [18]. In Fig. 1.1, a summary of 16 studies determines that the relative risk of developing colorectal cancer after consuming dietary fiber is approximately 0.86 ($p < 0.05$) [8]. Thus, there is a significant inverse association between developing colorectal cancer and consuming dietary fiber [8].

Despite many studies showing that dietary fiber has an inverse association with development of colorectal cancer, it is controversial. For example, fiber does not appear to confer a protective effect for individuals that develop colorectal cancer before the age of 65 [3, 5]. Confounding factors such as body mass index, income status and even marital status can muddle findings and result in the (statistical) loss of the protective effect [3, 19]. Regardless, based on the evidence, The American Institute of Cancer Research recommends that consumption of dietary fiber [20]. How fiber inhibits colorectal cancer development and the mechanisms that contribute to this protective effect are not well understood

Background: Current Pathophysiology of Colorectal Cancer

The twentieth century brought forth the current, “genetic mutation model,” of cancer [21]. In 1953, Carl Nordling’s applied mathematical modeling demonstrated for the first time, “that the cancerous cell contains not one, but a number of mutated genes,” [21]. Alfred Knudson further developed this theory by applying statistical analysis on childhood cases of retinoblastoma [22]. This directly established that with the accumulation of mutations or “hits” cancer can occur. As such colorectal cancer is a multistep process [23]. The majority of which, occur sporadically through a series of aberrant and stochastic somatic mutations [24]. Given the complexity of colorectal tumorigenesis, a brief review of current theories is needed to characterize sporadic tumorigenesis.

Loeb’s Mutator Phenotype

At this point in time, the idea that the accumulation of “hits” produced cancer required further explanation as it did not characterize all types of cancers. Therefore, a new model was adapted to help explain how tumors could acquire mutations that would promote survival. This model described that DNA polymerase could be altered and result in poor reading of the DNA and by extension assemble new, daughter DNA with copious mutations [25]. Normally, DNA rearrangement is tightly controlled, but in cancer cells this can be mutated and lead to aberrant changes to cell regulation checkpoints [25-27]. The altered DNA polymerase would show a high degree of infidelity resulting in a high mutation rate; deemed the “mutator phenotype,” [27]. The tumor cells with the mutator phenotype have point mutations, microsatellite instability, and loss of heterozygosity [26]. The subsequent genotypes of mutated cells have two fates; 1) lethal mutants that do not survive to replicate; 2) show an established selective advantage of traits that

are passed down to daughter cells [25]. These daughter cells display selective traits that promote cancer cell survival [25].

The evolutionary process of pruning daughter cells to promote survivability and confer selective advantage highlights the need for specific mutations. This allows them to bypass control systems that normally dictate healthy cell replication [27]. It is through genetic instability that mutations are acquired over generations [26-28]. These tumors are products of the original mutator phenotype with newly selected survival traits [26, 28]. This is thought to give rise to tumors that are highly heterogeneous [26, 27]. The pruning of advantageous daughter cells reveals that tumorigenesis takes time. This is of importance as the number of successful mutations is thought to take years. Consequently, number of “hits” will increase as a projection of time [24]. Hence, the rate of colorectal cancer incidence increases with age [5, 6].

Vogelstein’s model of adenoma to carcinoma

The seminal understanding of these selective advantages became apparent within the literature hereafter. Eric Fearon and Bert Vogelstein published their pioneering paper in June 1990 that focused on identifying these advantages and traits [51]. They defined our current understanding of colorectal tumor initiation, promotion, and progression [29]. They proposed that tumors arise from the activations of oncogenes and inactivation of tumor suppressor genes within colonic epithelial cells [29]. The progression of normal to neoplastic tissue is due mutations in four to five genes such as *Ras* and *Apc* genes [29]. Clinical evidence has determined that mutations in these genes allow cancer cells to evade regulatory signaling pathways [29]. The overall effect leads to net cell growth of tumors with selective advantages that avoid normal cell

cycle check points [29]. These tumors show a loss of regulatory functions [30]. For example, genetic mutations of regulatory proteins such as *p53*, *Apc*, *HIF-1*, and *PI3K* are common in many cancers [30]. The accumulation of these events escalate the transition from neoplasms to malignant tumors [29]. The Vogelgram (Fig. 1.2) demonstrates, over time, a theoretical process that hyperplastic colonocytes might utilize during tumorigenesis [29].

The common thread that becomes pervasive throughout the dissemination of the literature is that cancer is a genetic disease that results from the accumulation of stochastic mutations over the course of time. Cancer cells evolve by selective advantage through mutations that manipulate the tumor microenvironment to drive their malignant progression. The accumulation of hits and selective advantages are important in metastasis. Characterization of these traits and their effects are important for therapeutic purposes.

Hallmarks of Cancer

During the 1990's, the general understanding of tumorigenesis had shifted to a multistep process with four to seven stochastic, genetic aberrations resulting in malignancies [25, 29, 31-33]. Over the course of the next decade, research further characterized the heterogeneous nature of tumors resulting in the publication of Hanahan and Weinbergs' influential "Hallmarks of Cancer" in 2000. The "hallmarks" were distilled into "six essential alterations," that characterize cancer progression [34]. They described these traits as evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, and tissue invasion and metastasis [34]. A decade later, two more hallmarks and "enabling characteristics," were added [57]. These new hallmarks include deregulating cellular

energetics and avoiding immune destruction [57]. The enabling characteristics are genome instability mutations and tumor-promoting inflammation [35]. These hallmarks work in concert to create an ideal, heterotypic microenvironment that provides the selective advantages necessary for tumor survival [34-37]. Therefore, it is important and necessary to characterize these hallmarks as they may be utilized for therapeutic purposes.

Of the new hallmarks published in 2011, two are important within the focus of my research: the deregulation of cellular energetics and tumor promoting inflammation. It is known that cancer cells can deregulate cellular energetics by shifting their preference for nutrient metabolism [38]. Recent findings have shown that pro-inflammatory cytokines influence cancer cell metabolism [38-41]. Very little is available regarding the mechanism(s) by which pro-inflammatory cytokines influence cellular energetics, especially in colorectal cancer. Specifically, the effect that IL-1 β has on butyrate metabolism in the colonocyte.

Chronic Inflammation and Tumor Promotion

Inflammation can be characterized as the complex biological response to pathogenic or harmful stimuli [51]. It can be facilitated by a wide variety of mediators including pro-inflammatory cytokines as a response to harmful stimuli i.e. cancer cells [51]. Inflammation is considered an enabling characteristic of cancers and promotes the pathogenesis of many human carcinomas including breast, esophageal, pancreatic, and colorectal [35, 36, 42]. Pro-inflammatory cytokines are, in part, mediators of the inflammatory process [39, 41, 43, 44]. Serum levels of pro-inflammatory cytokines are elevated in colorectal cancer patients and are associated with a poor prognosis [45, 46]. Elevated pro-inflammatory cytokines, such as TNF- α

and IL-1 β , stimulate signaling pathways that propagate more inflammation [47]. This feed forward loop is known to enable tumorigenic activity such as invasion and metastasis [48]. Furthermore, TNF- α has been shown to alter nutrient utilization [49]. The long term effect of this is unknown but may promote cancer progression. It is to the end that the mechanisms by which pro-inflammatory cytokines alter cancer cell metabolism to promote survival are necessary to study.

The role of inflammation in tumorigenesis is strongly supported by research-based evidence [50-53]. The more robust associations arise in patients with inflammatory bowel diseases, such as Crohn's or ulcerative colitis [42]. These rank among the top three risks for developing colorectal cancer [41, 47, 54]. Other inflammatory disease states such as pelvic inflammatory disease or Hepatitis C are associated with increased risk of ovarian or liver cancer [42]. Often times animal models are used for discovering and validating inflammatory mechanisms that influence the transformation of normal tissue to neoplastic growth [42, 55].

For example, nude mice subcutaneously implanted with adenoma cells do not develop any malignant growths [56]. However, when the same cells are implanted with a small piece of plastic, mice show robust tumor growth [56]. Histological analysis of these tumors reveal a highly proliferative, fibrous stroma within the tumors [56]. The authors postulate, that the irritating piece of plastic promoted a localized immune response by stimulating an inflammatory response and thus promote the tumorigenic process. This suggests that inflammation is a necessary component for neoplastic cells to transform into tumors [56].

Moreover, individual pro-inflammatory cytokines, the mediators of inflammation, can be sufficient enough to induce carcinogenesis [57-60]. For example, polymorphisms in the IL-1 β gene are often accompanied by an increase in IL-1 β serum levels [61]. This is linked to an

increased risk of gastric cancer [59]. As such, IL-1 β transgenic mice develop spontaneous gastric inflammation, neoplasia, and eventually carcinoma [60]. Overexpression of IL-1 β in these mice causes an accelerated progression of gastric carcinoma [60]. These findings indicate that IL-1 β can stimulate cancer progression, suggesting that inflammation, induced by IL-1 β , may be sufficient to stimulate cancer initiation and progression.

Conversely, in the absence of inflammatory factors, tumorigenesis does not appear and the incidence of cancer is significantly reduced [50, 51, 62, 63]. For example, mutant TNF- α knockout mice develop fewer skin lesions and tumors upon exposure to chemical carcinogenesis [64-66]. Interleukin 1- β converting enzyme (ICE) knockout mice, show a reduction in colorectal tumorigenesis [67]. These findings indicate the pro-inflammatory cytokines facilitate cancer progression. Therefore, inhibition of their activity results in decreased tumor maturity and number [39, 68]. To this end, mice receiving anti-inflammatory pharmaceutical interventions display fewer, less mature tumors [41, 50, 62, 69]. Likewise, when the transgenic mice over-express IL-1 β are treated with IL-1RA, a natural antagonist of IL-1 β , display a significantly reduced inflammatory and neoplastic response [61]. Even dietary interventions are shown to reduce carcinogenesis. Mice fed tributyrin, a butyrate analog, are less susceptible to colonic adenoma formation in chemically induced colorectal carcinoma models [70]. This indicates that butyrate confers a protective effect. These data suggest that inflammatory factors, such as pro-inflammatory cytokines, enable carcinogenesis and as such attenuation of their signaling can reduce neoplastic incidence and progression. In short, elucidation of the mechanisms that influence tumorigenesis may provide important insight into colorectal cancer formation.

Interleukin-1

IL-1 β is part of the interleukin-1 cytokine family [71]. There are three forms of IL-1: IL-1 α , IL-1 β and IL-1RA derived from the same gene through alternative splicing [71, 72]. Clinical evidence suggests that the pro-inflammatory cytokine, IL-1 β , is associated with tumorigenesis [71-75]. IL-1 β stimulates invasion and metastasis in cancers [39, 76]. IL-1 β is elevated in the serum of colorectal cancer patients and is associated with poor prognosis [77-79]. Interestingly, the human body has two naturally occurring mechanisms by which IL-1 β activity can be restricted. The first being the expression of a decoy receptor that does not transduce signal and the second being the expression of IL-1RA which can block IL-1 β from binding to the IL-1RI receptor [71].

IL-1RA

Interleukin 1 receptor antagonist or IL-1RA can bind to the type I receptor and block any other agonists from binding. The binding of IL-1RA to the type I receptor is not fully understood, but it thought to have an important role in blocking the pleiotropic effect of IL-1 β [72, 80, 81]. Polymorphisms in the IL-1RA gene have been associated with colorectal cancer incidence [82]. The use of the recombinant form of IL-1RA, Anakinra, has demonstrated its value as a natural anti-inflammatory protein in animal and human models for decades [72, 73, 80, 81]. IL-1RA knockout mice display signs and symptoms consistent with arthritis, arteritis, and skin inflammation, thereby suggesting its role to combat the effects of IL-1 α and IL-1 β *in vivo* [83-86]. Likewise, deficiency in IL-1RA, due to homozygous mutations, results in skin lesions and bone malformations suggesting that it is necessary to combat the signal transduction

and inflammatory effects of IL-1 β and IL-1 α [83, 87]. Animal models that overexpress IL-1 β have accelerated progression of tumors [99], and is attenuated by IL-1RA administration [61]. These findings suggest that IL-1RA has its own mechanisms of action in addition to blocking IL-1 β 's signal transduction. IL-1RA's ability to block IL-1 β 's activity is of significance and has been identified as a potential therapy in the prevention and treatment of colorectal cancers [72, 73, 81, 88, 89].

IL-1 Receptors

There are two primary IL-1 receptors, interleukin type I and type II (IL-1RI and IL-1RII) [72]. Alternative splicing results in the two primary receptors [72, 90]. The functional receptor, IL-1RI, is expressed in high concentration on endothelial, smooth muscle and epithelial cells [72, 91]. It is expressed in colonocytes and in the HCT116 colorectal cancer cell line [92].

IL-1RII acts as an inert, “decoy,” or “sink” receptor and does not appear to transduce signal [72, 80, 81, 88, 93]. The type II receptor has a high affinity for IL-1 β compared to type I receptor. This suggests that any IL-1 β binding to the decoy type II receptor reduces the amount of IL-1 β binding to the (signal-transducing) type I receptor [72]. Healthy humans show a tenfold higher presentation of IL-1RII to IL-1RI [72]. The relative binding capacity for the three IL-1 ligands are as follows for type I receptor IL-1Ra > IL-1 α > IL-1 β and type II IL-1 β > IL-1 α > IL-1RA [72].

IL-1 β

IL-1 β is also known as endogenous pyrogen, granulocytic pyrogen, leukocytic pyrogen, leukocytic endogenous mediator, lymphocyte activating factor, hemopoietin-1, and osteoclast activating factor [71]. It was the first interleukin studied as a fever-inducing molecule [85]. It was cloned in 1984 [71, 94]. IL-1 β is synthesized in a precursor form before being cleaved by cytosolic IL-1 β -converting enzyme (ICE) to its active form [95]. It is considered a major upstream pro-inflammatory cytokine that acts as an “alarm” by inducing a cascade of chain reactions [88]. Upon cleavage, IL-1 β , is secreted from the cell where it can target its receptor, IL-1RI [71, 96].

IL-1 β Signal Transduction

IL-1 β activates IL-1RI upon binding as shown in Fig. 1.3. It stimulates an intracellular signal transduction cascade that targets the mitogen activated kinases (JNK, ERK $\frac{1}{2}$ and p38), their transcription factor c-Jun and the transcription factor NF- κ B via its subunits p65 and/or p50 [97]. IL-1 β is unique among the cytokines, in that the body produces a receptor antagonist to counteract IL-1 β 's effect [71, 93]. IL-1RA blocks IL-1 β from binding to IL-1RI, thereby inhibiting intracellular signaling [73, 80, 81, 93].

p38

p38, also known as mitogen-activated protein kinase (MAPK), is an intracellular target of IL-1 β signal transduction [96, 98-101]. The p38 MAPK is a family of serine/threonine protein kinases that play important roles in cellular responses to external stress signals such as inflammation [96, 102, 103]. p38 plays a role in cell cycle regulation, metabolism, survival pathways, and cell differentiation [98, 99,

101]. p38 is a 38 kDa protein that can be rapidly phosphorylated in response to many types of stimuli including IL-1 β [103]. There are four splice variants of p38 which include an α , β , γ , and δ [98, 99, 101]. Isoforms α and β are ubiquitously expressed [98]. However, the γ and δ are differentially expressed and are dependent on the tissue type [98]. Inhibition of p38 suppresses IBD and attenuates colorectal cancer progression [99, 101, 104, 105]. It is to this end that understanding the mechanisms by which p38 influences colorectal cancer progression are of importance.

p38 and Colorectal Cancer

One of the hallmarks of cancer is the evasion of regulatory signaling resulting in uncontrolled proliferation [35]. The accumulation of these hallmarks result in deregulation of signaling pathways that regulate cell cycle [34, 35]. p38 is just one of many pathways that regulates the balance between apoptosis and cell survival [98, 99]. It is often “hijacked” by cancer cells as a means of evading regulatory signals [99]. p38 is associated with tumor growth in many cancers including colorectal [105, 106]. For example, patient derived colorectal cancer xenografts treated with p38 inhibitors show reduced net growth as compared to their untreated counterparts [100]. This suggests that p38 supports net cell growth and overall tumorigenesis. Interestingly, p38 isoforms appear to have differing roles in tumorigenesis. For example, p38 γ knockout mice demonstrate rapidly increased tumorigenesis compared to their WT controls [103]. This alludes to a protective effect for p38 γ . Likewise, p38 α KO mice showed significantly more tumors and increased tumor burden size as compared to their WT controls [107]. These data reveal that p38 isoforms may suppress cancer progression. This may be linked to cancer cell metabolism, as p38 is associated with stimulating a metabolic shift in colorectal cancers that

sustains cancer cell proliferation [105, 108]. It is to this end that cancer cells demonstrate the ability to commandeer intracellular signaling pathways i.e. p38 to stimulate cancer cell metabolism.

IL-1 β and carcinogenesis

Inflammation and pro-inflammatory cytokines have long been implicated for their role in colorectal cancer pathogenesis [35, 39, 41, 42, 45, 73, 109-112]. “Smoldering” inflammation is a phrase often used to describe the subclinical inflammation that is associated with colorectal cancer [112, 113]. Smoldering inflammation supports the pathogenesis of colorectal cancer through a wide variety of mechanisms [111-114]. These include chemokine recruitment, angiogenesis, matrix remodeling, and tumor cell proliferation [114]. These survival mechanisms are, in part, controlled by pro-inflammatory cytokines signaling such as TNF- α or IL-1 β [111-116]. IL-1 β is a key modulator of these survival pathways and has been used *in vivo* numerous times to demonstrate its role in inflammation-induced tumor progression [59, 85, 86, 95, 96, 117-121]. For example, matrix metalloproteinases (MMPs) are enzymes required for normal extracellular matrix turnover and tissue remodeling [122]. MMP7 is associated with inflammatory processes that can contribute to tumorigenesis specifically colorectal cancers [123]. Metalloproteinase-7-deficient (MMP-7^{-/-}) mice have a three-fold higher intestinal baseline of IL-1 β (~20 vs 60 pg/mL) [123]. These mice are more susceptible to dextran sulfate sodium (DSS) induced colitis as compared to control mice [123]. Both wild type and mutant mice were treated with 4% DSS for 9 days, both developed severe colitis, however the mutant mice died on day nine due to the severity of their colitis [123]. This suggests that the increased

baseline inflammation (as denoted by the three-fold increase in baseline IL-1 β) induced an exacerbated response to the treatment and therefore expedited disease progression [123]. MMPs are thought to contribute to IL-1 β degradation [122]. This suggests, that the increase in baseline IL-1 β in the matrix metalloproteinase (MMP) KO may be a result of decreased IL-1 β turnover [109]. These findings suggest that IL-1 β expedited the progression and severity of DSS-induced colitis and therefore, appear to play a significant role in inflammation based disease models.

To illustrate this further, a single dose of IL-1 β prior to injection of tumor cells increases the colonization of malignant cells in the lungs [120, 121]. This demonstrates that IL-1 β encourages metastasis via stimulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) [83, 128]. These data exemplify IL-1 β 's ability to stimulate survival traits in cancer cells promote tumorigenesis. To this end, a reduction its signaling pathway seems imperative to inhibit or slow the progression of cancer. Interestingly, dietary nutrients, such as butyrate, reverse pro-inflammatory-cytokine induced adhesion molecule formation [124-126]. Butyrate also inhibits the activation NF κ B, an intracellular inflammatory signaling protein, by preventing its translocation to the nucleus [127, 128]. These functions may contribute to butyrate's anti-cancer effects. It is to the end that as IL-1 β and butyrate have overlapping cell signaling properties, it is important to understand the effect they have in concert.

Crossroads of Inflammation and Diet

Clinical research strongly suggests the protective effects that diet and dietary components have in preventing cancer [129]. For example, increased dietary fiber intake has been shown to

aid in the reduction of “flare-ups” in patients with CD and IBD [130]. The mechanism(s) of action is still not fully understood [133]. One mechanism may be butyrate; as butyrate enemas are one of the most effective treatments for IBD in the clinic [13, 131, 132]. Butyrate has been shown to mitigate inflammatory responses within colonocytes [126, 128]. Interestingly, butyrate metabolism has been shown to be down-regulated in IBDs and colorectal cancer [49, 133-136]. The long term effects of this are unclear, but may be associated with mitigating the pro-cancer effects of pro-inflammatory cytokines. Pro-inflammatory cytokines are found to be upregulated in IBDs and colorectal cancer [77, 137]. Pro-inflammatory cytokines have been shown to shift colonocyte metabolism away from butyrate utilization [49, 52, 110, 138]. Therefore, understanding the function of pro-inflammatory cytokine induced mechanisms that perturb butyrate metabolism could yield key insight into colorectal cancer pathology and prevention.

Dietary Fiber and Butyrate Metabolism

The gastrointestinal tract is inhabited by microorganisms that have a symbiotic/mutualistic relationship with its host [139]. These trillions of microorganisms ferment indigestible matter such as resistant starches, unabsorbed sugars, and polysaccharides [140, 141]. Within the colon, this indigestible matter can be fermented to form short chain fatty acids that provide 10-30% of the total ingested energy [140]. Without these microbes, as in the case of germ free mice, they must consume 10-30% more food to maintain the same body weight as conventionally raised mice (those with normal microbiota) [141]. Despite the germ free mice consuming more food, their epididymal fat pads are nearly half the size of their microbiome-laden counterparts [141, 142]. Suggesting the role of the microbiome and its fermentation

products such as butyrate, contribute to overall health is a significant one. It is to this end that changes in luminal butyrate concentration may affect human health. Recent evidence has demonstrated that there is a reduction in butyrate producing bacteria in patients with IBDs and colorectal cancer [135, 143]. The implications of this reduction are unclear, but may accentuate the unique role of butyrate in preventing IBD and colorectal cancer.

As early as the 1970's, observational research noted that Africans have much lower incidence of colorectal cancer and this is thought to be due to their increased fiber intake as compared to a Western diet (Fig. 1.4) [15]. A number of theories were proposed regarding this effect [32]. First, the dilution of carcinogens within the stool mass could lead to less interaction with the intestinal epithelia [32]. Second, there could be a decrease in transit time for the stool mass [32]. Third, fermentation of fiber could result in a reduction of colonic pH due to the production of short chain fatty acids [32]. This is supported by clinical research. For example, additional intake of 10-30 grams of fermentable fibers increases short chain fatty acids production and fecal bulk, resulting in shortened transit times [14].

SCFAs and colonocyte health

The fermentation of dietary fiber results within the colon in the formation of short chain fatty acids (SCFAs) [144-146]. SCFAs are organic fatty acids that vary in length from one to six carbon atoms in various carbon arrangement [14]. Acetate, propionate, and butyrate are found in high (mM) concentrations within the colon [145]. Fecal SCFAs are produced in the following order of acetate > propionate $\geq$ butyrate with approximately 60:20:20 for their molar ratio in the colonic lumen [14]. They are principal anions which aid in absorption, lower pH, and maintain

luminal osmolarity maintenance [14]. These SCFAs are significant sources of energy in the colon and suppress glucose and glutamine oxidation [145, 147]. They regulate colonocyte metabolism which in turn regulates colonocyte growth, differentiation, and proliferation [34]

Acetate

Acetate is the principal SCFA in the large intestine [14, 145, 147]. Acetate is readily absorbed in the colon. It is metabolized in the colon, but the majority (50-70%) is used by the liver for hepatic cholesterol synthesis and lipogenesis [14]. The expression of acetyl-CoA synthetase in adipose and liver catalyzes the formation of acetyl CoA; the first step in activation of lipogenesis [14]. In fact, subjects given rectal infusions of acetate show a dose dependent increase in the levels of serum cholesterol and triacylglycerols (TAGs) [14]. Accordingly, the formation of lipid products from acetate supplementation promotes cellular proliferation and increased oxidation in the cancerous colonocyte cell line, HT-29 [148]. The effects of acetate on lipogenesis can be further amplified in the presence of glucose with one and a half more acetate incorporated into lipid formation than when butyrate is present in the colonocyte [148]. Furthermore, acetate can increase histone acetylation, but is not a histone deacetylase inhibitor as butyrate is, and therefore, it does not possess the same growth inhibitory properties as butyrate in cancerous colonocytes [148].

Propionate

Propionate is a three-carbon SCFA produced in the colon [145, 147]. It is absorbed more readily than acetate in the colon [14]. The liver has been shown to have a 90% uptake of

propionate as opposed to 75% of acetate via the portal vein [14]. Furthermore, colonic infusions of propionate promote gluconeogenic pathways, whereas acetate increased serum cholesterol and glucagon, and reduced free fatty acids [149]. This suggests that propionate may play a role in cholesterol synthesis inhibition and regulate gluconeogenesis via its conversion to succinyl CoA in the mitochondria [14, 150].

Butyrate

Butyrate, a four-carbon fatty acid, plays a key role in colonocyte health and homeostasis as shown in Figure 1.5 [14, 145]. It is the primary energy source of colonocytes [145, 147]. Butyrate regulates the maturation and differentiation of colonocytes [147]. In the case of colorectal cancer, butyrate induces cell cycle arrest and apoptosis in cancerous colonocytes [14, 134, 151]. This is due to cancerous colonocytes having altered butyrate metabolism (suppression of oxidation), which changes the fate of butyrate and form diminishment of its anti-cancer properties [49, 152-155]. These mechanisms are not fully understood. However, evidence suggests that this may be, in part, due to the presence of pro-inflammatory cytokines which alter butyrate's metabolic fate [155]. Understanding the mechanisms that affect butyrate homeostasis in the colon are important to understand its role in prevention and treatment of colorectal cancer.

