Negative Regulation of Inflammation: Implications for Inflammatory Bowel Disease and Colitis Associated Cancer

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ABSTRACT

The ability to sense and respond to external environmental signals is closely regulated by a plurality of cell signaling pathways, thereby maintaining homeostasis. In particular, the inflammatory signaling cascade contributes to cellular homeostasis and regulates responses prompted by external stimuli. Such responses are diverse and range from a variety of processes, including tissue repair, cell fate decisions, and even immune-cell signaling. As with any signaling cascade, strict regulation is required for proper functioning, as abnormalities within the pathway are often associated with pathologic outcomes. A hyperactive inflammatory response within the gastrointestinal tract, for example, contributes to inflammatory bowel disease (IBD), presenting as Crohn’s disease or ulcerative colitis. Furthermore, as a chronic condition, IBD is associated with an increased risk for the development of colitis-associated cancer.

In order to resolve inflammation and thus restore homeostasis, negative regulation may be utilized to mediate the activity of inflammatory molecules. The mechanistic action of a specific negative regulator of interest, interleukin receptor associated kinase M (IRAK-M), is explored in detail within the present dissertation. Investigation of IRAK-M in mouse models of colitis, which mimics human IBD, and in mouse models of inflammation-driven tumorigenesis, which models colitis associated cancer, demonstrated that loss of this molecule contributes to host protection. Therefore, IRAK-M may be a suitable target for inhibition in order to advance therapeutic options for
human patients afflicted with a GI-related inflammatory disease, such as IBD and colitis associated cancer.

Furthermore, an *ex vivo* method that models the interaction of intestinal epithelial cells with microbes present in the GI tract was optimized and is described in the present dissertation. This method takes advantage of primary intestinal derived organoids, also termed “mini-guts”, which display similar features corresponding to intestinal tissue *in vivo*. For this reason, the use of “mini-guts” has several advantages, particularly for the enhancement of personalized medicine. The method discussed herein aims to normalize experimental conditions in order to enhance reproducibility, which can further be used to uncover microbial-epithelial interactions that contribute to a pathological state, such as IBD. Finally, this method of intestinal epithelial cell culture was utilized to evaluate the role of a protein, termed NF-κB inducing kinase (NIK), in intestinal epithelial cell growth and proliferation. Ultimately, *ex vivo* organoid culture can serve as an important model system to study the contribution of NIK in intestinal stem cell renewal, cancer progression, as well as in maintenance of the integrity of the gastrointestinal barrier.
Inflammation is a tightly regulated physiologic process that is employed by body systems such as the gastrointestinal (GI) tract to handle pathogenic insult, aid in wound healing, and help prevent infections. When abnormal inflammatory responses occur, this can lead to the progression of severe diseases such as ulcerative colitis and Crohn's disease. When inflammation persists in the GI tract, such as in inflammatory bowel disease, this can predispose patients to the development of inflammation-associated colorectal cancer. In order to improve the treatment options for patients afflicted with these maladies, this dissertation is aimed at studying the signaling pathways of the innate immune system that regulate such inflammatory responses. Furthermore, this body of work encompasses a detailed method for isolating and culturing intestinal stem cells, which can be applied in personalized medicine for patients with intestinal diseases. This method was utilized in this dissertation to study genetically modified intestinal stem cells, and can further be used to investigate the interactions of intestinal epithelial cells with pyogenic bacteria that contribute to inflammatory maladies in the GI tract.
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Attribution

Chapter 1 includes portions of previously published work that was co-first authored by myself, Dylan McDaniel and Veronica Ringel-Scalia. The portions that have been included in this dissertation from that publication were sections that I had written. Footnotes that reference the original publication have been included in Chapter 1.

Chapter 2 includes work that was a collaborative effort. Figures 2.1, 2.2, 2.4-2.10 contains data that was generated in Dr. Irving Allen's lab by myself and Dr. Allen, while figure 2.3 contains data contributed by Dr. Liwu Li's lab including: Yao Zang, Na Diao, Christina K. Lee, and Keqiang Chen. I wrote the original draft of the manuscript, including descriptions of the figures, with edits contributed by Dr. Liwu Li and Dr. Irving C. Allen. Yao Zang provided the data and figure legend for figure 2.3. I solely generated the data for figures: 2.7-2.10. Methodology as well as formal data analysis for the remaining figures contained contributions from myself, Yao Zang, Na Dial, Christina K. Lee, Keqiang Chen, Tanya LeRoith, Clayton C. Caswell, Daniel J. Slade, Richard Helm, Liwu Li, and Irving C. Allen.

Chapter 3 includes a step-by-step method with contributions from myself and Tara Srinivasan. I wrote the entire manuscript, and generated all of the figures. The remaining authors provided described in the method. Tara Srinivasan provided technical assistance, as well as scientific advice to help frame the method described.
Chapter 4 contains contributions from both myself and Kristin Eden. While working in collaboration with Kristin Eden on this project, I conducted the experiments for and generated figures 4.1 and 4.2, and wrote the entirety of chapter 4. Kristin Eden contributed to the methodology as well as formal data analysis for figure 4.1 and 4.2. The experiment that was conducted to generate Figure 4.3 contains equal contribution from myself and Kristin Eden.
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Chapter 1:
Introduction

The gastrointestinal (GI) system, similar to the integumentary system, has an important immunological role, and acts as a physical barrier that helps to prevent the access of microbes within the body. The cells of the GI system are renewed on a daily basis, which maintains proper barrier and physiologic function. Several cells of the immune system are located just beneath the epithelial cells of the GI system, mainly for protection due to the high probability of encountering a foreign pathogen if this site is breached. When a pathogen manages to invade host tissues, an immune response ensues to handle the invading microbes. Many immune cells will then produce inflammatory molecules that relay information to surrounding cells that directs a proper cellular defense reaction in response to the foreign microbes. Resolution of these inflammatory molecules must occur; otherwise continued production might lead to catastrophic tissue damage. As such, several regulatory molecules are produced by the cell to prevent hyperactive inflammation. The research in the present dissertation focuses on these molecules, specifically on a positive and a negative regulator of NF-κB signaling.

As a primary focus of this dissertation, a molecule induced by the cell to limit the inflammatory cascade, termed interleukin receptor associated kinase-M (IRAK-M), is evaluated with respect to its function and contribution to inflammatory related diseases in the GI system. Both epithelial and immune cells contribute to host defenses, but due to complexities that exist with in vivo animal models, the elucidation of precise
molecular mechanisms can be complicated. Therefore, in order to simplify and study the epithelial contribution to disorders such