Butyrate's concentration in the lumen of the colon

Gut microbiota contribute to the acquisition of energy within their human host by fermenting what would otherwise be non-digestible sources of carbohydrates into short chain

fatty acids such as acetate, propionate and butyrate as shown in Fig. 1.5 [133, 156-158]. Total short chain fatty acid contents can reach approximately 100 mM in the gut lumen [133, 158]. Butyrate is found in very high concentrations [mM] within the lumen of the colon [158]. A concentration gradient is formed as butyrate passes through the unstirred water layer and down into the crypt [158]. Thus, higher butyrate levels exist in the lumen and diminish as butyrate moves down the crypt structure. Colonocytes in the crypt function to lower butyrate levels by oxidizing it as an energy source [159]. Thereby, lowering the concentration as butyrate moves down the crypt which, in turn, promotes stem cell proliferation. Butyrate uptake by colonocytes is mediated by a solute carrier family 16 member (SLC16A1), also known as monocarboxylic acid transporter 1 (MCT1) [136]. Upon uptake in the colonocyte, butyrate can be utilized as an energy source or a HDAC inhibitor [145].

Butyrate as a primary energy source

Isolated colonocytes display different oxidative capacities depending on the metabolic substrate [145]. Radiolabeled glucose and/or butyrate contribute to 59% and 72%, respectively, of the oxygen consumption in isolated colonocytes [145]. These findings demonstrate that the colonocyte prefers butyrate as a primary energy source [145]. Following uptake by the colonocyte as shown in Fig. 1.6, butyrate is metabolized in the mitochondria to form two acetyl CoA molecules [136]. The resulting acetyl CoA molecules enter the Krebs Cycle to eventually produce ATP or contribute acetyl groups for lipogenesis or histone acetylation [136, 148, 160, 161]. In the presence of 5 mM glucose, butyrate is mainly used for oxidation [144]. However, at higher concentrations (>5mM) butyrate can play a unique role in histone modification by

inhibiting histone deacetylases [152]. Butyrate is unique among the short chain fatty acids for its dual functions that contribute to histone modification and colonocyte metabolism [162].

Butyrate and histone acetylation

Histone acetylation is an epigenetic mechanism that regulates gene expression as shown in Figure 1.7 [70, 133, 163, 164]. Histone acetylation is regulated by acetyl groups, histone acetyltransferases (HATs) and histone deacetylase (HDACs). HATs catalyze the addition of acetyl groups and HDACs catalyze the removal of acetyl groups from lysine tails within histones [13, 133, 156]. HDACs are classified into four classes (I-IV); butyrate inhibits classes I, II and III [162, 165, 166]. Interestingly, butyrate affects the activity of both HATs and HDACs [152, 162]. Butyrate provides acetyl groups that stimulate HAT activity. In addition, butyrate inhibits HDAC activity resulting in histone hyperacetylation [152, 162]. The resulting histone hyperacetylation regulates gene expression by altering chromatin organization [162, 167]. This allows induction or repression of certain genes, including cell cycle regulation proteins such as p21 [162, 168-171]. Butyrate's ability to hyperacetylate histones affects only 2% of gene expression [162]. Despite this small amount, that 2% is crucial in colonocyte health and regulation [13]. This transcriptional regulation promotes maturation and differentiation in normal, healthy colon cells [13]. Yet, in cancerous colonocytes butyrate induces cell cycle arrest and even apoptosis [162]. This dichotomy is known as the butyrate paradox [152, 172].

Butyrate's mechanism of growth restriction of cancerous colonocytes

Butyrate has been extensively studied for its growth arresting properties in cancerous colonocytes [13, 133, 156, 161, 167-171, 173-179]. For example, treatment of as low as 2 mM butyrate can inhibit cell proliferation in HT-29 cells, a colorectal cancer cell line [148]. Yet, acetate at concentrations as high as 10 mM does not produce this same effect [148]. In fact, it has the reverse effect, acetate stimulates tumor cell proliferation by increasing the available acetyl groups that are necessary for lipogenesis that contribute to new cell turnover [148]. Acetate is used elucidate butyrate's short chain fatty effects [14, 145, 148, 149, 156, 158], but it does not inhibit cell proliferation as butyrate does. Different effect between these two SCFAs highlight the fact that the mechanisms by which butyrate inhibits cancer cell proliferation need to be further clarified [156, 161, 173, 176-179].

Butyrate's ability to inhibit HDAC activity results in hyperacetylation and transcriptional regulation [13, 177]. Butyrate stimulates hyperacetylation of the *p21* promotor region resulting in increased p21 expression and subsequently cell cycle regulation in cancerous colonocytes [169-171, 174]. To separate butyrate's short chain fatty acid effect from its HDAC inhibition activity, trichostatin A (TSA) is often used [162]. TSA inhibits HDAC activity in a very similar manner to butyrate [13, 168]. As such, TSA and butyrate both stimulate expression of p21 through the same mechanism of HDAC inhibition [13, 162, 180]. Therefore, butyrate's ability to stimulate cell cycle regulation is dependent upon its ability to inhibit HDAC activity [169-171, 174, 181], not due to its short chain fatty acid effects on metabolism [148].

p21

At high concentrations (>5mM), butyrate can inhibit HDAC activity leading to elevated p21 expression in colorectal cancer cells [13, 152]. The eukaryotic cell cycle is controlled by two families of proteins: the cyclins and cyclin dependent kinases (CDKs) [171, 182]. These proteins, in part, regulate the progression of cell growth and cell cycle arrest [168]. For example, D cyclins regulate the G1-S transition phase and bind with its respective CDKs 4 and 6 before complexing with a retinoblastoma (Rb) protein [162]. This signals the cells to progress into the S phase [171]. However, in the presence of p21, this progression can be selectively inhibited causing cell growth arrest and even apoptosis through BAX/BIM mediated pathway as shown in Fig. 1.8 [171]. Many have demonstrated butyrate's unique ability to increase hyperacetylation in the promotor region of p21, thereby increasing its transcription/expression, and subsequently ability to obstruct cell cycle progression past the G1 phase in cancerous colonocytes [162, 169-171, 174].

Murine Models

Butyrate is a byproduct of dietary fiber fermentation, and therefore, its production is dependent upon the butyrate producing bacteria present within the microbiome. Patients with colorectal cancer often have less butyrate producing bacteria [135, 183, 184]. The effect of this dysbiosis is unclear.

Dietary fiber significantly contributes to host energy homeostasis and colonic health, in part, by being broken down into an energy source by colonic microbiota [70]. For example, germ free (GF) and conventionally raised (CR) mice (those with a complete microbiome) display

functional differences in energy metabolism [70]. GF mice must increase their intake by 10-30% as compared to CR mice to maintain body weight [141, 142, 185]. Colonocytes isolated from GF mice have significant differences in mitochondrial metabolism (i.e. diminished NADH/ATP production) and display a different metabolic phenotype compared to CR mice [70].

Interestingly, all of these differences are rescued upon butyrate supplementation [141]. These findings demonstrate that butyrate plays a fundamental role in colonic health and overall wellbeing in this animal model. Just as dietary fiber promotes overall colonic health it can also have a protective effect against colorectal cancer formation. To demonstrate these effects, chemical induction of colorectal cancer is used in murine models as they are highly reliable and are easily replicated. A common chemical induction model uses azoxymethane (AOM) to induce DNA mutations [186, 187]. AOM induced-tumors histologically represent many characteristics common with human colorectal cancers such as *Kras* mutations [187]. These tumors form over months [29, 187-189]. Dextran sulfate sodium (DSS) added to the drinking water dramatically reduces the latency period of tumor formation [186, 187, 190]. AOM/DSS murine models replicate an inflammation-based, colitis-like colorectal cancer model.

Dietary fiber and its by product butyrate have a protective effect against adenomatous polyp formation [70, 188, 190-192]. For example, germ free mice (GF) colonized with or without butyrate producing bacteria (BPB) show that the mice with BPB had a protective effect in developing tumors (Table 1.1) [70]. Briefly, mice underwent AOM/DSS treatment to induce tumorigenesis [70]. Throughout, mice were maintained on low or high fiber diets that were calorically matched [70]. GF with the BPB had significantly increased luminal butyrate levels and had significantly fewer tumors than control groups (1 tumor versus 3-4 tumors; Table 1.2)

[70]. This signifies that a high fiber diet protects against colorectal tumorigenesis in a microbiota and butyrate dependent manner [70].

To further confirm the tumor suppressive effects of the BPB, the butyrate producing species was genetically altered to limit the formation of butyrate [70]. In the second round of experiments, the experimental model showed significantly decreased luminal butyrate and lost the protective effect of butyrate resulting in increased tumor size and colorectal cancer progression[70].

However, when the same model is used conjunction with a tributyrin supplemented diet, a butyrate analog, the protective effect is rescued in a microbiota-independent manner [70]. In addition to this protective effect, these tumors showed significantly reduced size and histopathologic progression as compared to the control groups [70].

This study establishes that dietary fiber has protective These findings, however, do not support the role of an inflammation induced tumorigenesis model with butyrate suppressing neoplastic growth in a murine model [70, 192].

Translating to humans

Highly controlled animal model testing does not always directly translate to human outcomes. While research based evidence supports the protective role of dietary fiber and SCFAs [7, 9, 10, 193], its translation into the general population isn't always as clear. For example, many factors such as methodology, microbiome, age, gender, and animal model can confound dietary fiber's protective association [13, 14, 194]. However, there are association to suggest that butyrate is advantageous for humans. It is especially pronounced when using butyrate as a treatment for bowel diseases. For example, fecal samples of patients

with IBDs show reduced SCFA appearance [195]. Interestingly, when isolated, these colonocytes show differences in their butyrate oxidation capacity [153]. Moreover, patients undergoing surgical diversion of their colon frequently develop a condition known as diversion colitis [131]. Supplementation with butyrate enemas in diversion colitis patients, none of whom had previous evidence of CD, UC, or IBD, resulted in mitigation of inflammatory symptoms and remission [131]. Likewise, avoidance of dietary fiber has demonstrated increases of “flares” but patients with IBDs [196]. In CD patients, butyrate supplementation causes a decrease in pro-inflammatory cytokine expression [128]. These mechanisms by which dietary fiber and its fermentation byproduct, butyrate, influence pro-inflammatory cytokines signaling are necessary to illuminate the path of understanding colorectal cancer prevention and treatment.

Specific Aims

In general, the pro-inflammatory based molecular mechanisms that result in decreased butyrate utilization within cancerous colonocytes are not well defined. Identification of these mechanisms may aid in the understanding of colorectal cancer treatment and prevention. A major goal of this research project is to elucidate the effect of the pro-inflammatory cytokine, IL-1 β , on butyrate metabolism in the cancerous colonocyte. More specifically, this research focuses on the effect of IL-1 β on butyrate oxidation and its fate in the cancerous colonocyte. Therefore, these studies will address the following:

- 1) Determine whether colon cancer cell metabolism is affected by IL-1 β in reference to butyrate oxidation; and determine if other proinflammatory cytokines affect butyrate metabolism.

2) Elucidation the mechanism by which IL-1 β shifts colonocyte butyrate metabolism; and determine if it is specific to the colonocyte.

3) Interrogate downstream consequences of IL-1 β 's effect on butyrate metabolism.

The major hypothesis of this work is that butyrate metabolism is altered by the pro-inflammatory cytokine, IL-1 β , and the downstream effects may play an important role in colorectal cancer progression.

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Appendix

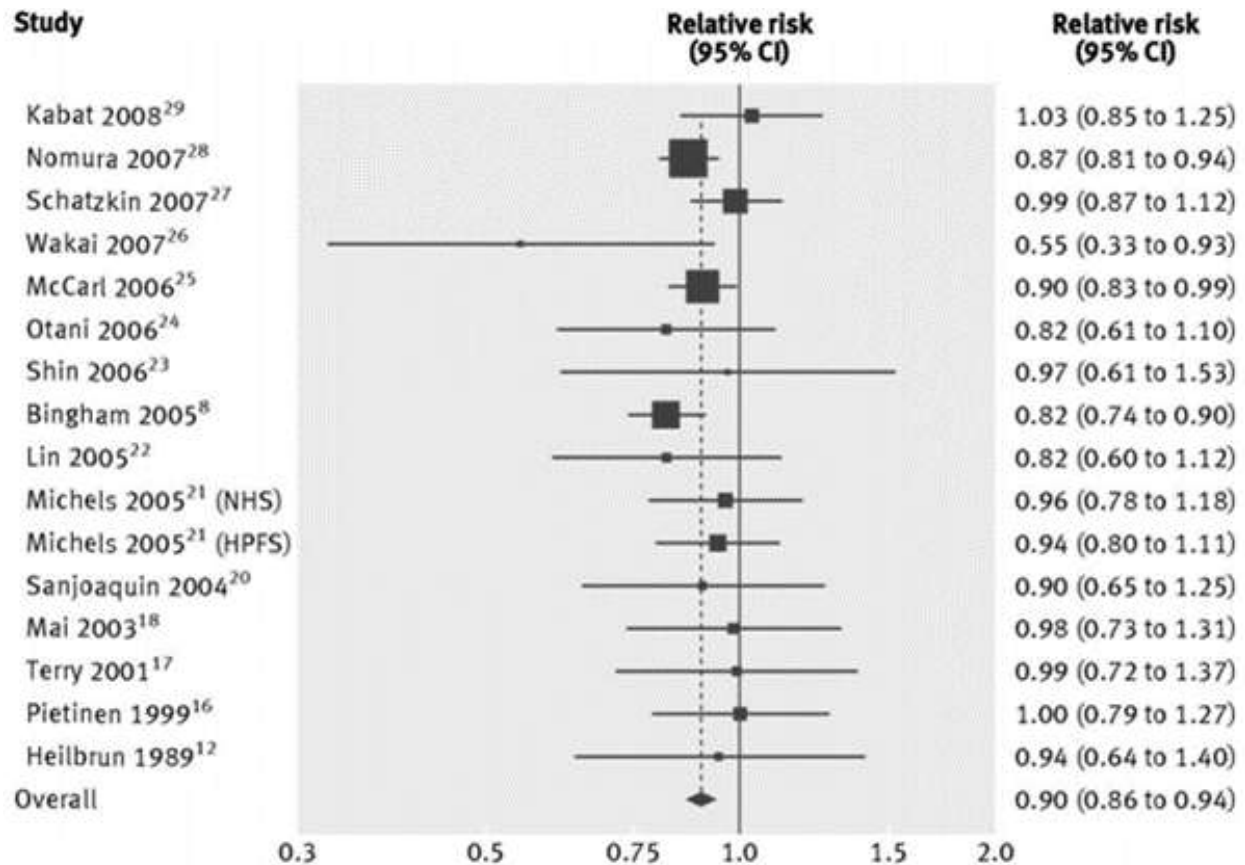


Figure 1.1. “Dose response analysis between for total dietary fiber intake,” Adapted from, “Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies,” by Aune et al [8]. A relative risk plot demonstrating that the increased consumption of dietary fiber has an inverse relationship of developing colorectal cancer.

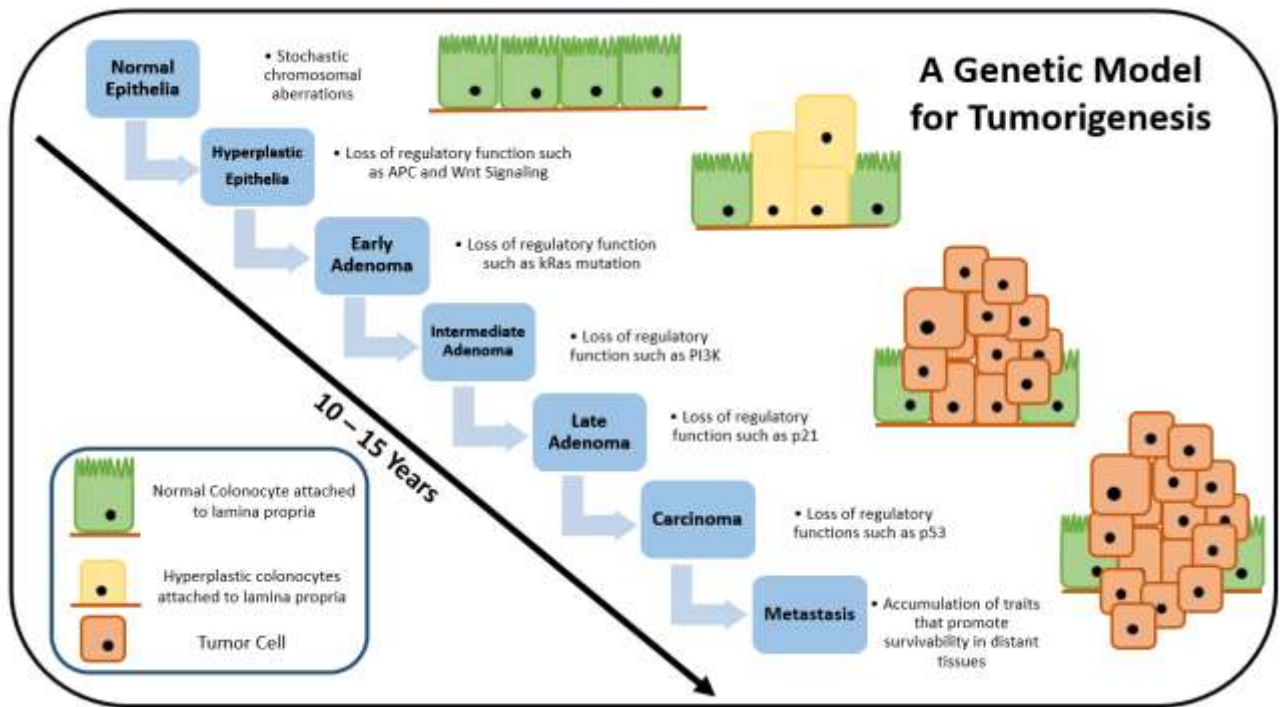


Figure 1.2. A Genetic Model for Colorectal Tumorigenesis. Adapted from Fearon and Vogelstein's seminal 1990 paper, "A Genetic Model for Colorectal Tumorigenesis," that illustrates the progressions of normal epithelia to malignant through the accumulation of genetic mutations resulting in metastasis.

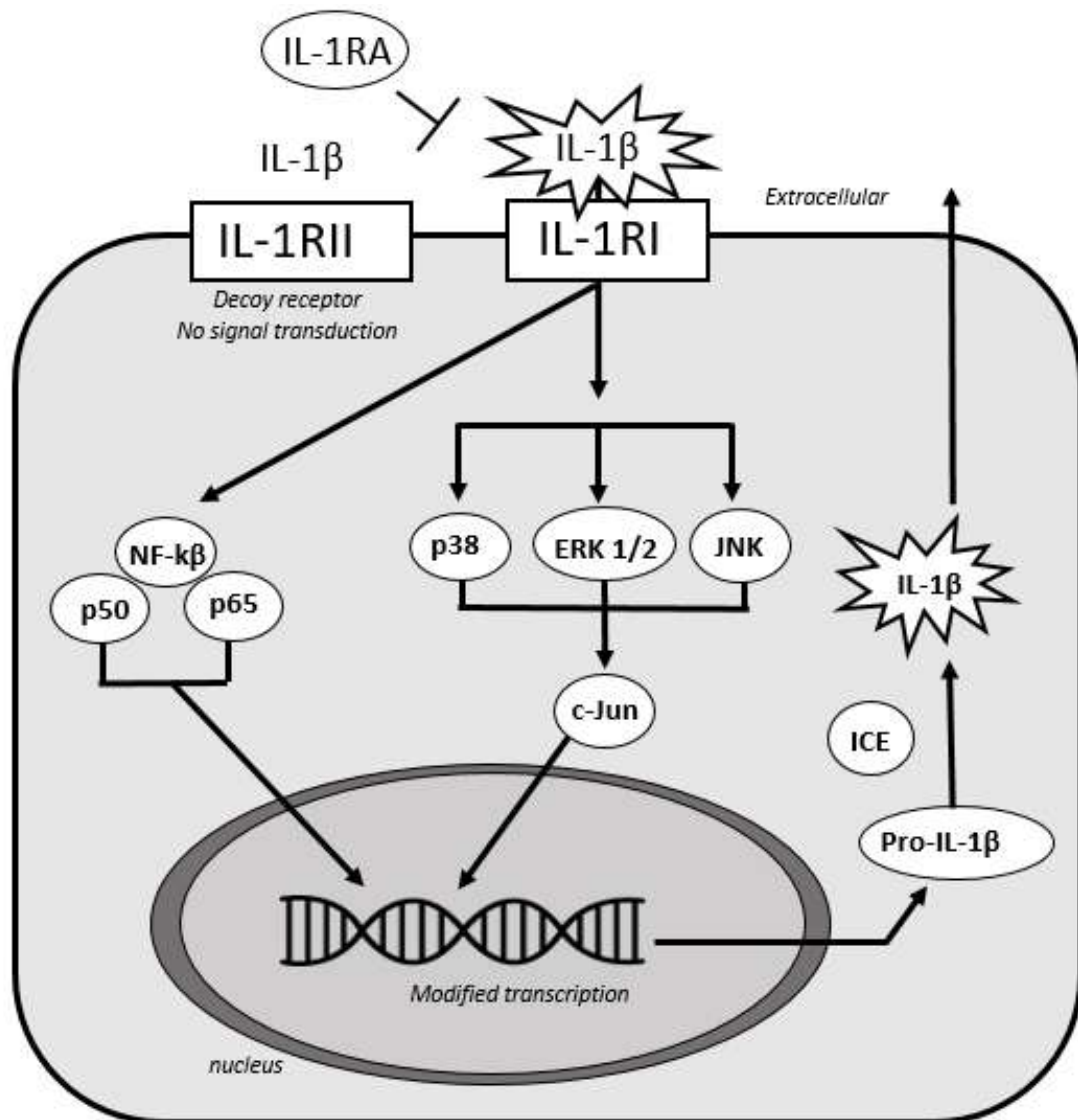


Figure 1.3. IL-1 β Signal Transduction Cascade. IL-1 β can operate through its type I receptor (IL-1RI) whereby it activates intracellular signaling pathways such as p38. Activation of these pathways can modify cell responses such as altered gene transcription.

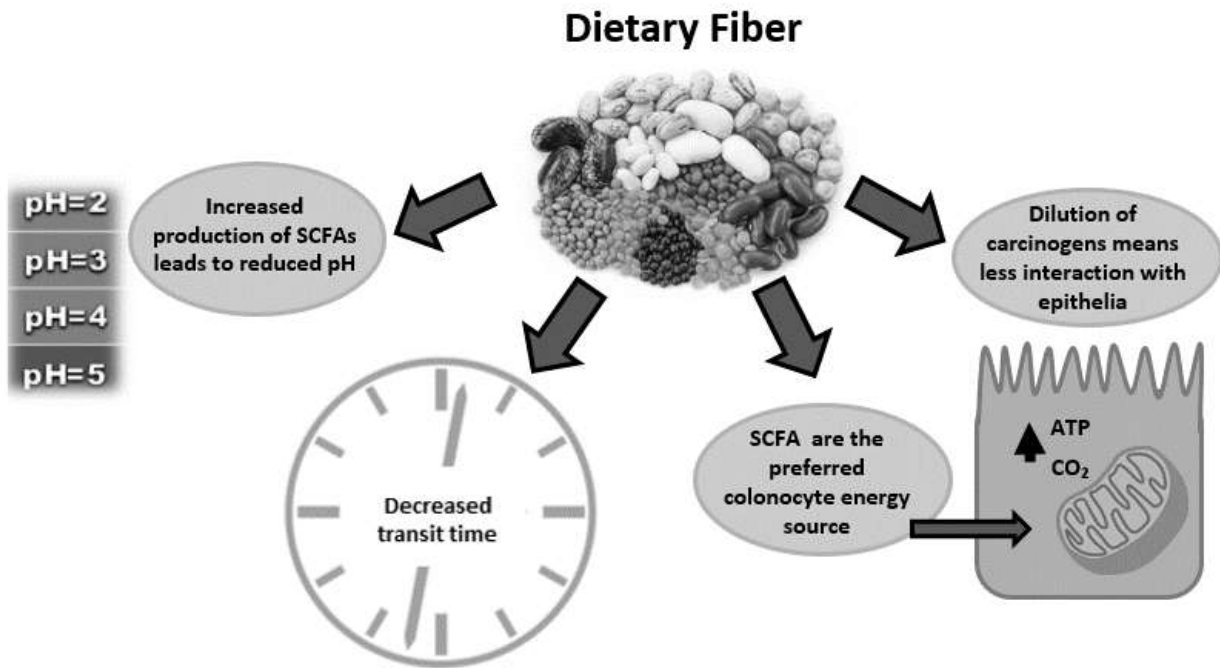


Figure 1.4. The proposed effects of fiber. Dietary fiber is proposed to have multiple effects within the colon. Some of these include increased short chain fatty acid production which can lead to a decrease in pH. Increased fiber intake is associated with decreased transit time in the colon. Dietary fiber fermentation leads to an increase in production of short fatty acids which are the preferred energy source of colonocytes. Finally, dietary fiber promotes dilution of carcinogens within the matter itself leading to decreased exposure along the luminal wall.

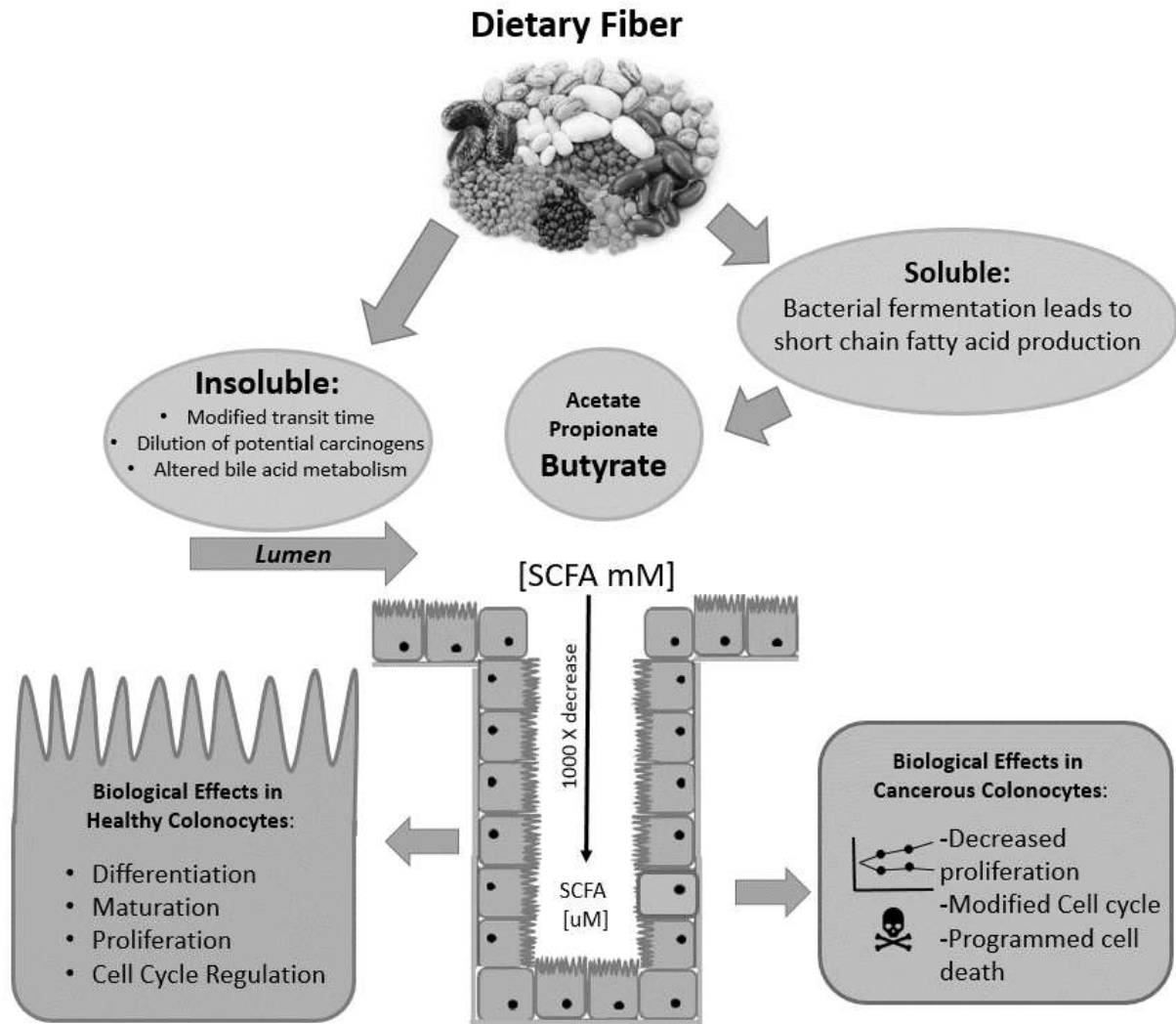


Figure 1.5. Fate of Dietary Fiber. Dietary fiber is fermented to form short chain fatty acids that can be utilized differently in normal versus cancerous colonocytes. For example, in healthy colonocytes, butyrate promotes differentiation and cellular maturation as it moves up the crypt. However, in cancerous colonocytes, butyrate can induce decreased proliferation, modified cell cycle which can lead to cell cycle arrest or even apoptosis [152].

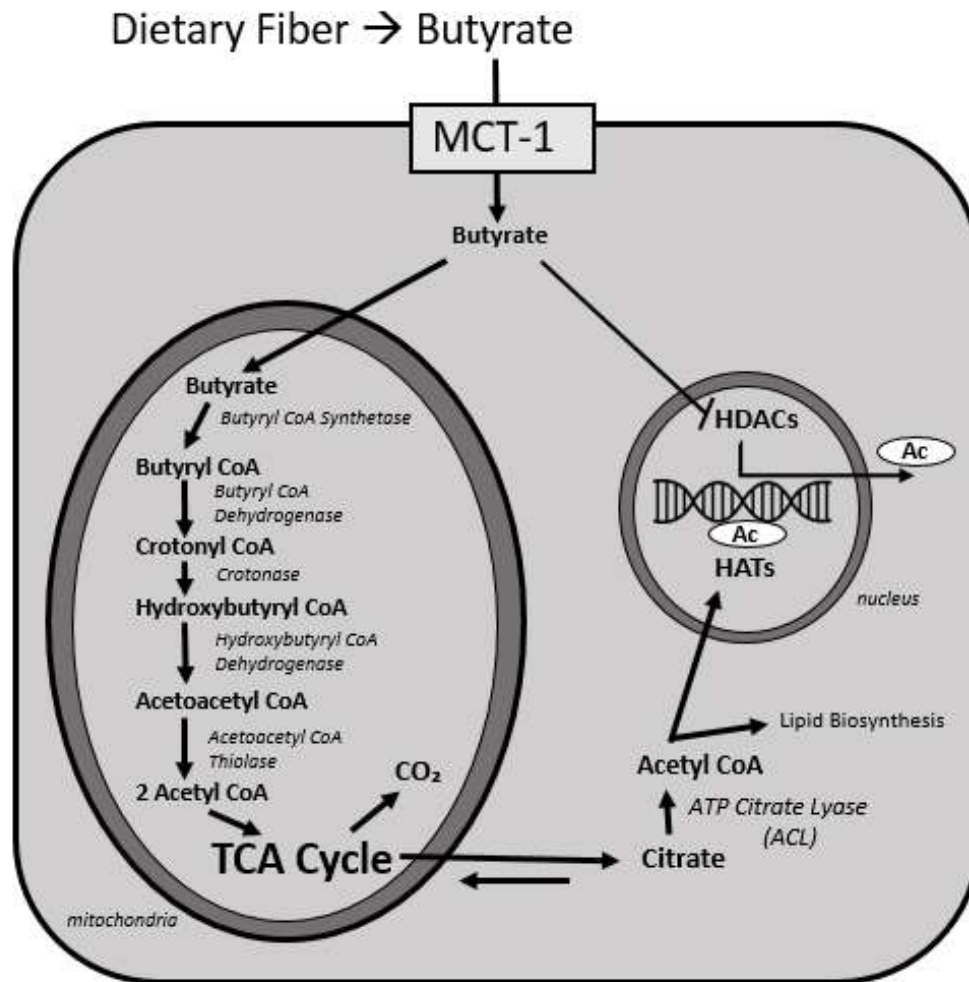


Figure 1.6. Butyrate metabolism in the colonocyte. Butyrate is metabolized to form acetyl CoA in the colonocytes. Acetyl CoA has many fates including histone acetylation through ATP citrate lyase dependent and independent mechanisms. Butyrate inhibits histone deacetylase activity by binding to the enzyme and inhibiting its activity, which removes acetyl groups from histones, thereby allowing hyperacetylation and subsequent gene transcription modification. Additionally, butyrate stimulates histone acetylase activity by providing acetyl groups as the substrate for this enzyme.

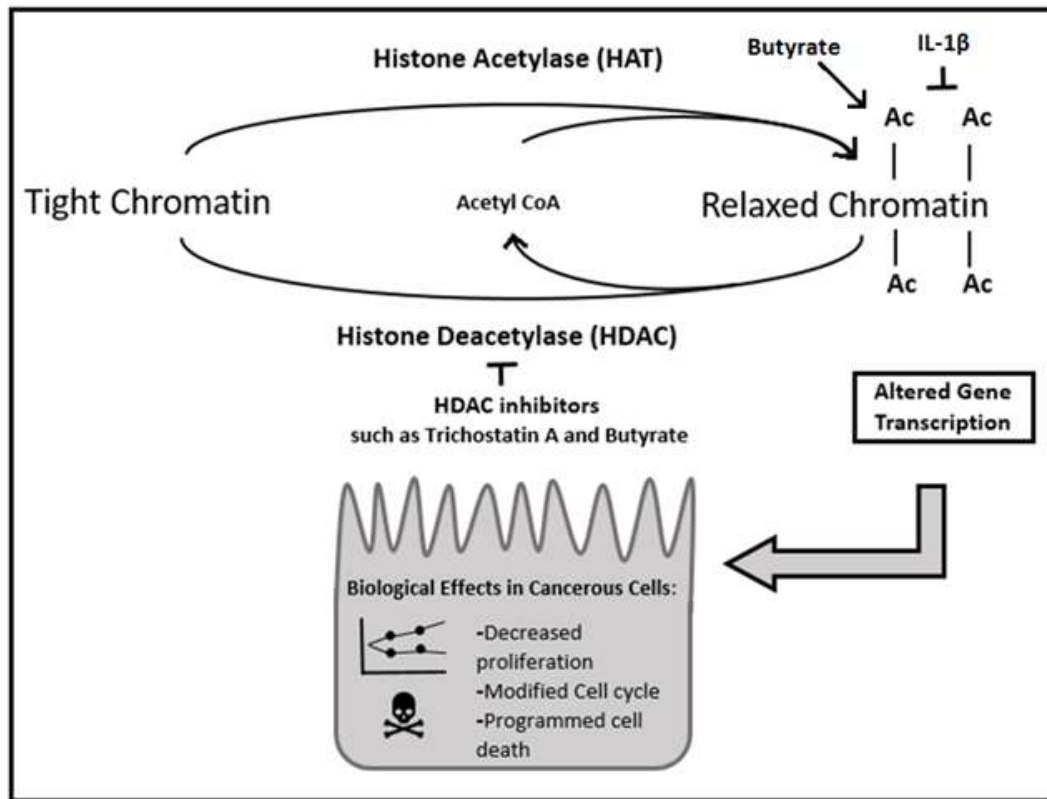


Figure 1.7. Butyrate's role in histone modification and downstream effects. Adapted from Donohoe et al article, "The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation," depicting the downstream pathways of butyrate oxidation.

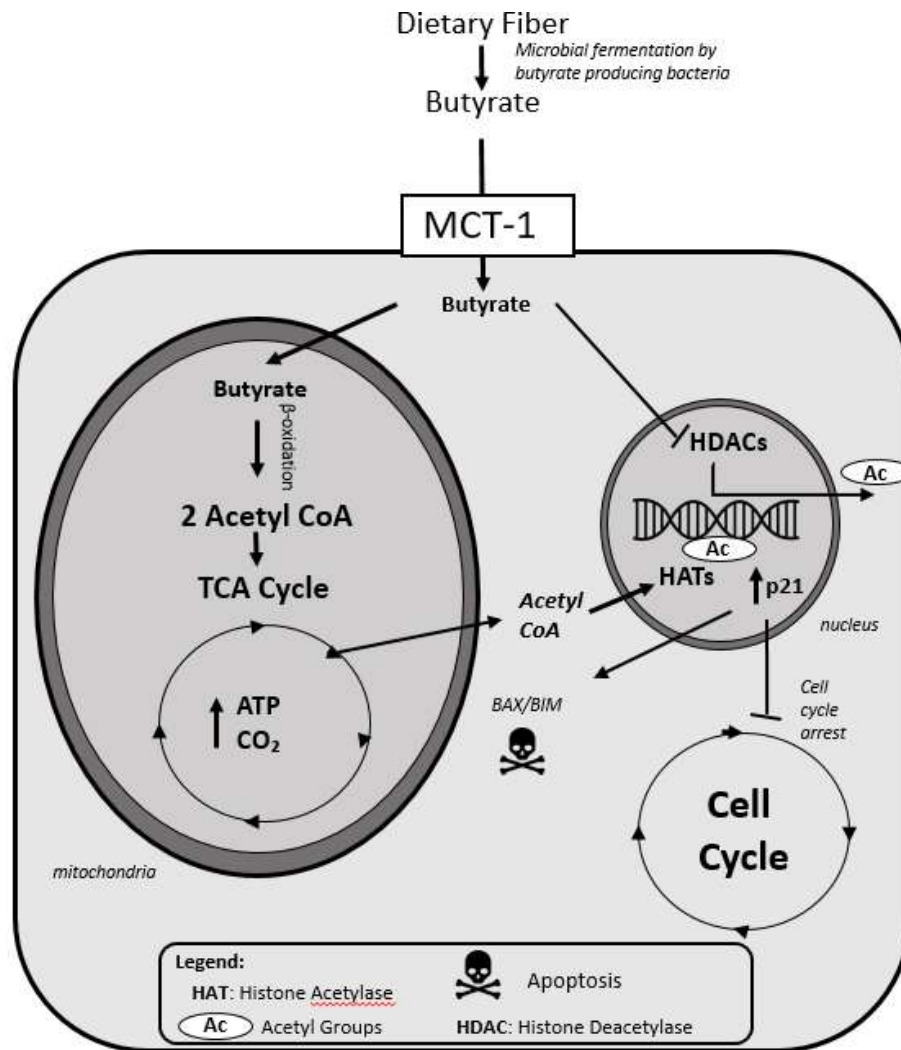


Figure 1.8. Butyrate mechanisms of growth restriction in cancerous colonocytes. Butyrate modifies cancerous colonocyte cell cycle by first providing acetyl groups and second by inhibiting histone deactylase (HDAC) activity allowing histones to become hyperacetylated. This hyperacetylation leads to gene transcription modification in promotor regions such as p21 and increases its transcription. Translation of this protein yield cell cycle arrest and apoptosis via BAX/BIM mediated pathway.

Table 1.1. A diet high in fiber has a tumor suppressive effect in a microbiota and butyrate dependent manner. Using AOM/DSS model to induce tumorigenesis, a diet high in fiber and with butyrate producing bacteria showed a significantly reduced tumor load as compared to control groups.

Low Dose (1 AOM/2 DSS)				
Fiber Content	High	Low	High	Low
BPB	-	-	+ with <i>B. fib</i>	+ with <i>B. fib</i>
Mouse	GF	GF	GF	GF
Average tumor #	3-4	3-4	1	3-4

Table 1.2. Luminal butyrate provides a protective effect against colorectal tumorigenesis. A diet high in fiber and with butyrate producing bacteria show a protective effect against colorectal tumorigenesis demonstrating a microbiota and butyrate dependent mechanism. Furthermore, a diet supplemented with tributyrin, demonstrates a butyrate dependent mechanism that significantly suppresses colorectal tumorigenesis.

High Dose (5 AOM/3 DSS)							
Fiber Content	High	Low	High	Low	High	Low	Tributyrin
BPB	-	-	+ with B. fib	+ with B. fib	+ with mut. B. fib	+ with mut. B. fib	-
Mouse	GF	GF	GF	GF	GF	GF	GF
Average tumor #	8-11	8-11	3	8-11	8-11	8-11	2

CHAPTER II

Characterization of the Pro-Inflammatory Cytokine IL-1 β on Butyrate Oxidation in Colorectal Cancer Cells

This chapter is revised based on a paper published by Megan Johnstone and Dallas Donohoe,

Full Title: Characterization of the Pro-Inflammatory Cytokine IL-1 β on Butyrate Oxidation in Colorectal Cancer Cells

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My primary contribution to this paper include (i) development of the problem into work, (ii) identification of the study objectives, (iii) design and conducting experiments, (iv) gathering and analyzing results, (vi) assembling contributions into a manuscript, (vii) aiding in the writing.

Abstract

Cancer, in part, is driven, by alterations in cellular metabolism that promote cell survival and cell proliferation. Identifying factors that influence this shift in cellular metabolism in cancer cells is important. Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine that has been reported to be elevated in colorectal cancer patients. While much is known towards the effect of dietary nutrients on regulating inflammation and the inflammatory response, which includes cytokines such as IL-1 β , far less is understood how cytokines impact nutrient fate to alter cancer cell metabolism. Butyrate, a nutrient derived from the fermentation of dietary fiber in the colon, is the preferential exogenous energetic substrate used by non-cancerous colonocytes, but is used less efficiently by colorectal cancer cells. To test whether IL-1 β alters colonocyte energy metabolism, we measured butyrate oxidation in HCT116 colorectal cancer cells with and without IL-1 β . We hypothesize that IL-1 β will push cancerous colonocytes away from the utilization and oxidation of butyrate. In this study, we demonstrate that pretreatment of colorectal cancer cells with IL-1 β diminished butyrate oxidation and NADH levels. This effect was blocked with the interleukin receptor antagonist A (IL-1RA). Moreover, IL-1 β suppressed basal mitochondrial respiration and lowered the mitochondrial spare capacity. By using inhibitors to block downstream targets of the interleukin-1 receptor pathway, we show that p38 is required for the IL-1 β -mediated decrease in butyrate oxidation. These data provide insight into the metabolic effects induced by IL-1 β in colorectal cancer, and identify relevant targets that may be exploited to block the effects of this cytokine.

Background

There is a great body of evidence supporting a direct role of inflammation in promoting cancer progression [1-3]. Patients with inflammatory bowel disease have an increased risk of developing colorectal cancer [4, 5]. Colorectal cancer patients have elevated levels of pro-inflammatory cytokines, including TNF α , IL-6, and IL-1 β [6, 7]. IL-1 β is an especially interesting culprit in colorectal cancer development as polymorphisms in its gene are associated with the disease [8-10]. Further supporting this cytokine's cancer promoting effect, IL-1 β knockout mice have an increased survival rate and decreased tumor growth in preclinical cancer models [11]. This also suggests that IL-1 β alone can drive tumorigenesis and cancer progression.

Diet is another factor that impacts the development of colorectal cancer [12-14]. Dietary fiber is thought to inhibit colorectal tumorigenesis, although this has been suggested to be, in part, due to the production of butyrate [15]. Butyrate is a short-chain fatty acid (SCFA) that is produced in the colonic lumen through the fermentation of fiber by resident bacteria. Importantly, butyrate serves as the primary energy source for colonocytes, and it can function as an histone deacetylase (HDAC) inhibitor at physiologically-relevant doses [16]. Towards this end, the oxidation of butyrate by the colonocyte appears to be important in regulating butyrate levels in the cell, which would subsequently regulate HDAC inhibition [16]. Colorectal cancer cells have diminished butyrate oxidation, and factors such as the Warburg effect and decreased intracellular carnitine may contribute to the altered metabolic fate of butyrate [15-17].

The cancerous colonocyte shifts cellular metabolism to increase glucose uptake and utilization. In the clinic, this can be confirmed through positron emission topography (PET) imaging demonstrating that colorectal tumors take up increased amounts of radiolabeled glucose [18, 19]. Glucose uptake is further increased as colorectal tumors acquire late stage mutations

such as *Kras* and progress toward metastasis, which suggests that changes in cellular metabolism in the cancerous colonocyte are important for colorectal cancer progression [20]. It is unclear whether IL-1 β enhances colorectal cancer development through altering cellular metabolism.

In this study, we find that IL-1 β suppresses the oxidation of butyrate in cancerous colonocytes. We further define the effects of IL-1 β on cancerous colonocytes and demonstrate that metabolism is shifted away from mitochondrial metabolism. However, glucose metabolism is also suppressed in these cancer cells when treated with IL-1 β . Finally, mechanisms that regulate the diminishment in butyrate oxidation are explored and p38 is identified as an important mediator.

Results

IL-1 β decreases butyrate oxidation in colorectal cancer cells

The Seahorse XF Extracellular Flux Analyzer is an instrument that measures oxidative (oxygen consumption) and non-oxidative (glycolysis or lactate production) components of cellular metabolism [21-23]. We sought to use this methodology to better understand how inflammatory cytokines such as IL-1 β impact cellular metabolism. Specifically, we wanted to test whether IL-1 β would alter the metabolic fate (i.e. oxidation) of butyrate in HCT116 cancerous colonocytes. We previously developed an experimental strategy using the Seahorse XF Analyzer to measure butyrate oxidation in actively respiring cells (Fig. 2.1A).

To test the effect of IL-1 β on butyrate oxidation, we pretreated HCT116 cells with IL-1 β and then measured the OCR. Cells that received butyrate showed a significant increase in OCR after the addition of 2DG when compared to cells that did not receive butyrate (Fig. 2.1B). This

is consistent with cells utilizing butyrate, when glucose utilization is inhibited by 2DG. The increase observed after 2DG injection was abolished in IL-1 β treated cells (Fig. 2.1C). Thus, IL-1 β suppressed butyrate oxidation in this colorectal cancer cell line.

Effect of IL-1 β and butyrate on NADH and ATP levels

The oxidation of fatty acids produces reducing equivalents such as NADH and FADH₂, which carry electrons to the electron transport chain complexes that drive ATP synthesis. Since butyrate oxidation was suppressed by IL-1 β , we tested whether NADH and ATP levels were impacted as a consequence. IL-1 β alone induced a significant decrease in NADH levels, but addition of butyrate rescued this deficit (Fig. 2.2A). This suggested that although butyrate oxidation was decreased by IL-1 β , overall NADH levels are not changed. Consistent with this interpretation, we observed no significant differences in ATP levels in cells treated with only IL-1 β or with IL-1 β and butyrate (Fig. 2.2B). The difference between IL-1 β alone decreasing NADH, and not ATP may be due to NADH utilization rather than NADH production. It was unclear whether IL-1 β affected mitochondrial function (i.e., basal respiration, spare respiratory capacity, proton leak, and ATP-coupled respiration).

IL-1 β alters mitochondrial function

To test whether IL-1 β influences mitochondrial function in HCT116 cells, we performed a mitochondrial stress assay. In the mitochondrial stress assay, cells are treated first with oligomycin, which blocks ATP synthase. As a result, protons (H⁺) are not able to cross from the inner mitochondrial space into the matrix and proceed down their electrochemical gradient.

Oxygen consumption drops and the amount of this decrease gives insight into the ATP coupled mitochondrial respiration. Next, FCCP is injected into the wells. FCCP is an ionophore, which allows protons to cross into the mitochondrial matrix. This uncouples respiration from ATP synthesis, and as such, oxygen consumption rises. The degree to which oxygen consumption rises is equivalent to the spare respiratory capacity. The higher the oxygen consumption rises then the more electrons are passing through the electron transport chain. Finally, antimycin and rotenone are injected into the wells to block all mitochondrial respiration and as a result the oxygen consumption drops. The level to which the oxygen consumption decreases indicates the contribution of non-mitochondrial metabolism.

Pretreatment of HCT116 cells with IL-1 β showed a drastic change in mitochondrial function as compared to non-treated cells (Fig. 2.3A). Both the basal and spare respiratory capacity was significantly reduced in HCT116 cells pretreated with IL-1 β (Fig. 2.3B). Furthermore, IL-1 β diminished the ATP coupled respiration, while increasing proton leak and non-mitochondrial metabolism (Figs. 2.3C & 2.3D). These data suggest that IL-1 β is decreasing mitochondrial metabolism in HCT116 cells.

Glycolytic activity is suppressed by IL-1 β

The mitochondrial stress test suggested that IL-1 β is pushing cellular energetics toward non-mitochondrial metabolism, which may represent glycolysis. Therefore, we tested whether IL-1 β altered glycolysis in the HCT116 cells. For the glycolysis assay, the extracellular acidification rate (ECAR) represents the amount of lactate secreted by the cancer cells. Lactate is a terminal product of glycolysis, and as such, the ECAR measurement is a proxy for glycolytic

activity. In the assay, glucose is injected, and higher glycolysis is indicated by elevated ECAR. Oligomycin is then injected to uncouple glycolysis from oxidative phosphorylation, and 2-deoxyglucose (2DG) is injected to fully block glucose utilization and glycolysis. IL-1 β treated HCT116 colorectal cancer cells showed a reduced ECAR response after glucose injection (Fig. 2.4A). This was consistent with IL-1 β decreasing both glycolysis and the glycolytic capacity in these colorectal cancer cells (Fig. 2.4B). Thus, we conclude that IL-1 β slows cellular metabolism in HCT116 cancer cells through suppressing mitochondrial metabolism and glycolysis.

Interleukin-1 receptor antagonist rescues butyrate oxidation in IL-1 β treated cells

IL-1 β binds and signals through the interleukin 1 receptor (IL-1R). The IL-1R receptor also binds to the endogenously produced interleukin 1 receptor antagonist (IL-1RA), which has been shown to inhibit IL-1 β signaling through the IL-1R [24]. To test whether IL-1 β is acting through the IL-1 receptor to suppress butyrate oxidation, IL-1RA was used to block IL-1 β binding to IL-1 receptor, and therefore, restore butyrate oxidation in the presence of IL-1 β . In these experiments, HCT116 cells were pretreated with IL-1 β alone or with IL-1RA for 45 min, and butyrate oxidation was measured. IL-RA completely rescued IL-1 β -mediated reduction in butyrate oxidation (Figs. 2.5A & 2.5B), while IL-1RA alone did not alter butyrate oxidation (Figs. 2.5C & 2.5D), suggesting this interaction was specific to IL-1 β . We confirmed that IL-1RA blocked NF κ B activation via IL-1 β (as judged by phosphorylation at Ser536) (Fig. 2.8A).

IL-1 β decreases the oxidation of butyrate through p38 and NF κ B

NF κ B is a downstream target of the IL-1 receptor. Caffeic acid phenethyl ester (CAPE) is a selective pharmacological inhibitor of NF κ B phosphorylation and activation [25]. In our experiments, we used CAPE to probe the involvement of NF κ B in mediating the effect of IL-1 β on suppressing butyrate oxidation. As expected, in HCT16 colorectal cancer cells, CAPE blocked the activation of NF κ B induced by IL-1 β (Fig. 2.9A). However, CAPE only partially rescued butyrate oxidation in cancerous colonocytes pretreated with IL-1 β (Figs. 2.6A & 2.6B). This suggested that NF κ B has a role in suppressing butyrate oxidation, however other downstream molecules to the IL-1 receptor appear to be involved.

IL-1 β also signals through p38 via the IL-1 receptor. Treatment of HCT116 cells with SB203580, a p38 inhibitor, blocked the activation and phosphorylation of p38 by IL-1 β (Fig. 2.8B). In addition, SB203580 completely rescued butyrate oxidation in HCT116 colorectal cancer cells pretreated with IL-1 β (Figs. 2.6C & 2.6D). The p38 inhibitor fully rescued the effect on butyrate oxidation, which suggests that p38 is a key mediator.

Discussion

The effect of IL-1 β on cellular metabolism in the cancerous colonocyte has not been interrogated in detail. Non-cancerous colonocytes utilize butyrate, derived from the fermentation of dietary fiber, as their primary energetic substrate [26]. Cancerous colonocytes shift toward elevated glycolysis and glucose utilization [18, 19, 27, 28]. IL-1 β decreases the capacity of cancerous colonocytes to use butyrate, and may represent one mechanism by which this cytokine alters metabolism to promote cancer progression.

In this study we measured the acute metabolic response to IL-1 β in actively respiring HCT116 colorectal cancer cells. The concentration of IL-1 β in this study was 1 ng/mL, which is within the physiological range of this cytokine. Blood levels of IL-1 β are reported between 100 ng/mL and 1 pg/mL depending on the conditions and external stimuli [29]. Previous work in colorectal cancer cell lines utilized radiolabelled butyrate and measured its conversion to $^{14}\text{CO}_2$ after a given incubation or exposure time. [30, 31]. Both tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ) were demonstrated to suppress butyrate oxidation. Interestingly, TNF- α and IFN- γ activate p38, which we found was important in mediating IL-1 β 's suppressive effect toward butyrate oxidation. A model constructed from our results illustrates that IL-1 β activates NF κ B and p38 pathways to regulate post-transcriptional mechanisms to inhibit butyrate oxidation (Fig. 2.7).

p38 has been previously linked to colorectal tumor initiation and development [32-34]. Specifically, knockout of the γ isoform of p38 inhibited tumorigenesis in a colitis-associated mouse model of colorectal cancer [34]. In addition, the p38 α isoform has also been shown to inhibit colorectal tumorigenesis. We demonstrate that a p38 inhibitor rescued the effect of IL-1 β on butyrate oxidation. Our data allude to p38 playing a role in shifting the colorectal cancer cell away from butyrate utilization or oxidation. p38 has also been associated with resistance to chemotherapeutic drugs in colorectal cancer cells [35, 36]. Therefore, p38 represents an interesting chemotherapeutic target that could be exploited to push the colorectal cancer cell back toward butyrate utilization and enhance the cancer cell's response to chemotherapeutic drugs.

Inflammation and diet are two factors that interact to affect colorectal cancer development [37-39]. Although much is known as to how nutrients impact inflammation, far less

is understood how inflammatory mediators impact nutrients and their metabolism. Here, we have identified a mechanism whereby IL-1 β stimulates p38, a MAPK kinase, to suppress the oxidation of butyrate in HCT116 colon cancer cells. Our data suggest that IL-1 β signals through IL-1R, to dampen mitochondrial metabolism, which includes butyrate oxidation. These findings warrant further investigation into IL-1 β 's ability to alter cellular metabolism and determine whether therapeutic strategies such as using IL-1RA or p38 inhibitors can be used to block these effects while also inhibiting colorectal cancer progression.

Methods

Cell culture, transfections, and treatment conditions

HCT116 cells (ATCC, CCL-247) were grown in DMEM formulated with 5 mM glucose and 10% FBS. Briefly, cells were incubated for three hours in media (DMEM, 10% FBS, 5 mM glucose) with one or a combination of 1 ng/mL IL-1 β , 10 μ M caffeic acid phenethyl ester (NF-kB inhibitor; Tocris Cat # 2743), 10 μ M SB203580 (p38 inhibitor; Cell Signaling Cat #5633). All experiments used 1 ng/mL of IL-1 β and 5 mM butyrate (final concentration).

Flux experiments

XF²⁴ Analyzer (Seahorse Bioscience) was used to measure the % change in the oxygen consumption rate (OCR). Experiments were conducted following manufacturer guidelines. Cells were split and seeded (identical cell number per well) into XF²⁴ cell culture microplates (Seahorse Bioscience, 100777-004). Before running assay, cells were incubated in KHB (no

glucose) with or without treatment condition in a 37°C non-CO₂ incubator for 45 minutes. After this time (15 minutes prior to running plate on Seahorse Analyzer), the KHB is exchanged with fresh KHB (2.5 mM glucose), and the same treatments as described above. Seahorse experiments were run with identical conditions (unless otherwise noted). Butyrate at 5 mM final concentration was injected and the change in OCR was measured from baseline (%OCR). Next, 2-deoxyglucose (Sigma, D8375) was injected and %OCR was measured again. Finally, sodium azide was injected to block oxidative metabolism.

In the mitochondrial stress assay, cells are treated first with oligomycin, which blocks ATP synthase. As a result, protons (H⁺) are not able to cross from the inner mitochondrial space into the matrix and proceed down their electrochemical gradient. Oxygen consumption drops and the amount of this decrease gives insight into the ATP coupled mitochondrial respiration. Next, FCCP is injected into the wells. FCCP is an ionophore, which allows protons to cross into the mitochondrial matrix. This uncouples respiration from ATP synthesis, and as such, oxygen consumption rises. The degree to which oxygen consumption rises is equivalent to the spare respiratory capacity. The higher the oxygen consumption rises then the more electrons are passing through the electron transport chain. Finally, antimycin and rotenone are injected into the wells to block all mitochondrial respiration and as a result the oxygen consumption drops. The level to which the oxygen consumption decreases indicates the contribution of non-mitochondrial metabolism.

Western Blotting

Proteins from HCT116 cells were extracted with RIPA buffer (Cell signaling, #9806), 1 mM PMSF (Cell signaling, #8553) and phosphatase inhibitor cocktail (Cell signaling, #5872). Protein concentrations were measured by Bradford assay. Gel electrophoresis and transfer were conducted using standard protocol for Western blot. Antibodies that were used in experiments included p38 (Cell Signaling, Cat # 9212S), phospho p38 (Cell Signaling, Cat # 9211S), p65 (Cell Signaling, Cat # 8242P), phospho p65 (Cell Signaling, Cat # 3033P), and β -actin (Sigma, Cat# A1978). Chemiluminescence detection was performed with the Odyssey Fc and bands were quantified with Image Studio Software (LI-COR Biosciences, Lincoln, NE).

NADH and ATP Assays

NADH levels in cells were measured with a NADH Assay Kit (Biovision, K334-100). ATP levels in HCT116 cells were measured with an ATP Fluorometric Assay Kit (Biovision, K354-100). For both assays, conditions were kept identical to the Seahorse experiments, and assays were performed as described by the manufacturer. Prior to each assay, cells were deproteinized immediately before the assay with a PCA deproteinization kit (Biovision, K808-200).

Statistical Analysis

For biochemical assays, Seahorse Experiments, and Western blots, the differences between experimental groups were determined by a two-way ANOVA analysis followed by a Tukey post-

hoc test. All data are expressed as mean $\pm$ SE. Significant differences were determined by $p < 0.05$, and are noted in figure legends.

LIST OF ABBREVIATIONS

Interleukin-1 β , IL-1 β ; interleukin receptor antagonist A, IL-1RA; tumor necrosis factor alpha, TNF α ; interleukin-6, IL-6; short-chain fatty acid, SCFA; histone deacetylase, HDAC; positron emission topography, PET; Krebs-Henseleit Buffer, KHB; oxygen consumption rate, OCR; extracellular acidification rate (ECAR); 2-deoxyglucose, 2-DG; nuclear factor kappa-light-chain enhancer of activated B cells, NF κ B; c-Jun N-terminal kinases, JNK; extracellular signal-regulated kinases, ERK; mitogen-activated protein kinase, MAPK

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Appendix

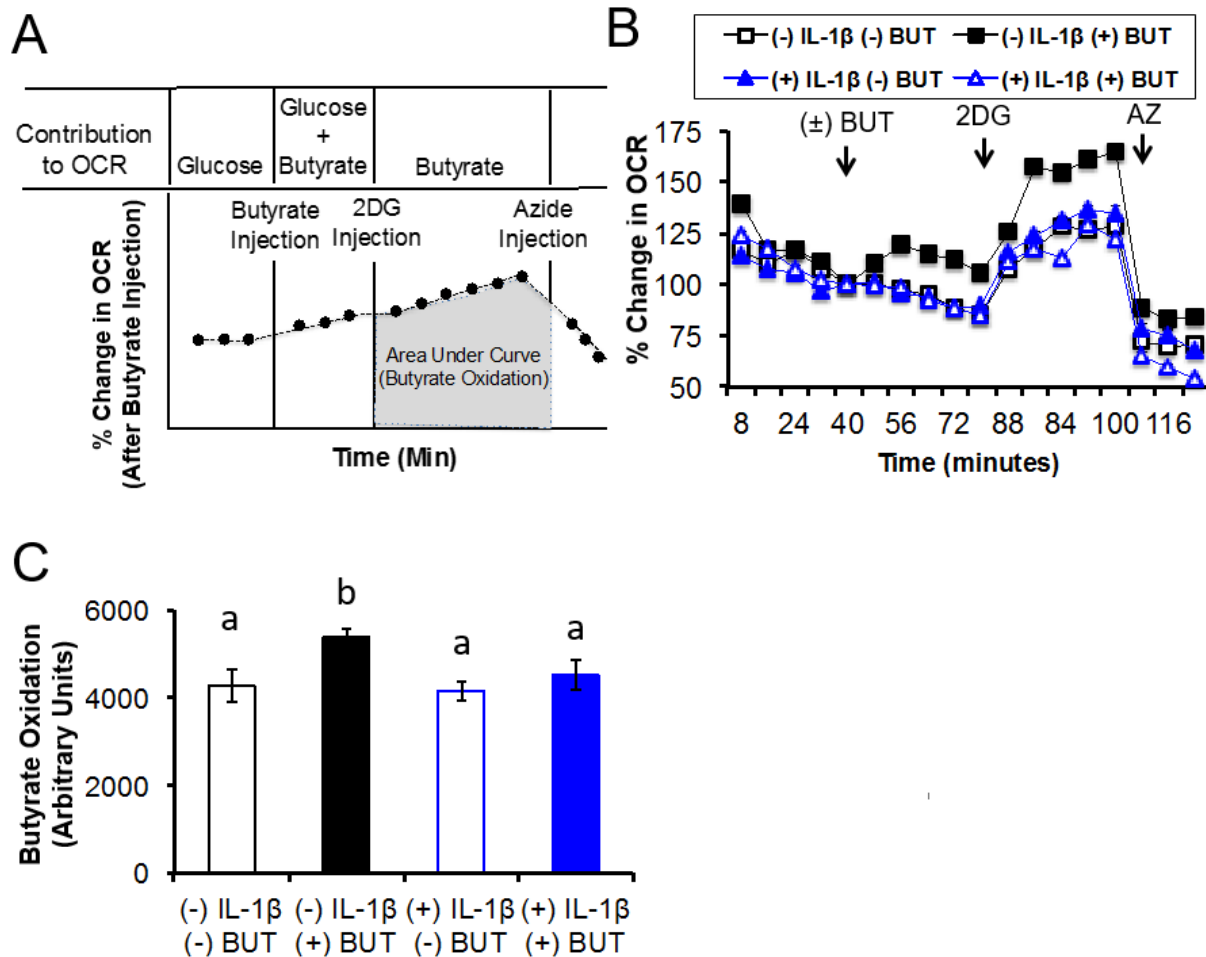


Figure 2.1. Butyrate oxidation is suppressed by IL-1 β in HCT116 cancerous colonocytes. (A) Schematic representation illustrating rationale used to measure butyrate oxidation with the XF²⁴ Analyzer. (B) Percent change in oxygen consumption rate (OCR) relative to baseline for HCT116 cells treated +/- IL-1 β followed by injection of butyrate. Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (C) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection. These measurements represent the butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Bars with the same superscript are not significantly different at $p < 0.01$. Error bars are $\pm$ SEM.

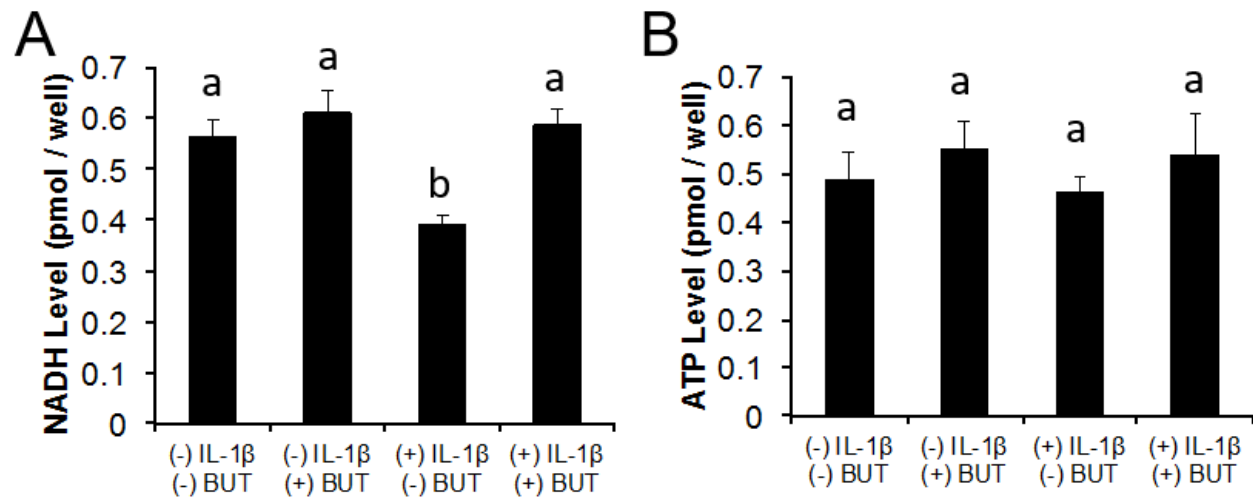


Figure 2.2. IL-1 β lowers NADH, but ATP levels are not significantly changed. (A) NADH levels and (B) ATP levels measured in HCT116 cells treated +/- IL-1 β and butyrate. Data points represent a standard mean where n = 6 per condition. Bars with the same superscript are not significantly different at p < 0.05. Error bars are +/- SEM.

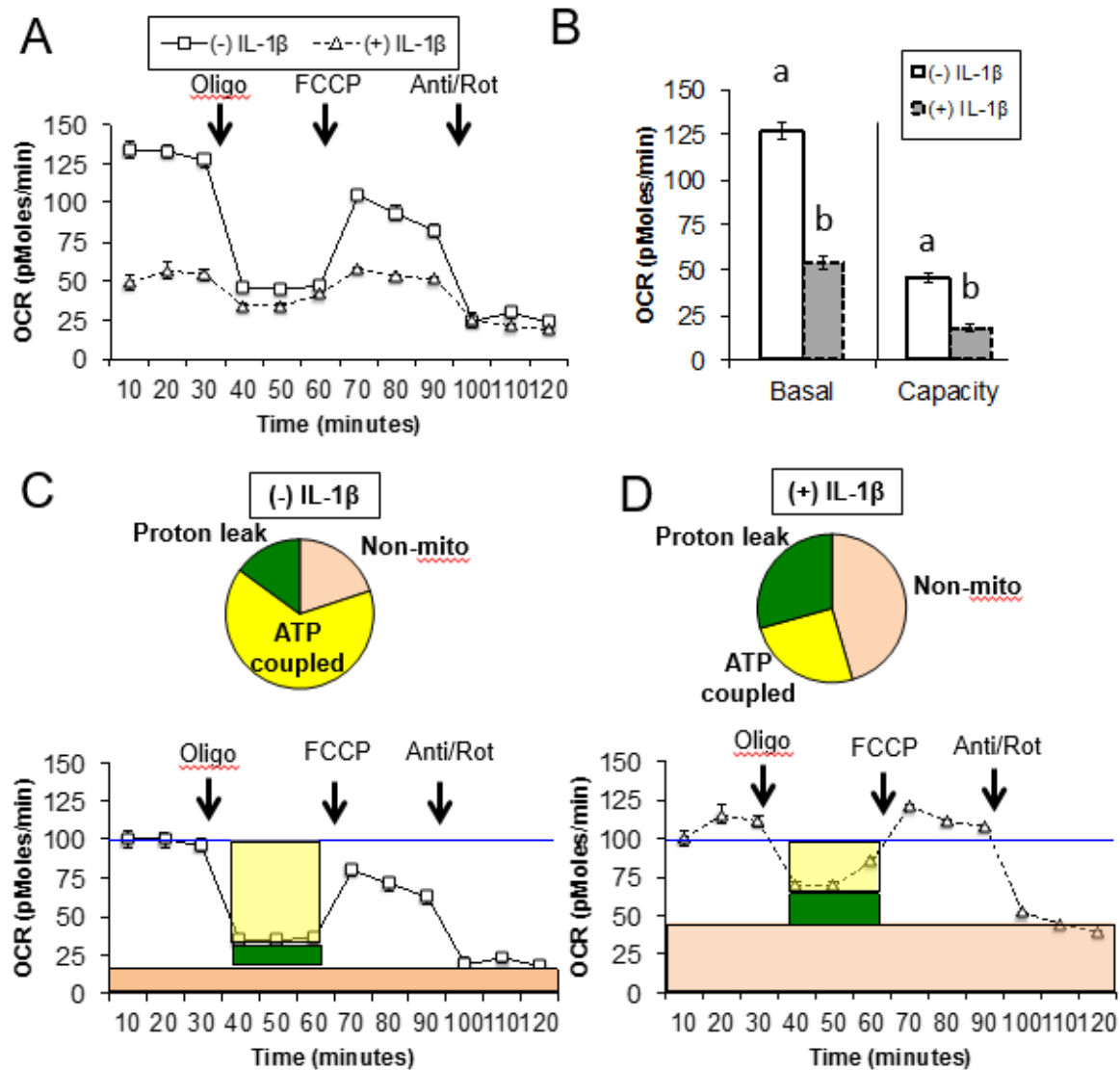


Figure 2.3. IL-1 β disrupts mitochondrial function. (A) OCR response over time. Oligomycin was injected to determine the ATP coupled respiration. FCCP was injected to establish the maximal respiration. Antimycin/Rotenone were injected to define mitochondrial and non-mitochondrial respiration. (B) Calculated basal respiration and spare respiratory capacity. (C) Contribution of proton leak, ATP-coupled respiration, and non-mitochondrial respiration to OCR. For these experiments HCT116 cells were treated with or without IL-1 β . Each data point represents the mean value $\pm$ SEM where $n = 8$ per condition. Bars with the same superscript are not significantly different at $p < 0.05$.

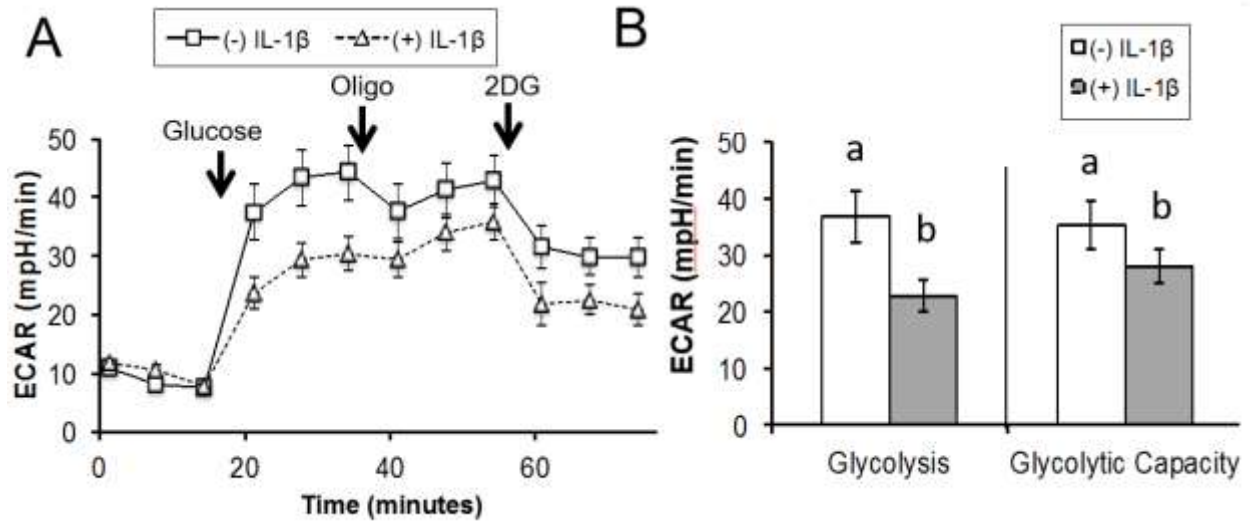


Figure 2.4. Glycolysis is diminished in IL-1 β treated colorectal cancer cells. (A) Extracellular acidification rate was measured after injection of glucose, oligomycin (Oligo), and 2-deoxyglucose (2DG). (B) Glycolysis was calculated from the ECAR response to glucose subtracted from the baseline ECAR before glucose injection. Glycolytic capacity represents the ECAR measurements before glucose injection subtracted from the ECAR measurements after oligomycin injection. For these experiments, HCT116 cells were treated with or without IL-1 β 45 min prior to assay. Each data point represents the mean value $\pm$ SEM where $n = 8$ per condition. Bars with the same superscript are not significantly different at $p < 0.05$.

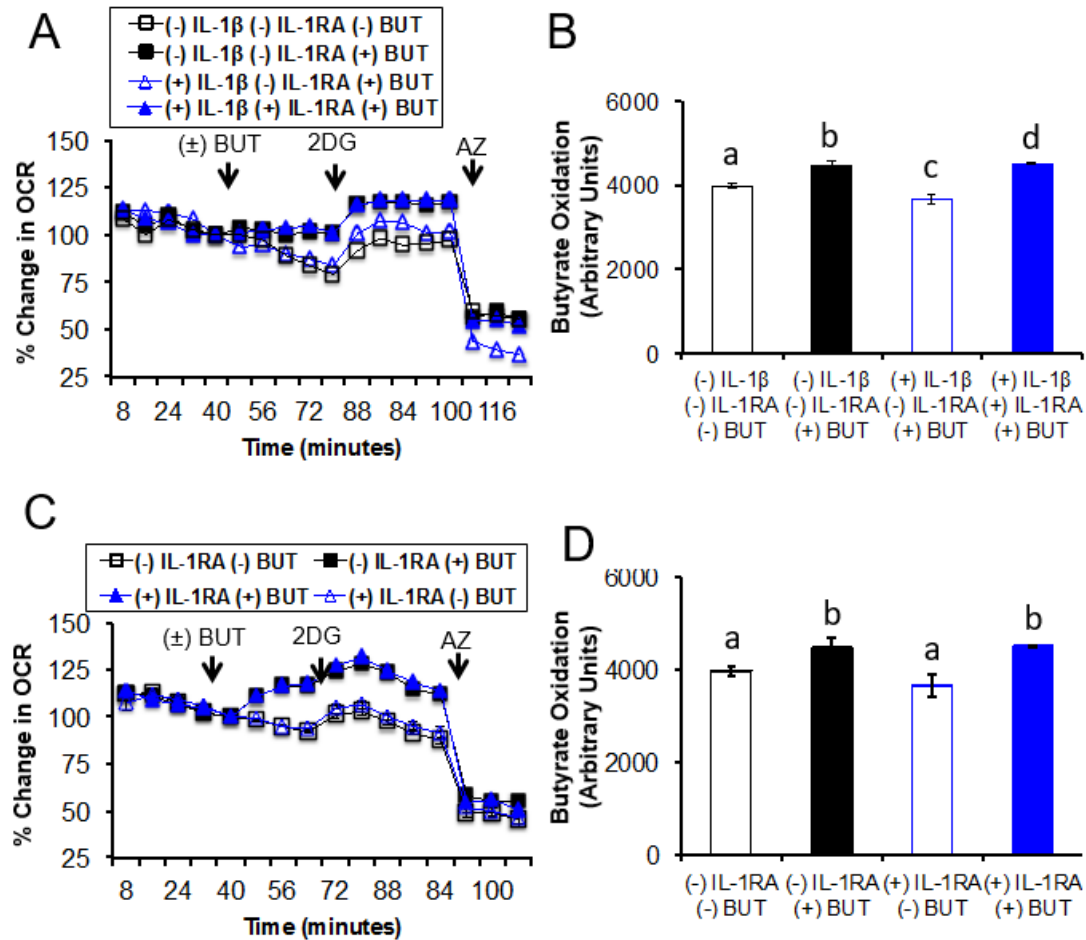


Figure 2.5. IL-1RA completely reverses IL-1 β suppression of butyrate oxidation. (A) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without IL-1 β or IL-1RA. Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (B) Area under the curve analysis is from OCR measurements taken after 2DG injection, but before azide injection. (C) Percent change in oxygen consumption rate (OCR) relative to baseline when pretreated with and without IL-1RA. (D) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection. For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per condition. Bars with the same superscript are not significantly different at $p < 0.05$. Error bars are $\pm$ the SEM.

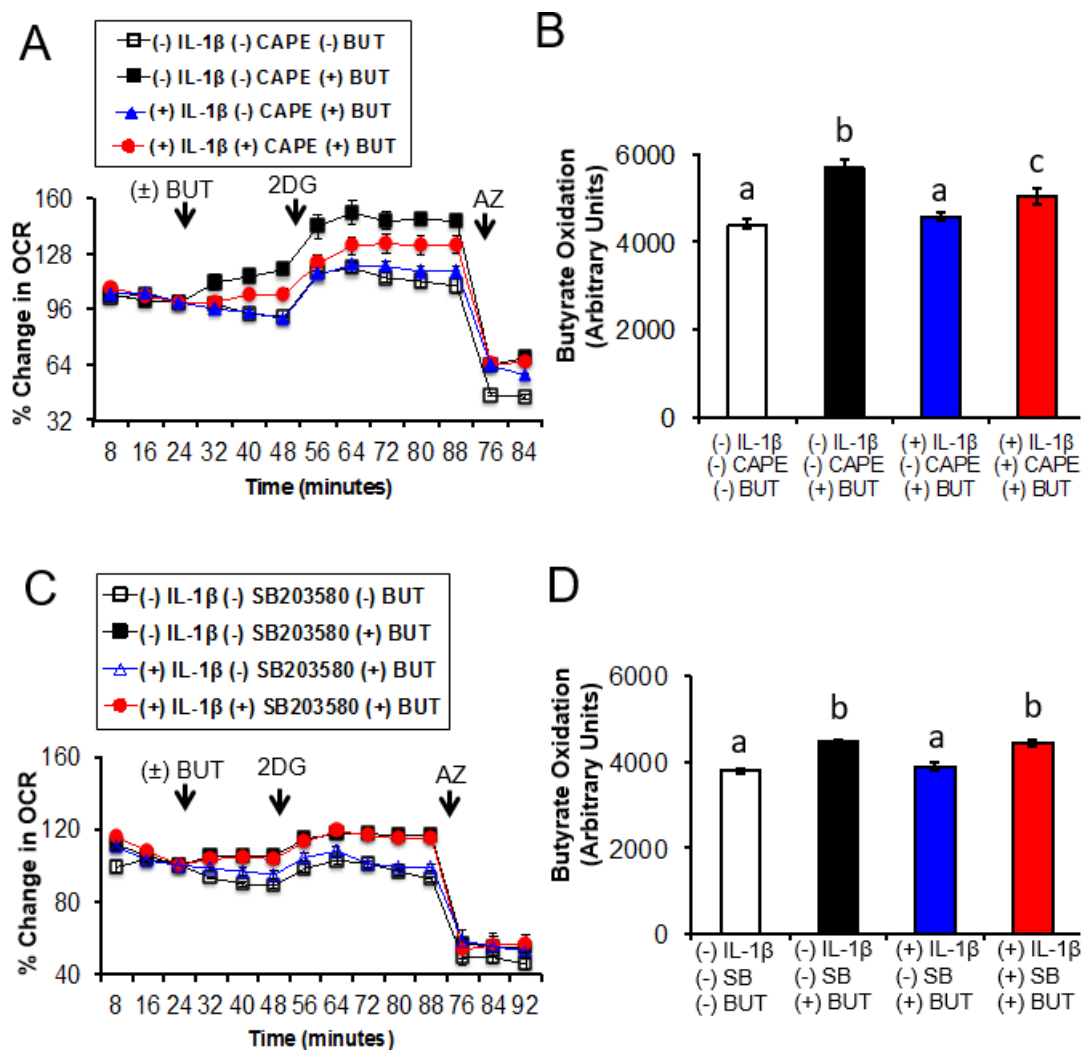


Figure 2.6. Inhibition of MAPK p38 completely rescues the diminished oxidation induced by IL-1 β . (A) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells pretreated with and without IL-1 β or caffeic acid phenethyl ester (CAPE). Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (B) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection. These measurements represent butyrate oxidation (arbitrary units). (C) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells pretreated with and without IL-1 β or SB203580. (D) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection. For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per condition. Bars with the same superscript are not significantly different at $p < 0.01$. Error bars are $\pm$ the SEM.

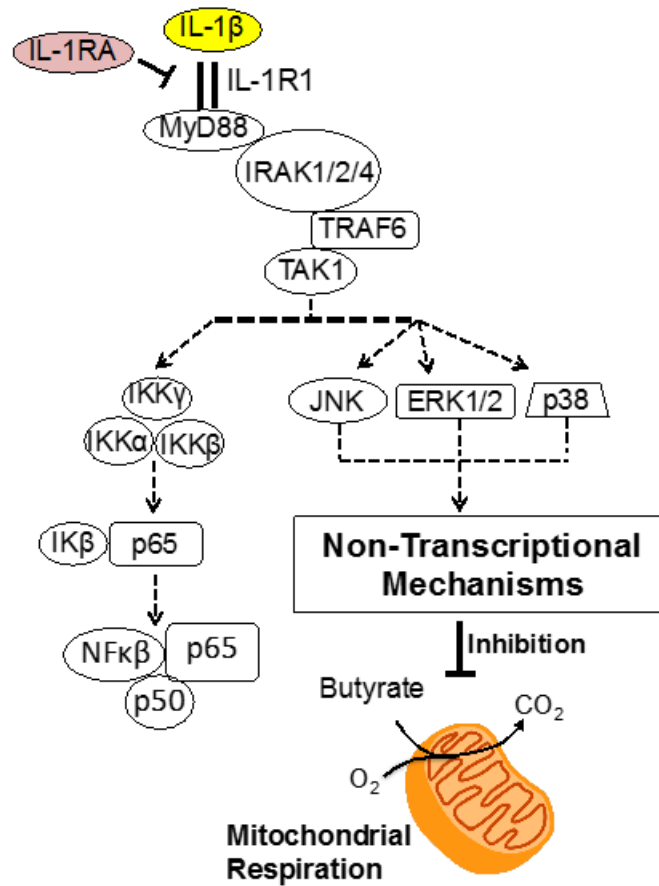


Figure 2.7. Downstream signaling pathways and targets of IL-1R1. Model showing the downstream signaling pathways that are activated by IL-1 β binding to interleukin-1 receptor 1 (IL-1R1). In this model, IL-1 β can signal through NF κ B and/or MAP kinases that include p38. Activation of these pathways by IL-1 β can be blocked by IL-1RA. Model was adapted from [40].

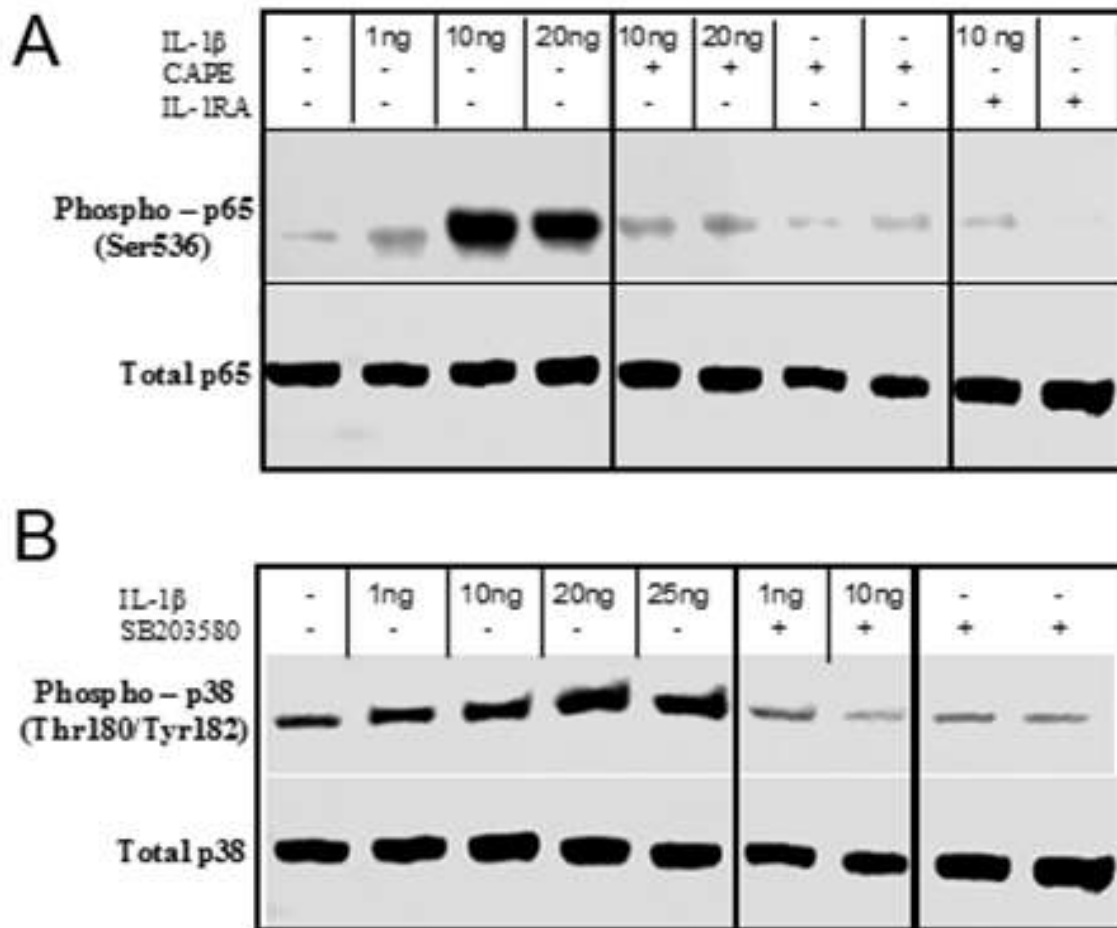


Figure 2.8. Inhibition of IL-1 β activation of p65 and p38. Representative western blot showing (a) phospho – p65 with total p65 and, (b) phospho – p38 with total p38. Conditions were kept identical to butyrate oxidation experiments and cells were treated for 45 minutes with cytokines or compounds. Experiments were repeated 2 more times to confirm results.

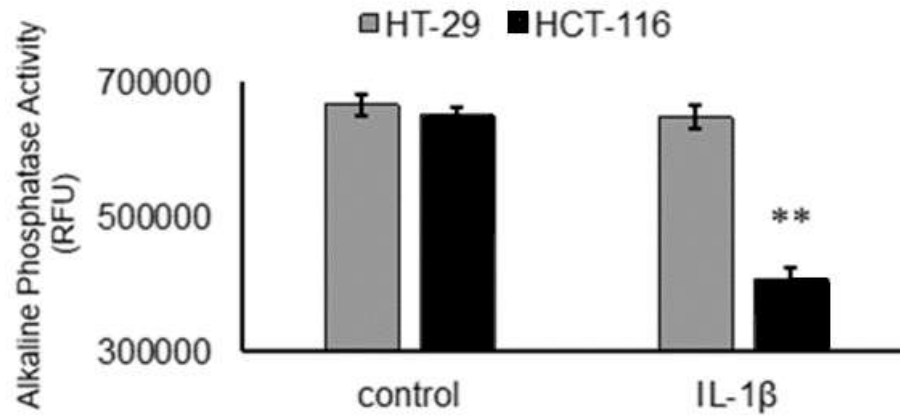


Figure 2.9. IL-1 β decreases alkaline phosphatase in HCT116 but not HT-29 colorectal cancer cells. Histogram showing effect of IL-1 β treatment on alkaline phosphatase activity, which serves as a marker of cellular differentiation. Conditions were kept identical to butyrate oxidation experiments and cells were treated for 45 minutes with IL-1 β . Error bars are +/- SEM. **p < 0.01

CHAPTER III

Characterization of the metabolic effects induced by pro-inflammatory cytokines on cancer cell lines

This chapter is revised based on experiments by Megan Johnstone and Dallas Donohoe,

Full Title: Characterization of the real-time metabolic effects mediated by pro-inflammatory cytokines on cancer cell lines

Authors: Megan Johnstone¹ and Dallas R. Donohoe¹

My primary contribution to this paper include (i) development of the problem into work, (ii) identification of the study objectives, (iii) design and conducting experiments, (iv) gathering and analyzing results, (vi) assembling contributions into writing this paper.

Abstract

Background: Cancer is, in part driven, by the tumor microenvironment that can promote cell survival and cell proliferation. Identifying factors that influence this shift in cellular metabolism in cancer cells is important. Inflammation is described as an “enabling characteristic” of cancer and is, in part, mediated by pro-inflammatory cytokines. Serum levels of pro-inflammatory cytokines are increased in many types of cancers. Much is known regarding the regulatory effects of diet on the inflammatory response. However, far less is understood regarding the effect of inflammation i.e. cytokines impact on nutrient utilization in cancer cell metabolism.

Literature suggests that inflammation drives cancer progression and may ultimately promote cancer cell survival. We hypothesized that IL-1 β , IL-6, and TNF- α would perturb cancerous cell nutrient metabolism to promote cell proliferation and survival.

Methods: Butyrate and glucose oxidation in colorectal, melanoma, and prostate cancer cells with and without IL-1 β , IL-6, and TNF- α pretreatment was measured with a Seahorse XF²⁴ Analyzer.

Results: Pretreatment of colorectal, melanoma, and prostate cancer cells with pro-inflammatory cytokines affected glucose and butyrate oxidation. In non-colon cancer cells, IL-1 β did not affect butyrate oxidation, but did increase endogenous lipid oxidation as compared to untreated cells. The colorectal cancer cells displayed opposing effects in the presence of pro-inflammatory cytokines. For example, butyrate oxidation was not suppressed when pretreated with IL-1 β in HT-29 colorectal cancer cells. In contrast, HCT-116 colorectal cancer cells displayed suppressed butyrate oxidative capacity in the presence of IL-1 β and TNF- α . Furthermore, IL-6 enhanced butyrate oxidation in HCT116 colorectal cancer cells. These data suggest that pro-inflammatory cytokines modify nutrient metabolism in cancer cells.

Conclusions: These data provide insight into the metabolic effects induced by pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in cancer cells, and identify therapeutic options that may be targeted to block the effects of these cytokines.

Background

The link between cancer and inflammation was first described by Virchow in the mid-nineteenth century [1, 2]. The inflammatory response is a known promotor of many cancers, including colorectal cancer [3-7]. Pro-inflammatory cytokines, mediators of inflammation, such as IL-6, IL-1 β and TNF- α , are elevated in many cancers [8-12]. These pro-inflammatory cytokines stimulate angiogenesis, adhesion factors, and alterations of nutrient metabolism in cancer cell lines [13, 14]. A shift in nutrient metabolism could alter cancer cell physiology to promote survival in cancer cells.

In addition to inflammation, diet promotes or inhibits cancer development [15-19]. Multiple lines of evidence show that diet or nutrients can potentially suppress the inflammatory response [4, 17, 20]. Conversely, the mechanisms by which inflammatory factors influence nutrient metabolism have yet to be explored in detail. Pro-inflammatory cytokines such as IL-1 β and TNF- α have been shown to shift cancerous colonocytes away from butyrate metabolism [14]. This shift away from butyrate metabolism is important in colonocytes as butyrate regulates cell maturation and differentiation [21]. Furthermore, in cancerous colonocytes, butyrate can induce apoptosis [22]. Therefore, insight into cytokine-induced molecular mechanisms that mediate a shift in nutrient utilization away from butyrate metabolism may reveal pathways that stimulate the transition from normal to neoplastic growth in the colon.

We utilize non-invasive, novel technique to measure the real-time response that pro-inflammatory cytokines can have on cancer cellular metabolism. Here we find that the pro-inflammatory cytokine, IL-1 β , alters the utilization of glucose, but not butyrate in two non-colorectal cancer cell lines. This suggests that effect of IL-1 β is cell type dependent. We also observe that TNF- α displays a similar effect on butyrate oxidation as IL-1 β in HCT116

colorectal cancer cells. Interestingly, the colorectal cancer cell line HT-29, did not mimic the butyrate suppressive effect in the presence of IL-1 β .

Results

IL-1 β effect on butyrate oxidation in A375 cells, a melanoma cell line.

Our strategy to measure butyrate oxidation is shown in Fig. 3.1A. After obtaining the baseline oxygen consumption rate (OCR), which is exclusively due to glucose oxidation, butyrate is injected into the wells. After injection of butyrate, glucose and butyrate contribute to the OCR. To separate glucose and butyrate oxidation, we inject 2-deoxyglucose (2DG), to inhibit glucose oxidation. Therefore, only butyrate is able to contribute to OCR as it is the only exogenous energetic substrate available.

To test the effect of IL-1 β on butyrate oxidation, we pretreated A375 cancerous melanocytes with IL-1 β and then measured the change in OCR (Fig. 3.1B). Pretreatment with IL-1 β did not affect butyrate oxidation in A375 cancer cells (Fig. 3.1C). Whereas, IL-1 β pretreatment led to increased OCR with non-treated cells (Fig. 3.1C).

IL-1 β effect on butyrate oxidation in CWR22Rv1, a castrate resistant prostate carcinoma cell line

Pretreatment with IL-1 β had no effect on butyrate oxidation in CWR22Rv1 cells (Fig. 3.2A & 3.2B). However, pretreatments with IL-1 β again (as seen in the melanoma cancer cell

line) show a significant increase in oxidation as compared to cells without any IL-1 β pretreatment (Fig. 3.2B).

IL-1 β effect butyrate oxidation in HT-29, a colorectal cancer cell line.

We sought to test if IL-1 β elicited a similar response on butyrate oxidation as it did in the HCT116 colorectal cancer cells by using HT-29 cells, another colorectal cancer cell line. HT-29 cells pretreated with IL-1 β displayed an elevated OCR upon butyrate injection (Fig. 3.3A). However, upon 2DG injection, cells pretreated with IL-1 β do not show any difference in butyrate oxidation as compared to cell without IL-1 β treatment (Fig. 3.3B). This indicates that colorectal cancer lines respond differently to IL-1 β and these difference may reveal insight into how IL-1 β suppresses butyrate oxidation in another colorectal cancer cell line.

Other pro-inflammatory cytokines alter butyrate oxidation in HCT116 cells

IL-6 effect on butyrate oxidation in HCT116s, a colorectal cancer cell line

Since we determined that the exogenous substrate oxidation response to IL-1 β was cell type dependent we next sought to test whether this response was cytokine dependent. Thus, we chose use HCT116 cells as they displayed a modified butyrate oxidation in response to IL-1 β pretreatment. We first used IL-6 which is a pro-inflammatory cytokine that is also shown to be upregulated in colorectal cancer [5, 23, 24]. HCT116 cells pretreated with IL-6 had a significant increase in butyrate oxidation as compared to butyrate treatment alone (Fig. 3.4A & 3.4B).

Therefore, IL-6 demonstrates that it has the opposite effect on regulating butyrate oxidation as compared to IL-1 β .

TNF- α effect butyrate oxidation in HCT116 cells, a colorectal cancer cell line

Previous work has shown that TNF- α diminishes butyrate oxidation and uptake in HT-29 cells [14]. We next sought to examine if TNF- α would produce similar suppression of butyrate oxidation in the HCT116 cell line. HCT116 cells pretreated with TNF- α had a significant decrease in butyrate oxidation as compared to cells without TNF- α treatment (Fig. 5A and 5B). Therefore, TNF- α is able to mimic the effect of IL-1 β on butyrate oxidation in this colorectal cancer cell line.

Discussion

Research shows that tumors can selectively modify their metabolism and nutrient utilization to increase their chances of survival [25, 26]. For example, glucose transporter expression on the cell surface of many cancers is elevated to facilitate glucose utilization [25, 27]. This results in increased glucose uptake, thereby shifting metabolism away from mitochondrial oxidation and towards glycolysis [28]. Similarly, prostate cancer cells increase glutamine utilization in order to increase ATP production [26]. These alterations confer a selective advantage that ensures survival and proliferation of cancer cells [29]. Previous work from our lab has demonstrated that the pro-inflammatory cytokine, IL-1 β , suppresses butyrate oxidation in HCT116, a colorectal cancer cell line. This is thought to, in part, cause a suppression of butyrate's anti-cancer effects. Thus, it is important to characterize the effect that IL-1 β may

stimulate in cancer cell metabolism. As IL-1 β diminished butyrate oxidation in HCT116 colorectal cancer cells, we sought to determine if this effect occurred in non-colon cancer cell lines. Therefore, analyzed a melanoma and prostate cancer cell line to identify whether IL-1 β exerted a similar effect on butyrate oxidation.

The melanoma cell line, A375, did utilize butyrate as denoted by the significant increase in oxygen consumption rate after butyrate injection. However, pretreatment with IL-1 β failed to reduce butyrate oxidation, suggesting that the IL-1 β induced effect observed in HCT116 cells is cell type dependent. The same trend is observed in the CWR22Rv1 prostate cancer cell line. Butyrate oxidation is increased, but is not significantly suppressed by IL-1 β . Yet IL-1 β treatment after 2DG injection also showed a significantly increased OCR rates as compared to control groups. These findings suggest that IL-1 β significantly induces endogenous lipid oxidation in the absence of glucose oxidation.

Both non-colorectal cancer cell lines, A375 and CWR22Rv1, oxidized butyrate. While, IL-1 β pretreatment did not suppress butyrate oxidation, it did increase OCR as compared to non-treated cells. In the presence of 2DG, a glycolytic inhibitor, cells that were pretreated with IL-1 β had a significantly increased OCR as compared to groups that did not receive IL-1 β pretreatments. This indicates that the pro-inflammatory cytokine, IL-1 β , stimulates a shift in metabolism towards endogenous lipid oxidation in the absence of glucose. This is important as cancer cells often outgrow their oxygen supply [28, 30, 31]. To survive, exogenous and endogenous fatty acid oxidation may be used as a source of energy [26]. The regulation of lipid metabolism by cancer cells are crucial to their survival [32]. Importantly, cancer cells have been shown to stimulate lipolysis, fatty acid uptake and β -oxidation from local adipose depots [32,

33]. Our data support the complex, dynamic shift in cancer cell metabolism that IL-1 β can stimulate to maintain their metabolic potential for cell proliferation in the absence of glucose.

Using HT-29 cells, another colorectal carcinoma cell line, IL-1 β pretreated cells did not suppress butyrate oxidation. Whereas, IL-1 β pretreatment did suppress butyrate oxidation in the colorectal cancer cell line HCT116. This may be due to differences in differentiation, genetic mutations in these two colorectal cancer cell lines [34]. For example, when both HT-29 and HCT116 cells are implanted in Matrigel; only the HCT116 are characterized as “highly aggressive” [34]. They have little to no capability to differentiate [34]. Whereas the HT-29 cells were able differentiate and form lumens within the Matrigel [34]. This phenotypic difference translates into animal models when HT-29 and HCT116 are used as xenografts. Xenografts of HT-29 and HCT116 in mice grow at similar rates, but display significant differences in vascular architecture and proliferation [35]. HT-29 cells display more complex, mature vasculature [35]. Furthermore, differences in the concentration of pro-inflammatory cytokines may affect cancer’s metabolic profile. Others demonstrate that TNF- α suppresses butyrate oxidation in HT-29 cells [14]. However, this effect is only seen at five and ten-times the amount of (50-100 ng/mL) of pro-inflammatory cytokine used in our experiments [14]. These data demonstrate that even though these are both colorectal cancer cell lines, their differences in geno- and phenotype expression can influence their nutrient metabolism.

In HCT116 cells, other pro-inflammatory cytokines also have a significant effect on cancer cell metabolism. IL-6 has been implicated for its role in promoting angiogenesis, proliferation and migration in colorectal cancer [13]. Pretreatment with IL-6 significantly increases butyrate oxidation in HCT116 as compared to only butyrate-treated cells. It is unknown the long-term effect this may have, but butyrate has also been shown to promote colorectal

cancer cell growth and proliferation in early stage colorectal cancer [36]. Interestingly, IL-6 has been shown to induce lipolysis in insulin resistant cells or glucose deprived cells [37]. Therefore, we hypothesize that in the absence of glucose IL-6 may induce increased lipolysis and subsequent β -oxidation of available substrates-butyrate and endogenous fatty acids. This may be further explained when comparing IL-6 pretreated cells to cells that received no treatment as the display a significantly increased OCR.

TNF- α suppresses butyrate oxidation in HCT116 as well as HT-29 cells [14]. Furthermore, the oxidation and uptake of butyrate were significantly decreased in the presence of TNF- α [50ng/mL] [14]. Cancer cells are known to change their nutrient utilization to increase their chances of survival [26, 32, 38, 39]. These data establish that pro-inflammatory cytokines modulate cancer cell nutrient metabolism. We hypothesize that these modulations will promote cancer cell survival by modifying metabolism to enhance cell proliferation and suppress cell cycle arrest. This will provide a framework for further characterizing the effect that pro-inflammatory cytokines elicit from cancer cell metabolism. Furthermore, it may provide an opportunity for further exploration of therapeutic approaches that inhibit pro-inflammatory cytokines effects on cancer cell metabolism.

Conclusions

Inflammation has long been linked to cancer initiation and progression [1, 3, 7, 40, 41]. More recently, inflammation has been linked to altering nutrient utilization in cancer cells to promote cancer cell survival [26]. As such, nutrients play a significant role not only as an energy source, but also as functional regulators of cells. Here we have identified that pro-inflammatory

cytokines, mediators of inflammation, alter nutrient utilization in a variety of cancer cell lines. These findings necessitate the need to further investigate the mechanisms by which pro-inflammatory cytokines transduce their signal. These findings may aid in the development of therapeutic strategies to prevent or inhibit tumorigenic progression.

Methods

Cell culture

HCT116 (ATCC, CCL-247), HT-29 (ATCC, HTB-39), and A375 (ATCC, CRL-1619) cancerous cells, were grown in DMEM formulated with 5mM glucose and 10% FBS. 22Rv1 (ATCC, CRL-2505) were grown in RPMI formulated with 5mM glucose and 10% FBS.

Flux experiment

XF²⁴ Analyzer (Seahorse Bioscience) was used to measure % change in the oxygen consumption rates (% OCR) in HCT116, A375, 22Rv1, and HT-29 cells. Experiments were conducted following manufacturer guidelines. Cells split and seeded (at an identical cell number per well) into XF²⁴ cell culture microplates (Seahorse Bioscience, 100777-004) and grown to 90% confluency in DMEM with only 10% FBS to minimize lipogenesis. Before running the seahorse assay, cell plates are incubated with 1x KHB (a substrate limited media without glucose with 50 μ M carnitine, with or without IL-1 β [1ng/mL], TNF- α [10 ng/mL], or IL-6[5ng/mL]) in non-CO₂ incubator at 37°C for 45 minutes, then replaced with fresh KHB with 2.5 mM glucose, 50 μ M carnitine, and corresponding cytokines as described above 15 minutes prior to assay. Seahorse

experiments were run with identical conditions. Butyrate at 5mM final concentration was injected and the change in OCR was measured from baseline (%OCR). Next, 2-deoxyglucose (2DG) (Sigma, D8375) was injected at a final concentration of 5 mM and %OCR was measured again. Finally, 10% sodium azide was injected to inhibit oxidative metabolism.

Authors' contributions

MJ and DRD conceived the study design. MJ performed experiments. MJ and DRD prepared manuscript. All authors read and approved final manuscript.

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Appendix

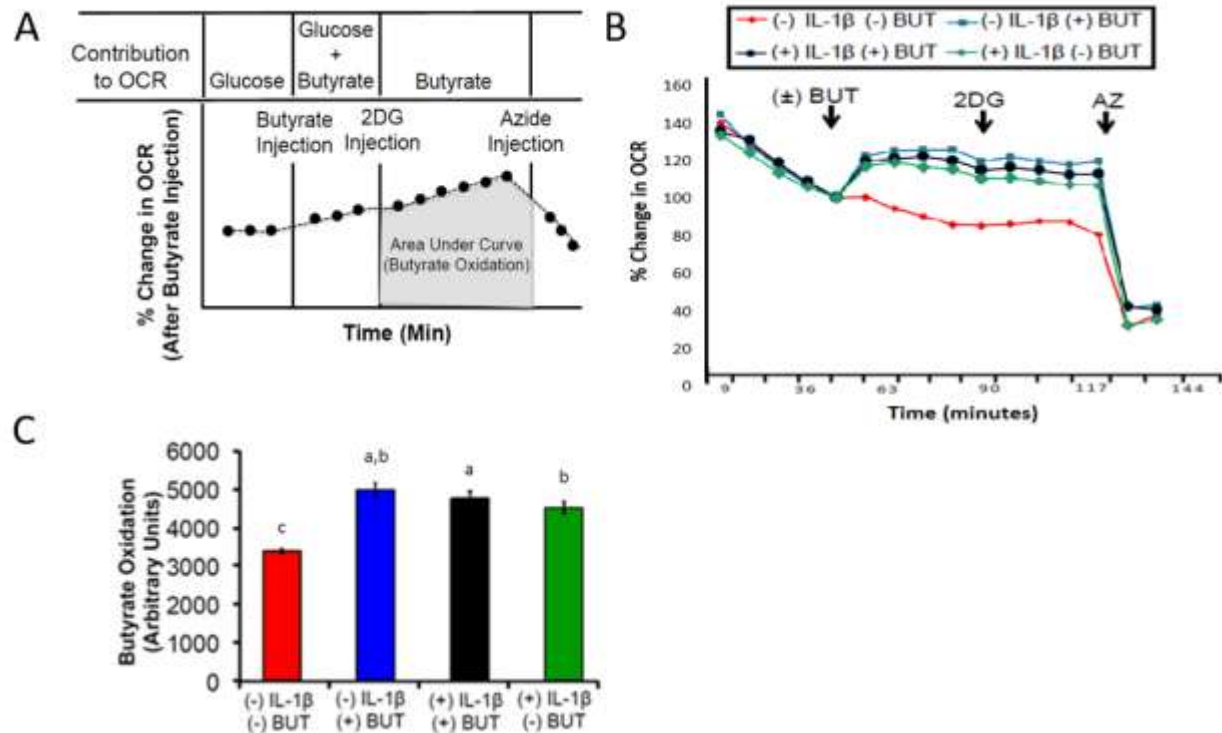


Figure 3.1. Butyrate oxidation is unaffected by IL-1 β [1 ng/mL] in A375 cells, a melanoma cell line. **A.** Schematic representation illustrating the exclusive measurement of butyrate oxidation with XF²⁴ Analyzer using a glucose analog, 2-deoxyglucose (2DG). **B.** Percent change in oxygen consumption rate (OCR) in A375 cells pretreated with or without IL-1 β (final concentration 1 ng/mL) and with or without butyrate (final concentration 5 mM). **C.** Average OCR measurements of butyrate oxidation taken after 2DG injection, prior to sodium azide injection. These measurements represent butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. Bars with different letters are significantly different at $p < 0.01$. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Error bars are $\pm$ the SD.

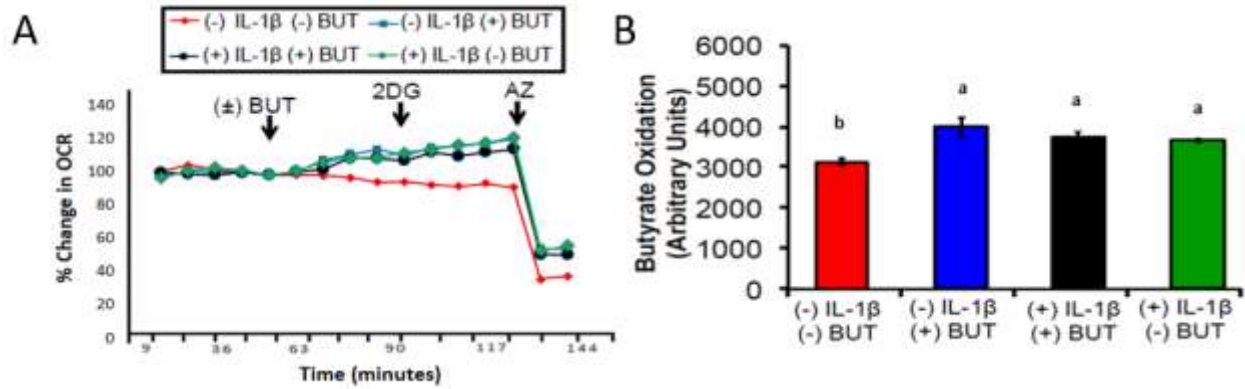


Figure 3.2. Butyrate oxidation is unaffected by IL-1 β in CWR22Rv1, a prostate carcinoma cell line. **A.** Percent change in oxygen consumption rate (OCR) over time in CWR22Rv1 cells pretreated with or without IL-1 β (final concentration 1 ng/mL) and with or without butyrate (final concentration 5 mM). **B.** Average OCR measurements of butyrate oxidation taken after 2DG injection, prior to sodium azide injection. These measurements represent butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. Bars with different letters are significantly different at $p < 0.01$. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Error bars are $\pm$ the SD.

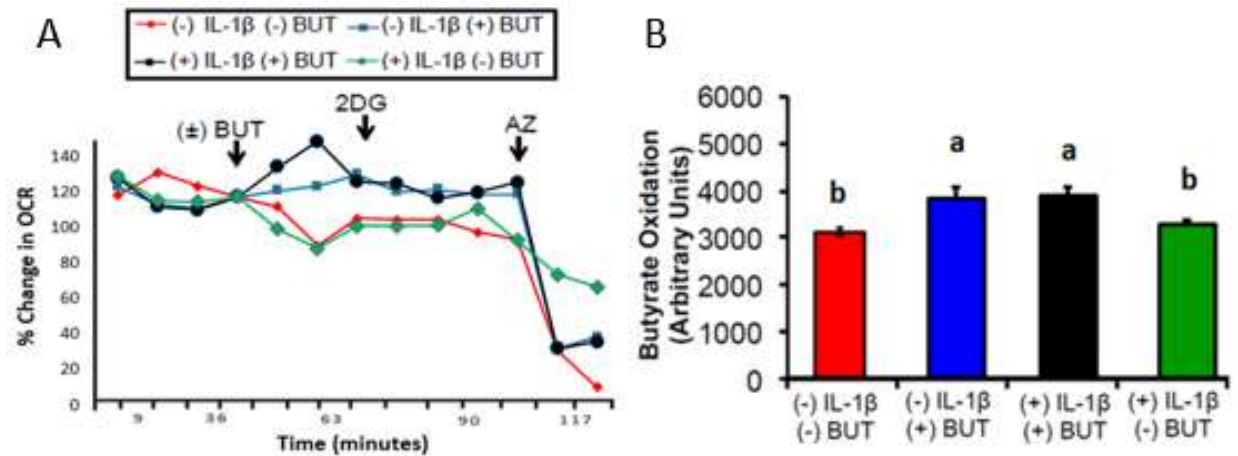


Figure 3.3. Butyrate oxidation is unaffected by IL-1 β [1 ng/mL] in HT-29, a colorectal cancer cell line. **A.** Percent change in oxygen consumption rate (OCR) over time in HT-29 cells pretreated with or without IL-1 β (final concentration 1 ng/mL) and with or without butyrate (final concentration 5 mM). **B.** Average OCR measurements of butyrate oxidation taken after 2DG injection, prior to sodium azide injection. These measurements represent butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. Bars with different letters are significantly different at $p < 0.01$. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Error bars are $\pm$ the SD.

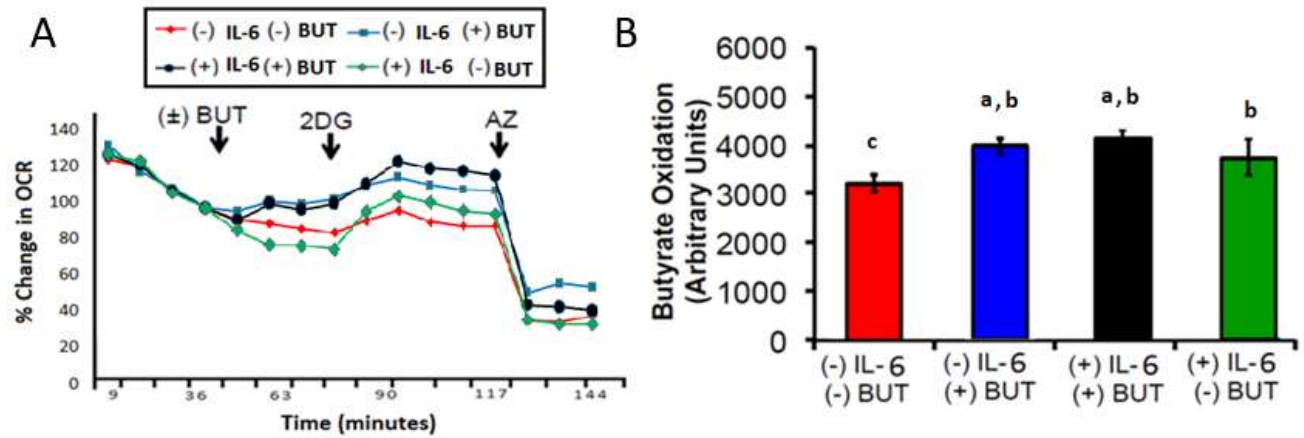


Figure 3.4. Butyrate oxidation is enhanced by IL-6 in HCT116 cells, a colorectal carcinoma cell line. **A.** Percent change in oxygen consumption rate (OCR) over time in HCT116 cells pretreated with or without IL-6 (final concentration 5 ng/mL) and with or without butyrate (final concentration 5 mM). **B.** Average OCR measurements of butyrate oxidation taken after 2DG injection, prior to sodium azide injection. These measurements represent butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. Bars with different letters are significantly different at $p < 0.01$. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Error bars are $\pm$ the SD.

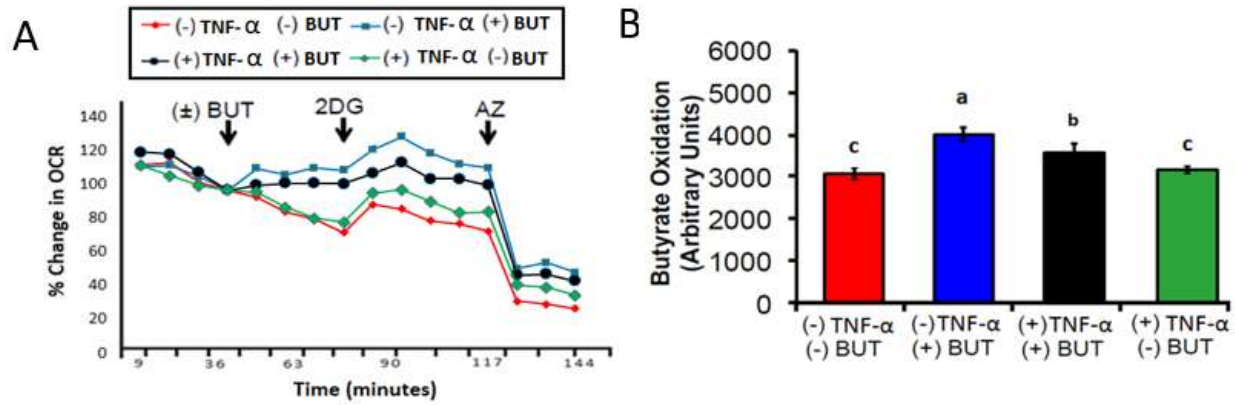


Figure 3.5. Butyrate oxidation is diminished by TNF- α in HCT116s, a colorectal carcinoma cell line. **A.** Percent change in oxygen consumption rate (OCR) over time in HCT116 cells pretreated with or without TNF- α (final concentration 10 ng/mL) and with or without butyrate (final concentration 5 mM). **B.** Average OCR measurements of butyrate oxidation taken after 2DG injection, prior to sodium azide injection. These measurements represent butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. Bars with different letters are significantly different at $p < 0.01$. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Error bars are $\pm$ the SD.

CHAPTER IV

Interleukin-1 beta regulates p21 induction by butyrate in colorectal cancer cells through a p38 mechanism

This chapter is revised based on a paper published by Megan Johnstone and Dallas Donohoe.

Full Title: Interleukin-1 beta regulates p21 induction by butyrate in colorectal cancer cells through a p38 mechanism

Authors: Megan E. Johnstone¹, Natalie Bennett¹, Haley Porter¹, and Dallas R. Donohoe¹,

My primary contribution to this paper include (i) development of the problem into work, (ii) identification of the study objectives, (iii) design and conducting experiments, (iv) gathering and analyzing results, (vi) assembling contributions into a paper, (vii) co-writing.

Abstract

Background: Inflammation is described as an “enabling characteristic” of cancer. Pro-inflammatory cytokines can mediate the inflammatory response and have been found to be elevated in many cancers. Pro-inflammatory cytokines have been shown to stimulate a variety of tumorigenic properties including altering nutrient utilization to promote cancer cell survival and proliferation. Previously, we have shown that IL-1 β , a pro-inflammatory cytokine, suppresses butyrate oxidation in the colorectal cancer cell line, HCT116. The long term effect of this remains unclear, but butyrate is known to suppress colorectal cancer cell growth, in part, by induction of p21. To this end, we hypothesized that IL-1 β would perturb butyrate metabolism decreasing p21 expression and inhibiting cancer cell proliferation.

Methods: The effect of IL-1 β on cell numbers was measured with an automated handheld cell counter. HDAC levels in HCT116 cells was measured with biochemical assays. The action of cytokines and pharmacological inhibitors were confirmed on downstream targets by immunoblotting. Furthermore, a Seahorse XF²⁴ Analyzer was used in conjunction with various pharmacological compounds to assay mitochondrial function

Results: Pretreatment of colorectal pro-inflammatory cytokine, IL-1 β suppresses butyrate’s ability to induce p21 expression. This results in increased cell proliferation as compared to butyrate treatment alone. Here we show that IL-1 β affects butyrate utilization via its receptor and a p38 mediated mechanism. This effect was completely blocked with the p38 selective inhibitor 203580 and p21 expression was rescued resulting in butyrate induced cell cycle arrest. These data suggest that IL-1 β modifies butyrate metabolism in colorectal cancer cells to promote cancer cell proliferation.

Conclusions: These data provide insight into the effects induced by pro-inflammatory cytokine, IL-1 β , the colorectal cancer cell line HCT116, and identify therapeutic targets that may be exploited to block the effects of IL-1 β .

Background

The short chain fatty acid, butyrate, is derived from microbial fermentation of dietary fiber in the colon [1, 2]. Butyrate slows cell proliferation in cancerous colonocytes, in part, by inhibiting histone deacetylases (HDACs) [3-5]. HDACs facilitate the removal of acetyl groups from histones and inhibition of HDACs results in hyperacetylation of histones [5, 6]. This hyperacetylation alters gene expression by promoting the relaxation of chromatin [4]. HDAC inhibition represents one mechanism by which butyrate represses or induces specific genes that regulate cell cycle progression [4]. It is through the induction of the cell cycle regulatory protein, p21, that butyrate is able to, in part, inhibit cancer cell proliferation [7-10]. Therefore, upregulation of p21 by butyrate results in cell cycle arrest in cancerous colonocytes [4, 11, 12]. Outside of elevating histone acetylation, it is unknown what upstream factors regulate this interaction between butyrate and p21.

Previously, it was found that the pro-inflammatory cytokine IL-1 β decreased the oxidation of butyrate in HCT116 colorectal cancer cells. The significance of this diminished butyrate oxidation is yet unexplored. The oxidation of butyrate has been previously associated with mediating colorectal cancer cell cycle regulation and apoptosis through induction, in part, of p21 [4, 13]. Accordingly, the suppression of butyrate oxidation as mediated by IL-1 β would block butyrate induced p21 expression, which would result in altered cell proliferation in butyrate-treated cells.

Here, we demonstrate that IL-1 β diminishes butyrate's anti-proliferative activity in HCT116 cancerous colonocytes. Specifically, HCT116 cancerous colonocytes treated concomitantly with butyrate and IL-1 β showed increased cell proliferation and net cell growth as compared to butyrate treatment alone. This suggests that IL-1 β dampens butyrate's anti-

proliferative effect, reduces upregulation of p21 by butyrate, and promotes colorectal cancer progression [14]. Decreasing the capacity of butyrate to induce p21 may represent one mechanism as to how IL-1 β stimulates cancer cell growth and proliferation.

Results

IL-1 β suppresses butyrate's anti-proliferative effect in HCT116 cells

It is unknown whether IL-1 β changes the anti-cancer properties of butyrate on colorectal cancer cells. For initial experiments, we tested the effect of IL-1 β on butyrate-mediated diminishment of cell growth, cell proliferation, and cell viability. In this regard, butyrate significantly reduced cell growth (Fig. 4.1A and B), cell proliferation (Fig. 4.1C), and cell viability (Fig. 4.1D) as compared to groups that were not treated with butyrate (control and IL-1 β). Interestingly, co-incubation with IL-1 β blocked the butyrate mediated decrease in cell growth, proliferation, and viability. These findings suggest that IL-1 β suppresses the cell cycle arrest induced by butyrate. Previously, the cell cycle protein p21 has been implicated in regulating butyrate's inhibitory actions on the cell cycle [7, 8]. Therefore, we next sought to test whether IL-1 β impacted p21 induction by butyrate.

IL-1 β suppresses p21 expression in the presence of TSA and butyrate

Butyrate and trichostatin A (TSA) are known to induce p21 expression [8, 9]. Butyrate and TSA induce p21 expression via HDAC inhibition. In agreement with what others have found, treatment of HCT116 colorectal cancer cells with butyrate (Fig. 4.2A) or TSA (Fig. 4.2B)

resulted in a rapid upregulation of p21 protein [5, 7-9, 15, 16]. However, butyrate treated cells simultaneously incubated with IL-1 β displayed a significant reduction in p21 expression as compared to cells treated with only butyrate or TSA. Given that TSA and butyrate inhibit HDACs and this effect of IL-1 β treatment to suppress p21 was observed with both butyrate and TSA suggests that HDAC inhibition may be important in mediating this effect. Therefore, IL-1 β may affect the ability of butyrate to increase histone acetylation as an HDAC inhibitor.

IL-1 β increases histone deacetylase activity in butyrate-treated cells

Butyrate regulates p21 through inhibition of HDACs, which results in global histone acetylation and histone acetylation at specific promoters [7, 8]. It is unclear whether IL-1 β alters histone acetylation or changes histone acetylation in cells treated with butyrate. HCT116 colorectal cancer cells treated with only IL-1 β showed no change in HDAC activity when compared to controls (cells not treated with IL-1 β) (Fig. 4.3A). In contrast, HCT116 cells treated with IL-1 β and butyrate had elevated HDAC activity as opposed to cells treated with butyrate only (Fig. 4.3B). This same effect was not observed in HCT116 cells treated with IL-1 β and TSA (Fig. 4.3C). Thus, suggesting that HDAC activity may be differentially affected by IL-1 β , and it may not entirely explain IL-1 β 's action to suppress p21 induction by butyrate or TSA.

IL-1 β affects global H3 acetylation in both butyrate or TSA treated cells

Since IL-1 β could alter butyrate's ability to inhibit HDACs, we next tested whether this culminated into a change in butyrate induced global histone acetylation. As expected, butyrate increased global pan H3 acetylation in HCT116 cells (Fig. 4.4A). Consistent with IL-1 β

interfering with HDAC inhibition by butyrate, HCT116 cells treated with IL-1 β and butyrate exhibited a reduction in H3 acetylation compared to butyrate alone (Fig. 4.4B). Surprisingly, TSA, which also increased H3 acetylation (Fig. 4.4C), showed a similar response (Figure 4.4D). Thus, even though IL-1 β didn't alter HDAC activity in the case of TSA, it still was able to dampen overall global induction of H3 pan acetylation by TSA. This may allude to discordance between how IL-1 β interacts to affect TSA compared to butyrate, in reference to p21 induction.

IL-1 β significantly affects gene expression and protein turnover

The fact that butyrate and TSA may regulate p21 protein expression through similar, but also distinct mechanisms, suggested that both gene expression and protein turnover may be important. Thus, RT-PCR was used to test whether IL-1 β inhibited *p21* gene expression by butyrate. The *p21* gene was significantly induced by butyrate, and this induction was diminished when co-treated with IL-1 β (Fig. 4.5A). To determine how p21 protein turnover was impacted by butyrate and IL-1 β , cycloheximide was used to block gene expression. HCT116 colorectal cancer cells pretreated for 1 hour with cycloheximide showed elevated p21 protein expression (p21 increased over time) in cells treated with butyrate (Fig. 4.5B). This increase in p21 protein expression became apparent after 3 hours (Fig. 4.5C). In cells treated with both butyrate and IL-1 β , p21 protein expression did not increase (Fig. 4.5D). These data suggested that IL-1 β is altering both the p21 gene expression and protein turnover, which again alludes to a mechanism beyond just IL-1 β altering butyrate's ability to function as an HDAC inhibitor. The mechanisms by which IL-1 β impacted p21 would likely involve downstream signaling proteins of the interleukin-1 receptor (the major target of IL-1 β).

Inhibition of p38 rescues histone acetylation and p21 expression

We previously found that IL-1 β had the ability to decrease butyrate oxidation in colonocytes through the mitogen-activated protein kinase called p38. p38 is one downstream target of IL-1R that is activated by IL-1 β (Fig. 4.6A). p38 has been shown to positively regulate p21 through enhancing p53 levels [17]. Towards this end, p21 induction has been linked to mitochondrial dysfunction and reduced oxygen consumption [18], which are two outcomes we previously found from IL-1 β exposure in butyrate-treated HCT116 colorectal cancer cells. The reduced oxygen consumption was rescued by blocking the activation of p38 with a selective pharmacological inhibitor. Thus, in our model we propose that IL-1 β reduces p21 induction by butyrate through activation of p38, and it is possible to negate this effect with a p38 inhibitor, SB203580 (Fig. 4.6B).

Butyrate or IL-1 β activated p38; as judged by the increased phosphorylation of the protein (Fig. 4.6C) compared to untreated cells. HCT116 cells treated with the combination of butyrate and IL-1 β also showed enhanced p38 phosphorylation, albeit somewhat reduced when compared to butyrate or IL-1 β alone. Importantly, this activation of p38 by both IL-1 β and butyrate was completely abolished in the presence of SB203580, the p38 inhibitor. SB203580 also completely rescued the effect of IL-1 β on p21 induction in butyrate treated cells (Fig. 4.6D). Similar to p38 mediating the effect of IL-1 β on suppressing butyrate oxidation, these data suggest that activation of p38 by IL-1 β impacts p21 expression.

Inhibition of p38 corrects mitochondrial changes induced by IL-1 β in butyrate treated cells

To characterize how IL-1 β impacts mitochondrial function in regards to butyrate, we performed a mitochondrial stress assay. IL-1 β and butyrate treated HCT116 cells showed an elevated response to FCCP, which is an ionophore or uncoupling agent, as compared to butyrate alone (Fig. 4.7A). Treatment of cells with butyrate, IL-1 β , and the p38 inhibitor (SB203580) reduced the response to FCCP to even a lower value than butyrate alone. The major mitochondrial change that IL-1 β effected in butyrate-treated cells was the proton leak, where the combination of the two increased the mitochondrial proton leak compared to cells treated with only butyrate (Fig. 4.7B). This effect was completely rescued with the p38 inhibitor. Thus, p38 appears to mediate the mitochondrial changes induced by IL-1 β , including the elevated proton leak, in butyrate-treated cells.

Since the p38 inhibitor blocked IL-1 β 's effect on mitochondrial function, we tested whether this inhibitor would also restore butyrate's anti-cancer effects in HCT116 colorectal cancer cells. Thereby associating activation of p38 with mitochondrial alterations induced by IL-1 β in butyrate treated cells and a cellular outcome such as cell growth. The p38 inhibitor, SB203580, negated IL-1 β protective effect in butyrate-treated cells (Fig. 4.7C).

Discussion

It is well established that butyrate inhibits cell proliferation in cancerous colonocytes by inducing p21 expression via HDAC inhibition [2, 3, 9, 19]. Factors that disrupt butyrate metabolism will interfere with HDAC inhibition and butyrate's anti-cancer effects, including p21 expression. Previously, it was shown that IL-1 β suppressed butyrate oxidation. In this study, IL-

1 β is demonstrated to block the action of butyrate to decrease cell growth, cell proliferation, and cell viability, which taken together suggests that this cytokine may alter the beneficial effects of butyrate to suppress colorectal cancer. These findings are consistent with previous reports that pro-inflammatory cytokines can stimulate metabolic changes that support cell survival and proliferation [20-22].

IL-1 β is known to promote tumorigenesis by stimulating invasion and metastasis [14]. The mechanisms through which IL-1 β stimulates tumor progression remain elusive. Identification of specific molecular targets that mediate IL-1 β -stimulated tumorigenesis would facilitate the development of therapeutic options to improve prevention and treatment of colorectal cancer. One potential way IL-1 β could impact the effect of butyrate is through interfering with HDAC inhibition and histone acetylation, especially as it inhibits butyrate oxidation. Towards this end, IL-1 β negatively impacted HDAC inhibition by butyrate, but not by TSA. Previously, it was reported that diminished oxidation of butyrate is associated with increased butyrate levels in the cell, and enhanced HDAC inhibition [13, 23]. Based on these findings, it was predicted that IL-1 β , through suppressing butyrate oxidation, would increase butyrate's HDAC inhibitory properties and global histone acetylation. However, this was not the case. This may be due to the fact that previous studies found that butyrate accumulated over time (24 hours) and many of the outcomes measured in this study occurred within a 3-hour time window. In fact, the ability of butyrate to increase p21 protein was highest at 3 hours and went down over extended treatment times (data not shown and [8]). Another factor that may explain the decrease in HDAC inhibition caused by IL-1 β relates to IL-1 β impacting butyrate uptake, as this cytokine has been demonstrated to down regulate the butyrate transporter called monocarboxylate transporter 1 (MCT1) in colorectal cancer cells [24]. Therefore, diminished

uptake of butyrate would result in less HDAC inhibition. This is consistent with IL-1 β having a selective effect on HDAC inhibition for butyrate, and not effecting HDAC inhibition by TSA.

Butyrate not only induced the expression of the p21 gene, but also enhanced p21 protein stability or inhibited degradation of the protein (as judged by the elevated p21 protein levels in the presence of cycloheximide). These data confirm an earlier study that showed a similar increase in p21 mRNA when treated with butyrate and cycloheximide in HT-29 colorectal cancer cells [8]. This study attributes this inability of cycloheximide to stop p21 induction by butyrate with the association that p21 is an early response gene. However, in this study cycloheximide was treated concomitantly, whereas in our study we pretreated HCT116 cells 1 hour prior to butyrate exposure, and then protein levels were measured as opposed to mRNA. Thus, it is concluded that butyrate inhibits the turnover of p21 protein. Interestingly, IL-1 β completely blocked this effect, once again suggesting that IL-1 β is modifying p21 expression by more than just altering HDAC activity and gene expression.

Previously, p38 was shown to control p21 expression indirectly through regulating p53 levels in human epidermal keratinocytes [17]. HCT116 cancer cells are wild type for the p53 gene, and thus it is possible that through blocking IL-1 β activation of p38, p53 induction would also be inhibited, which would the upregulation of p21. p53 has also been shown to regulate mitochondrial respiration through altering the electron transport chain (ETC) and oxygen consumption [25]. This would be consistent with the wide range of mitochondrial perturbations induced by IL-1 β and would link help link the rescue of the p38 inhibitor with mitochondrial respiration. One issue with the role of p53 relates to the induction of p53 by p38 was reported after 24 or 48 hours activation, thus it is not clear whether 3 hours following p38 activation is a long enough period to achieve a significant change in p53 levels. Nevertheless, analyzing the

role of p53 toward mediating IL-1 β 's effects in butyrate treated colorectal cancer cells will be a priority in future studies.

p38 has already been shown to be a potential valuable target in colorectal cancer research. Inhibition of p38 inhibits colorectal tumor development and progression in a preclinical mouse model of colitis-induced colorectal cancer [26]. It is also targeted by important tumor suppressor proteins, which are known to block the disease [27]. Our data support the current literature that inflammation induced activation of p38 increases colorectal cancer cell growth and proliferation. These findings reveal novel insight as to how IL-1 β can modulate cancerous colonocyte nutrient metabolism to support cancer cell survival.

Methods

Cell culture

HCT116 cells (ATCC, CCL-247) were grown in DMEM formulated with 5mM glucose and 10% FBS. Briefly cells were treated for three hours in treatment media (DMEM, 10% FBS, 5mM glucose) with one or combination of the following: Butyrate [5mM], IL-1 β [1 ng/mL], SB203580, specific for all p38 isoforms [10uM] (Cell Signaling Cat # 5633), and Cycloheximide [10ug/mL] (Acros Cat #AC357420010).

Cell Viability Assay/ApoLive-Glo Multiplex Assay

Cell viability was measured according to manufacturer protocol (Promega, Cat #G6410). Briefly, cells were seeded at equal density in a 96-well plate. The assay measures live cell protease

activity as a measure of viability by using a fluorogenic, cell permeant, peptide substrate that is cleaved by proteases to generate a fluorescent signal proportional to the number of living cells. Conditions were kept identical to previous assay and performed as described in the manufacturer protocol.

Cell Proliferation Fluorescent Assay

Cell proliferation was measured with a fluorometric assay kit (Biovision K307-1000). Briefly, HCT116 cells were seeded at equal density in a 96-well. Prior to confluency cells were treated for 24 hours with corresponding treatments. Conditions were kept identical to previous assay and performed as described in the manufacturer protocol. Briefly, cells were lysed and a fluorescent nuclear dye was added 15 minutes prior to plate reading. Fluorescence was read on a plate reader with Ex/Em 480/538.

Cell Number

Cells were seeded at equal density the prior day to treatments. Cells were treated for 24 hours with corresponding treatments. Conditions were kept identical to previous assays and performed as described in the manufacturer protocol. Cells were then trypsonized, pelleted, resuspended and counted via a handheld automated cell counter, Cell Sceptor by Millipore.

HDAC inSitu Fluorescent Assay

HDAC levels were measured in HCT116 cells with a HDAC Fluorometric Assay kit (Biovision K339-100). Conditions were kept identical to previous assay and performed as described in the manufacturer protocol.

Western Blotting

Proteins from HCT116 cells were extracted with RIPA buffer (Cell signaling, #9806), 1mM PMSF (Cell signaling, #8553) and phosphatase inhibitor cocktail (Cell signaling, #5872). Protein concentrations were measured by Bradford assay. Gel electrophoresis and transfer were conducted using standard protocol for Western blot. Antibodies that were used included p38 (Cell Signaling, Cat # 9212S), phospho p38 (Cell Signaling, Cat # 9211S), p21(Santa Cruz, Cat # sc-469), β -actin (Sigma, Cat #A1978), pan-acetylated-histone H3 (Active motif, Cat# 39139), total Histone H3 (Active motif, Cat# 39763), and β -actin (Sigma, Cat# A1978).

Chemiluminescence or fluorescent detection was performed with the Odyssey Fc and bands were quantified with Image Studio Software (LI-COR Biosciences, Lincoln, NE).

Flux experiment

XF²⁴ Analyzer (Seahorse Bioscience) was used to measure % change in the oxygen consumption rates (% OCR) in HCT116 and HT-29 cells. Experiments were conducted following manufacturer guidelines. Cells (HCT116 cells) split and seeded (at an identical cell number per well) into XF²⁴ cell culture microplates (Seahorse Bioscience, 100777-004). Before

running seahorse, cell plates are incubated with KHB (no glucose with 50 μ M carnitine, with or without IL-1B, CAPE, SB, IL-1RA) in non-CO₂ incubator at 37°C for 45 minutes, then replaced with fresh KHB with 2.5 mM glucose, 50 μ M carnitine, and corresponding treatments as described above 15 minutes prior to assay. Seahorse experiments were run with identical conditions (unless otherwise noted). Butyrate at 5mM final concentration was injected and the change in OCR was measured from baseline (%OCR). Oligomycin(1 μ M/mL), FCCP (1 μ M/mL, Rotenone and Antimycin (1 μ M/mL) were injected from MitoStress test kit (Seahorse Bioscience, #103015-100).

Statistical Analysis

For biochemical assays and Western blots, the differences between experimental groups were determined by ANOVA followed by a Tukey post-hoc test. All data are expressed as mean $\pm$ SE.

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Appendix

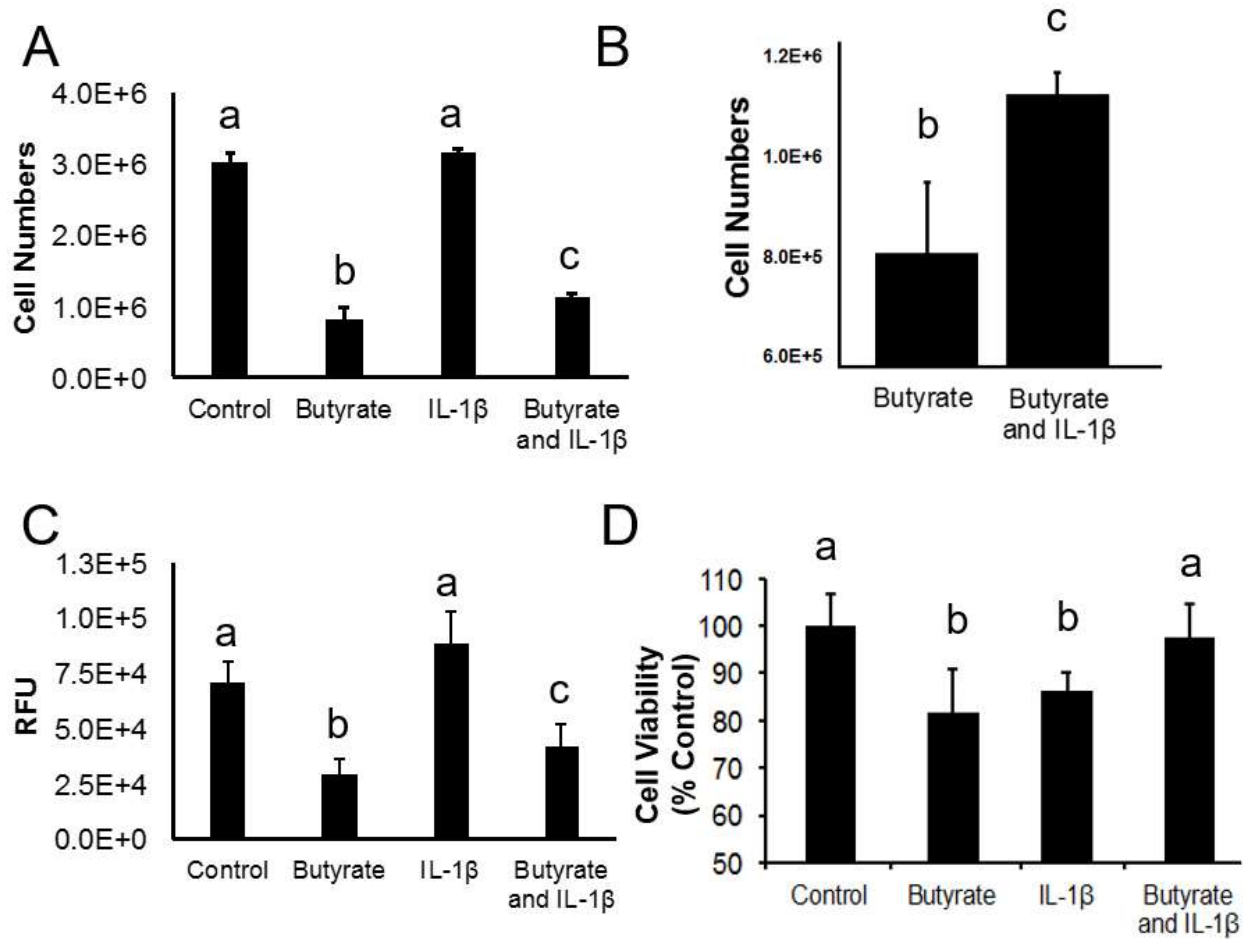


Figure 4.1. IL-1 β suppresses butyrate's anti-proliferative effect in HCT116 cells. **A.** Cell number measured in HCT116 cells. **B.** Cell number measured in butyrate and butyrate with IL-1 β treated HCT116 cells. **C.** Relative fluorescent units indicative of cell proliferation. **D.** Relative fluorescence as a percentage of control measuring cell viability. Data points represents a standard mean where n = 3+ per condition. Error bars are +/- SEM. Bars with different letters are significantly different at p<0.05.

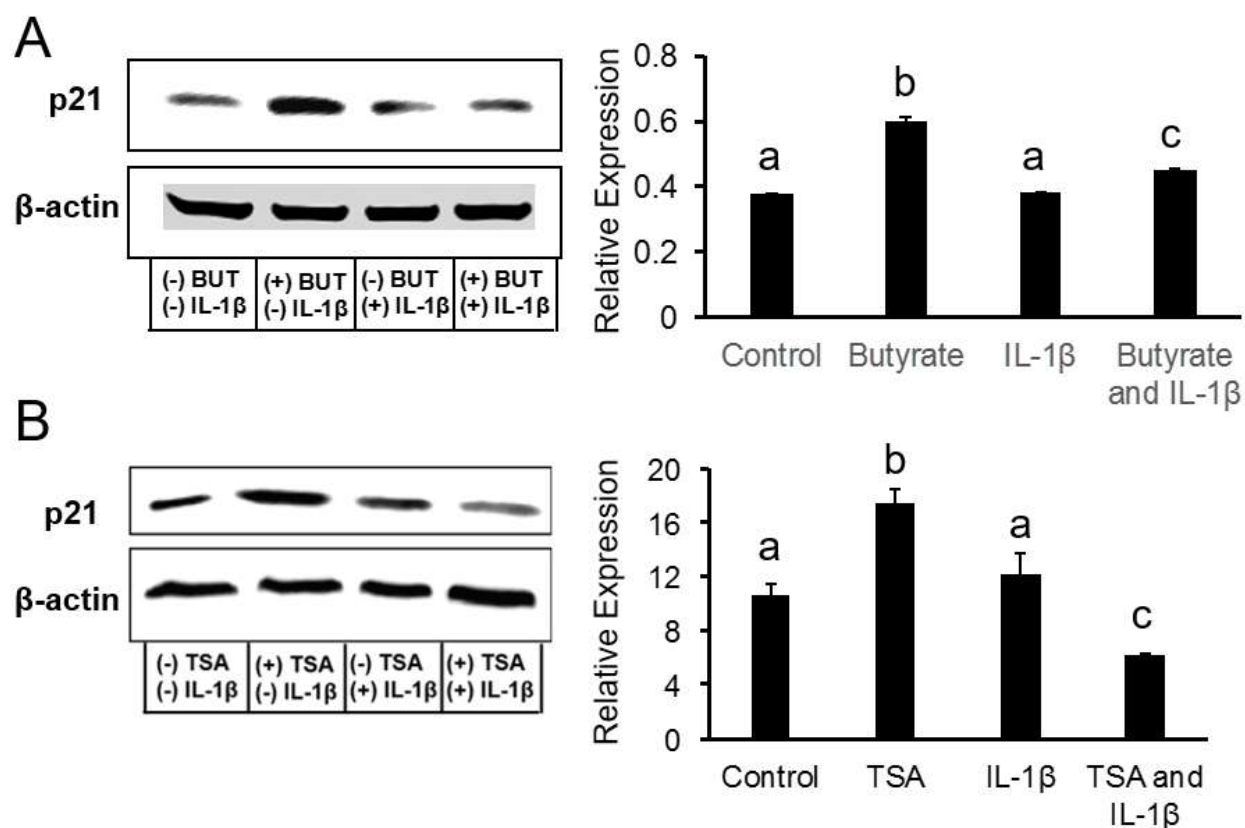


Figure 4.2. IL-1 β suppresses p21 expression in the presence of TSA and butyrate. A. Western blot and quantification of butyrate induced p21 expression. **B.** Western blot and quantification of TSA (trichostatin A) induced p21 expression. Data points represents a standard mean where $n = 3+$ per condition. Error bars are $\pm$ SEM. Bars with different letters are significantly different at $p < 0.05$.

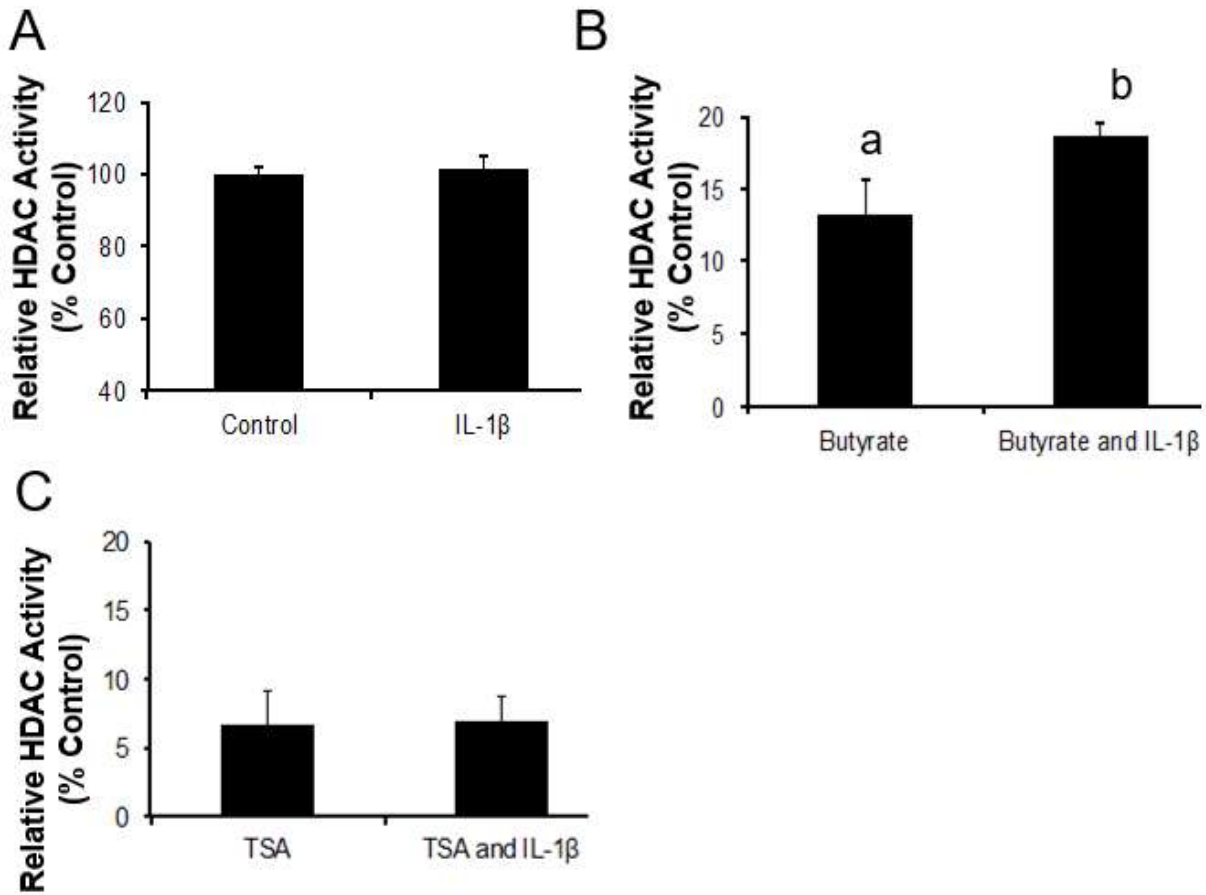


Figure 4.3. IL-1 β increases histone deacetylase activity in butyrate-treated cells. **A.** Relative fluorescent HDAC activity in control versus IL-1 β treated cells. **B.** Relative fluorescent HDAC activity in butyrate versus IL-1 β and butyrate treated cells **C.** Relative fluorescent HDAC activity in TSA versus IL-1 β and TSA treated cells Data points represents a standard mean where n = 5+ per condition. Error bars are +/- SEM. Bars with different letters are significantly different at p<0.05.

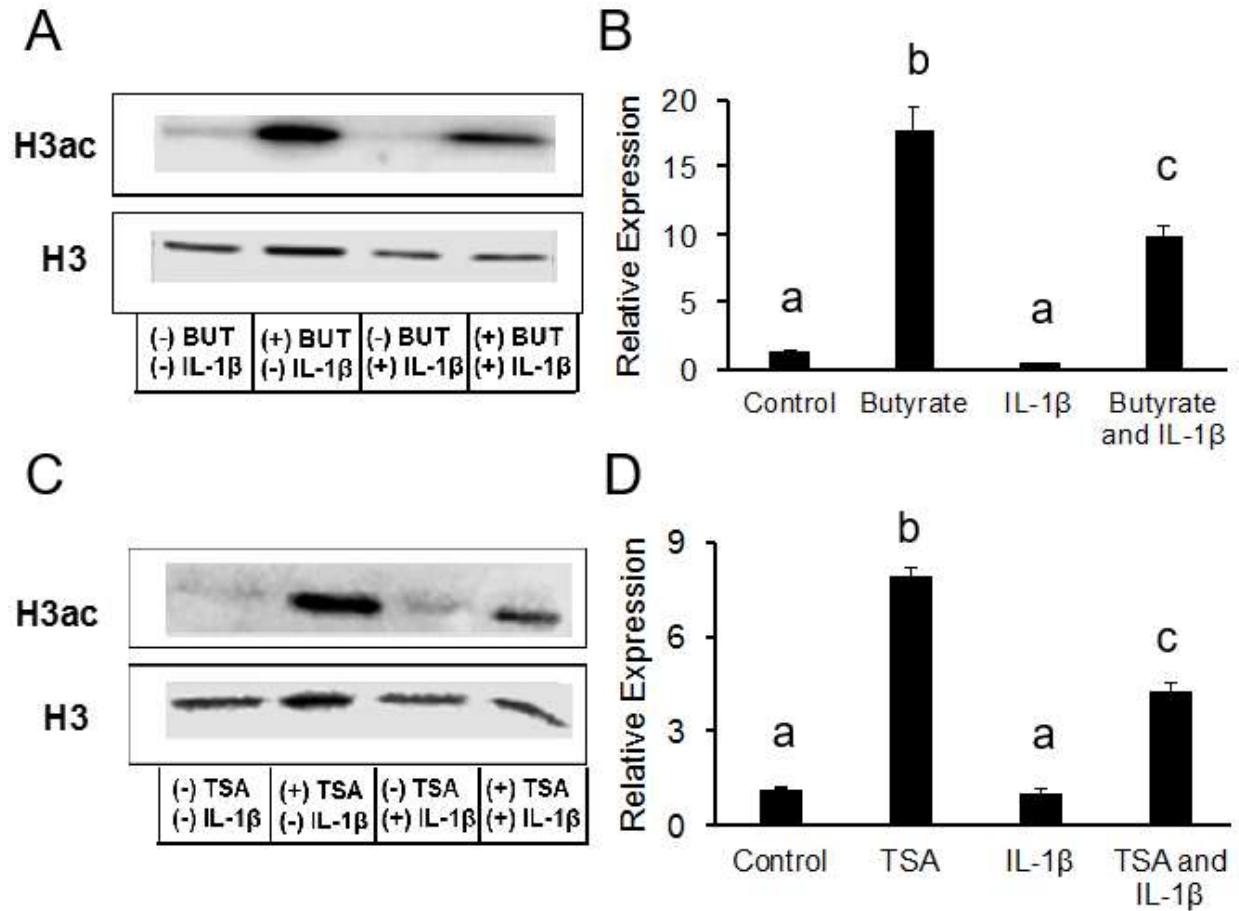


Figure 4.4. IL-1 β affects global H3 acetylation in both butyrate or TSA treated cells. A. Western blot and quantification of butyrate induced H3ac expression. **B.** Western blot and quantification of TSA (trichostatin A) induced H3ac expression. Data points represents a standard mean where $n = 3+$ per condition. Error bars are $\pm$ SEM. Bars with different letters are significantly different at $p < 0.05$.

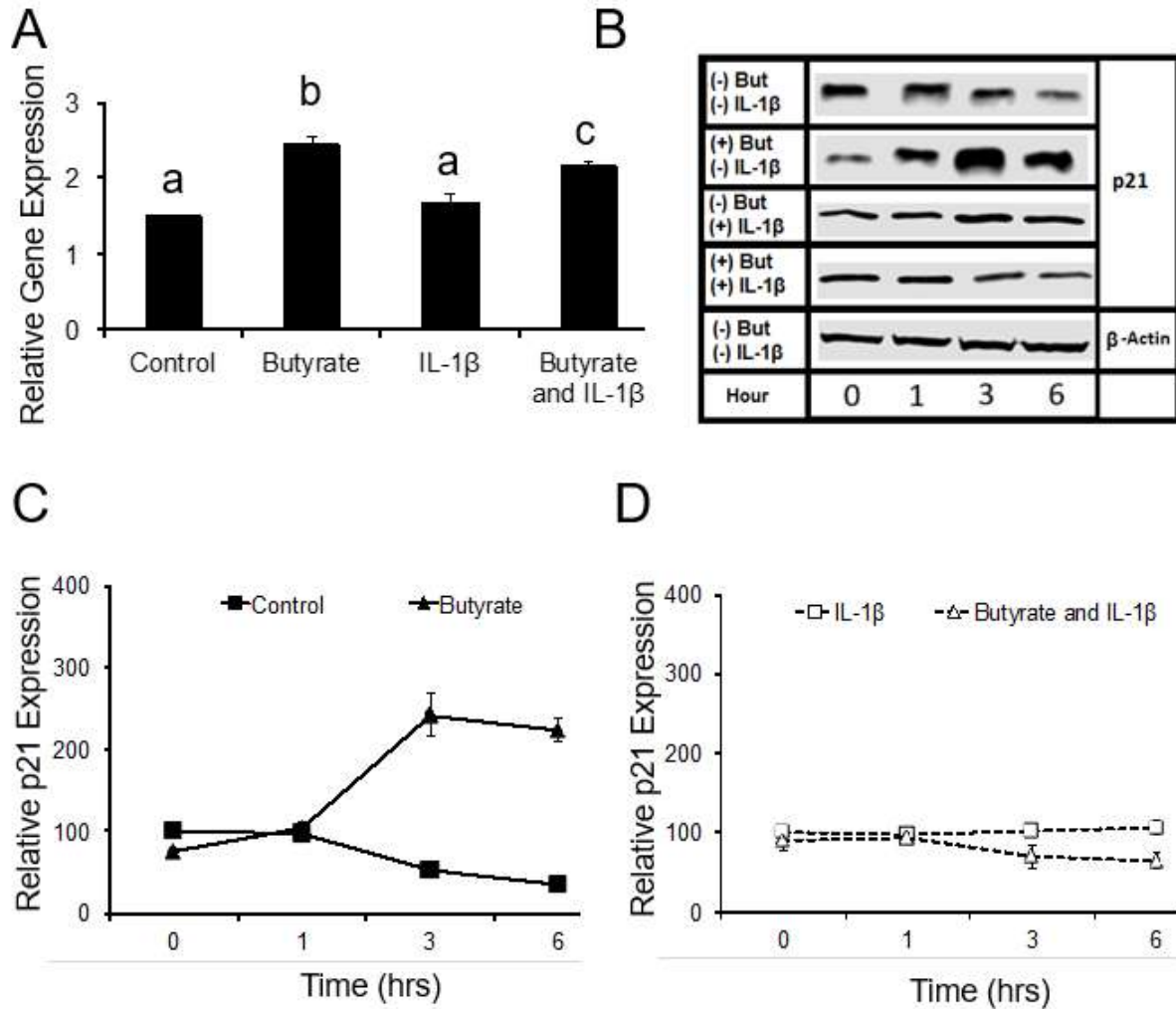


Figure 4.5. IL-1 β significantly affects gene expression and protein turnover. A. RT-PCR was used to test *p21* gene expression B. Western blot of p21 with cycloheximide pretreatment C. quantification of cycloheximide induced p21 turnover expression comparing control and butyrate groups D. quantification of cycloheximide induced p21 turnover expression comparing IL-1 β and IL-1 β and butyrate groups. Data points represents a standard mean where n = 3+ per condition. Error bars are +/- SEM. Bars with different letters are significantly different at p<0.05.

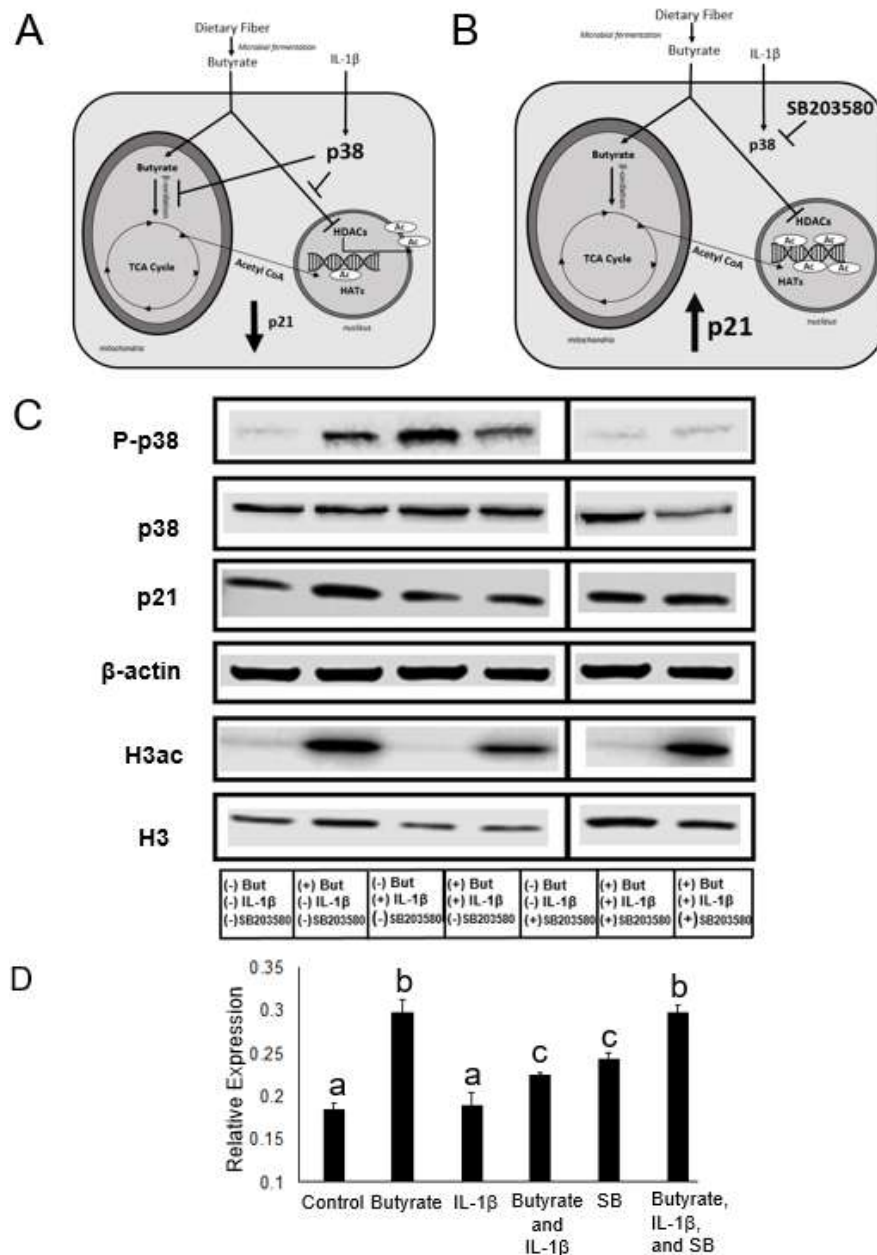


Figure 4.6. Inhibition of p38 rescues histone acetylation and p21 expression. **A.** Model representing IL-1 β downstream target, p38, and effect on butyrate metabolism **B.** Model representing inhibition of p38 rescues butyrate induced p21 expression **C.** Western blot assay was employed to detect the expression of P-p38, p38, p21, β -actin, H3ac, and H3. **D.** Quantification of p21 expression corrected with β -actin. Data points represent a standard mean where $n = 3+$ per condition. Error bars are $\pm$ SEM. Bars with different letters are significantly different at $p < 0.05$.

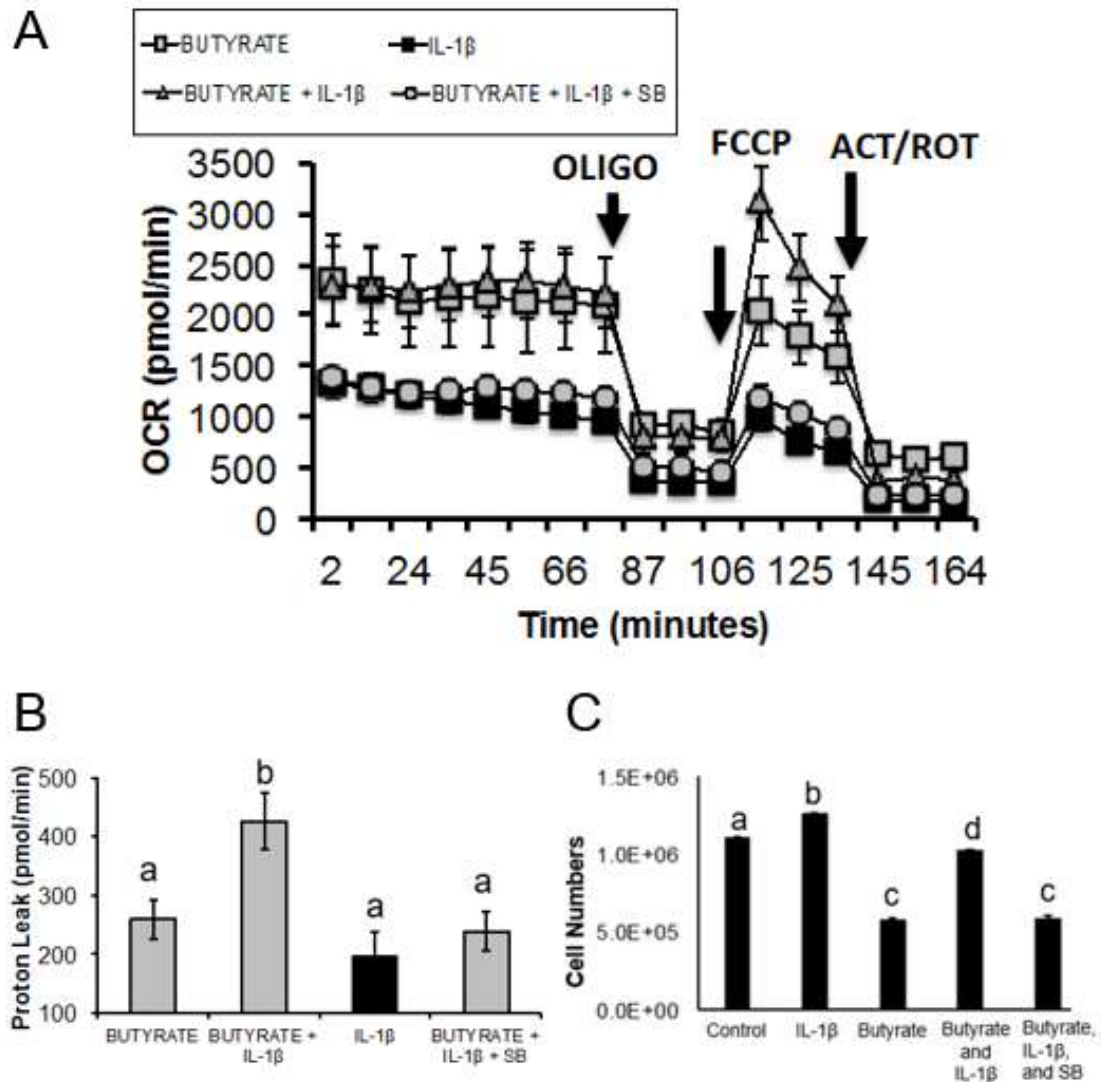


Figure 4.7. Inhibition of p38 rescues histone acetylation and p21 expression. **A.** Model representing IL-1 β downstream target, p38, and effect on butyrate metabolism **B.** Model representing inhibition of p38 rescues butyrate induced p21 expression **C. Western blot D.** quantification of p21 expression. Data points represents a standard mean where n = 3+ per condition. Error bars are +/- SEM. Bars with different letters are significantly different at p<0.05.

CHAPTER V

General Discussion

Discussion

Previous studies examining the link between inflammation and cancer progression have pointed towards pro-inflammatory cytokines, in part, as key mediators of this effect. Pro-inflammatory cytokines are elevated in the serum of many cancers [1-3]. They have been shown to stimulate cancer cell survival mechanisms such as angiogenesis, cell proliferation, and even alter nutrient utilization [4-7]. For example, butyrate, a short chain fatty acids derived from fiber fermentation in the colon, is the preferred energy source of colonocytes and increases colonocyte differentiation which may counter tumor proliferation [8]. *Ex vivo* studies using colonic biopsies from healthy patients undergoing routine colonoscopy were incubated with the pro-inflammatory cytokine, TNF- α , were found to significantly reduce butyrate oxidation [9]. These findings have been confirmed by *in vitro* studies in which TNF- α and IL-1 β significantly reduced butyrate oxidation in colorectal cancer cell lines [10, 11]. Indeed, the effects of pro-inflammatory cytokines appears to have a profound effect on butyrate oxidation in colonocytes.

The present data indicate that the pro-inflammatory cytokine, IL-1 β , suppresses butyrate oxidation in colonocytes. It is thought that factors that disrupt butyrate metabolism will interfere with its anti-cancer effects. The mechanisms by which this pro-inflammatory cytokine alter nutrient utilization have not been fully characterized. Emerging data have identified that pro-inflammatory cytokines affect mitochondrial metabolism and subsequently, nutrient metabolism to promote cancer cell survival [7, 12, 13]. Our studies demonstrate the pro-inflammatory cytokine, IL-1 β , suppresses mitochondrial oxidation of butyrate and this results in butyrate-resistant cell proliferation in the HCT116 cancerous colonocyte cell line.

Effect of pro-inflammatory cytokines, IL-1 β , on mitochondrial metabolism

Butyrate is known to be oxidized in the mitochondria. More specifically, it undergoes β -oxidation to form two acetyl CoA that is used for almost exclusively for oxidative phosphorylation [14, 15], whereas less than 2% is found in cellular lipids and histones. Regardless, this study and others indicate that by controlling intracellular concentrations of butyrate, such as by suppressing uptake or oxidation, cancer cells can diminish butyrate's growth inhibitory effects [10, 15]. In this study, we describe that pro-inflammatory cytokines may also be able to alter butyrate's fate and preserve cancer cell proliferation. Although this effect has been seen in other models, the timeline and treatment conditions have not been well described [9, 10]. We demonstrate that IL-1 β has an immediate effect on butyrate oxidation and more importantly on mitochondrial function. In another study using hepatocytes, IL-1 β was found to perturb mitochondrial function resulting in a decreased oxygen consumption coupled with upregulated proton leak [13]. In agreement, we see in Chapters 2 and 4, that IL-1 β inhibits oxygen consumption and upregulates proton leak. We validated these effect by measuring NADH and find that it is also diminished in the presence of IL-1 β . This suggests that IL-1 β perturbs mitochondrial function and may facilitate a shift in metabolism towards upregulated glycolysis. However, in our findings we did not see this shift towards increased glucose consumption. On the contrary, we find that IL-1 β suppressed glycolytic capacity as well. This suggests that IL-1 β may promote cancer cell survival through another means.

We postulate that by inhibiting IL-1 β , we would be able to rescue butyrate's growth inhibitory effects. To do so we sought to determine the pathway that IL-1 β would most likely use. Towards this end we find that IL-1 β utilized the interleukin-1 type I receptor (IL-1RI). IL-1 β is unique among the cytokines in that it has its own naturally expressed receptor antagonist,

interleukin-1 receptor antagonist (IL-1RA) [16, 17]. Population studies find polymorphisms in the *IL-1RA* gene are associated with an increase in cancer incidence [18]. *In vivo* studies demonstrate that IL-1RA is able to negate cancer promoting effects of IL-1 β and reduce cancer progression in murine gastric cancer models [19]. Thus, it appears that IL-1RA would be able to suppress IL-1 β signal transduction. The present studies show that IL-1RI is the receptor that IL-1 β is using and that IL-1RA is able to inhibit IL-1 β 's signal transduction resulting in rescued butyrate oxidation. Furthermore, in Chapter 2, we find that neither butyrate or glucose oxidation is affected by IL-1RA. This suggests that IL-1RA did not appear to perturb cancer cell metabolism.

We next turned our focus to IL-1 β 's mode of intracellular signal transduction. Previous studies examining the link between IL-1 β and cancer cell progression have pointed towards NF κ B [20, 21]. NF κ B has been linked to promoting tumorigenesis in many cancers [20]. Furthermore, NF κ B has been linked to regulating energy homeostasis by regulating cytochrome c oxidase [22]. For this reason, we chose to inhibit NF κ B by using a selective inhibitor, caffeic acid phenethyl ester (CAPE). While we show that CAPE did block phosphorylation and activation of NF κ B, it failed to rescue butyrate oxidation. This suggests that NF κ B, while activated by IL-1 β , is not a necessary component in mediating IL-1 β 's effect on butyrate oxidation.

p38 has been previously linked to colorectal tumor progression [4, 23-25]. Specifically, inhibition of p38 in patient derived xenografts slows tumor growth [24]. *In vivo* studies using nude mice models of gastric adenocarcinoma indicate that IL-1 β activates p38 thereby promoting metastatic cancer progression [26]. *In vitro* studies using hepatocytes find p38 aids in the regulation of hepatocyte metabolism [27]. These findings allude to p38 playing a role in cancer metabolism and suggest a pathway in which IL-1 β may utilize to shift colorectal cancer cell

metabolism away from butyrate utilization. In Chapter 2, the present data indicate that the selective inhibition of p38, by SB203580, rescues butyrate oxidation. Furthermore, in Chapter 4, we find that inhibition of p38 results in a rescue of mitochondrial function. Our data demonstrate that IL-1 β , in a p38-dependent mechanism, perturbs mitochondrial function resulting in decreased butyrate oxidation. The mechanisms by which IL-1 β perturbs butyrate utilization have not been well characterized and may represent an interesting therapeutic target that could be exploited to enhance butyrate utilization and therefore augment butyrate's anti-cancer effects. To this end we postulated that IL-1 β may have downstream functional effects on suppressed butyrate metabolism such as suppressing butyrate growth inhibitory properties.

Downstream consequences: Butyrate resistant cell proliferation

Previous studies have demonstrated the protective effect of butyrate on colorectal cancer cell progression and have pointed to its role cell cycle regulation as the mediator of this effect. *In vivo* studies using murine models receiving a high fiber or tributyrin (a butyrate analog) diet displayed decreased incidence of colorectal neoplasia [28, 29]. *In vitro* studies corroborate these findings and demonstrate butyrate's ability to suppress cell proliferation in a p21-dependent manner [30]. Therefore, this would suggest that butyrate has a profound effect on colorectal cancer cell proliferation and any interruption of its growth inhibition effect may result in cancer cell survival.

Butyrate is known to induce histone acetylation that, in part, induces expression of p21 [30-32]. More specifically, increased expression of p21 aids in the regulation of cell cycle and can restrict cell cycle progression in the G1 phase or increased expression of p21 can stimulate

apoptosis via a BAX/BIM mediated pathway [30-35]. Previous *ex vivo* studies in cardiac myocytes have shown IL-1 β to inhibit p21 expression, which enhances cell proliferation [36]. The mechanism by which IL-1 β mediates this effect was not characterized, but alluded to that fact that p21 expression was upregulated within the first eight hours of treatment [36]. Consistent with these results we see that butyrate stimulates an increase in p21 expression in the first three hours. Others have found that p21 is required for butyrate induced growth arrest [30]. To this end we find that IL-1 β suppresses p21 in our cancerous colonocytes in the presence of butyrate and results in butyrate resistant cell proliferation.

In Chapter 2, we described that IL-1 β could suppress butyrate oxidation. Previous studies have shown that diminished butyrate oxidation results in increased intracellular butyrate concentrations and leads to enhanced histone deacetylase (HDAC) inhibition [29, 37]. Towards this end we predicted that IL-1 β , by suppressing butyrate oxidation, would enhance global histone acetylation and therefore increase butyrate's growth inhibitory properties. However, we find that IL-1 β suppresses global histone acetylation. We next predicted that IL-1 β would impact butyrate's HDAC inhibition activity. We find that IL-1 β negatively impacted HDAC activity in the presence of butyrate, but not by trichostatin A (TSA). This may be due to the fact that previous studies have measured butyrate induced HDAC activity at a later time point and our studies looked at these effects within a three-hour window. Additionally, others have shown that pro-inflammatory cytokines downregulate butyrate uptake [10]. This is consistent in findings of biopsies from patients with ulcerative colitis and inflammatory bowel disease treated with inflammatory cytokines show diminished butyrate uptake and oxidation [14, 38].

Butyrate exclusively arrests cancer cell growth through increased p21 expression. The mechanisms that regulate p21 are either dependent or independent of the tumor suppressor

protein p53 [30]. The levels of p21 are highly controlled to ensure cell cycle checkpoints [33]. Previous studies show that p38 is linked to regulating p21 expression indirectly of p53 and in human epidermal keratinocytes [39]. HCT116 cancer cells are wild type for p53 and therefore perturbing p53 activity through p38 may represent a pathway for regulating p21 expression. In Chapter 2 we find that inhibition of p38 rescued butyrate oxidation and therefore in Chapter 4, we employed the same methodology and found that inhibition of p38 resulted in a rescue of butyrate induced p21 expression. Furthermore, this rescued butyrate's ability to induce growth arrest resulting in a negation of IL-1 β 's protective effect in butyrate-treated cells. The ability of IL-1 β to confer selective resistance to butyrate in these cancerous colonocytes is a prime example of how cancer cells modulate their environment to ensure survival. Ultimately, this confers a significant, selective advantage to cancerous colonocytes that promotes net cell growth. These findings reveal novel insight as to how IL-1 β can shift nutrient metabolism to support cancer progression.

Pro-inflammatory cytokines acutely perturb cancer cell metabolism

Pro-inflammatory cytokines influence alterations in mitochondrial function [7, 13, 27]. In Chapters 2 and 4, we see the direct, real-time effects that the pro-inflammatory cytokine, IL-1 β , can have on mitochondrial oxidation. The bioenergetics of tumor cells or oncobioenergetics is influenced by a variety of factors and dynamically change in response to the cellular microenvironment [12, 40, 41]. The use of FDG-PET/CT (fluorodeoxyglucose-positron emission tomography/computerized tomography) scans are used to trace, visually, the uptake of a glucose analog, FDG, and detect metabolically active malignant tissue [42, 43]. These types of studies exploit the metabolic nature of cancer and are used to locate and stage cancer [42]. These studies

show that many types of cancer cells have a phenotypic oncobioenergetic signature. We hypothesize, due to our previous studies, that pro-inflammatory cytokines may also affect cancer cell metabolism in other cancer cell lines and may have a metabolic phenotype. By characterizing the effects of pro-inflammatory cytokines on cellular metabolism we may reveal insight as to how cytokines can influence cancer cell metabolism.

Previous *in vitro* studies using prostate cancer cells lines determine that the aggressiveness of the cancer cell line has a phenotypic oncobioenergetic profile [7]. The authors postulate that the metabolic profile may be a useful parameter in predicting cancer invasiveness i.e. aggression [7]. In Chapter 3, the data indicates that IL-1 β does not affect butyrate oxidation in non-colon cancer cells. However, IL-1 β does affect endogenous substrate oxidation. *In vitro* studies using late stage prostate cancer cell lines associated increased oxidative phosphorylation with increased invasiveness [7]. In addition, *in vivo* murine studies demonstrate that IL-1 β promotes invasiveness and tumorigenesis [44-46]. This may be due to the ability of IL-1 β to enhance cancer cell metabolism to promote cancer progression by modifying mitochondrial function. We find that IL-1 β stimulates non-colon cancer cells to increase endogenous substrate oxidation in the absence of glucose. This may mimic the tumor microenvironment in that tumors often outgrow their blood supply and must rely on endogenous substrate utilization [6, 47, 48]. This may further allude to the fact that IL-1 β is associated with stimulating angiogenesis, adhesion molecules and metastasis as a way to support cancer cell survival [16, 41, 49]. More studies like these are necessary to characterize the effects of pro-inflammatory cytokines on cancer cell metabolism.

We find the differences in butyrate oxidation may be due to tissue type and/or the differentiation capacity of these cancer cells. TNF- α has been shown to suppress butyrate oxidation in HT-29

cells [10]. Accordingly, in Chapter 2, we tested if IL-1 β would have the same effect and we found that HT-29 colorectal cancer cells did not respond to IL-1 β 's effect on butyrate oxidation as the HCT116 cancer cell line did. Whereas, TNF- α also suppressed butyrate oxidation in HCT116 cells. We postulate that this is due to genetic and differentiation differences as demonstrated by the higher baseline levels of alkaline phosphatase found in HT-29 cells. Likewise, others have found dissimilar disease pathology in HT-29 versus HCT116 cells, even though both are colorectal cancer cell lines [50]. Our data represent a real-time view of pro-inflammatory cytokines affecting cellular metabolism in a cytokine and cell type dependent manner. By reviewing Table 5.1 and 5.2, we can see that a variety of pro-inflammatory cytokines have differing effects on butyrate and endogenous substrate oxidation.

Future Directions

We find that pro-inflammatory cytokines stimulate shifts in nutrient utilization to promote cancer cell survival. Identification of the mechanisms associated with this need to be more clearly understood. In the data presented here we have demonstrated that IL-1 β , acting through its receptor, suppressed butyrate oxidation in cancerous colonocytes which affected cell cycle and proliferation rates through a p38 mediated mechanism. These studies provide a comprehensive analysis of the effects that pro-inflammatory cytokines have on butyrate metabolism in cancerous colonocytes.

Nevertheless, there is still more to be understood about the effect of pro-inflammatory cytokines have on cancer cell metabolism. Especially, considering that inflammation is an enabling characteristic of cancer initiation and progression. Furthermore, we have demonstrated

here that IL-1 β can modify cancer cell metabolism to increase chances of survival. Therefore, we should seek to find our studies replicated and verified to validate our original research. To this end it would be advantageous to determine which p38 isoform contributes IL-1 β ability to stimulate the shift in nutrient utilization. Furthermore, exploration methodologies that perturb IL-1 β 's signal in more complex system to test the pro-inflammatory cytokine butyrate suppression effect is produced in these systems.

Conclusion

This dissertation project represents a comprehensive analysis of pro-inflammatory cytokines effects on butyrate metabolism in the colorectal cancer cell line, HCT116. We demonstrate that IL-1 β perturbed butyrate metabolism resulting in increased cancer cell proliferation in the colorectal cancer cell line HCT116. This project has identified two targets through which IL-1 β is mediating its signals; IL-1RI and p38. Ultimately, IL-1 β confers a selective advantage via p38 to avoid butyrate-induced p21 expression. These types of advantages can lead to heterogeneous tumor populations filled with cells that have specialized traits to promote tumorigenesis. Furthermore, these findings reveal novel insight as to how IL-1 β can modulate cancerous colonocyte metabolism to support cancer cell proliferation

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Appendix

Table 5.1. Pro-inflammatory cytokine effect on butyrate oxidation in different cancer cell lines. (-) suppresses butyrate oxidation. (+) enhances butyrate oxidation. (NE) no effect. (ND) not determined.

	IL-1β	TNF-α	IL-6
HCT116	-	-	+
HT-29	NE	-	ND
A375	-	ND	ND
22Rv1	-	ND	ND

Table 5.2. IL-1 β 's effect on endogenous substrate oxidation in different cancer cell lines. (-) suppresses butyrate oxidation. (+) enhances butyrate oxidation. (NE) no effect. (ND) not determined.

	IL-1β
HCT116	+
HT-29	NE
A375	+
22Rv1	+

VITA

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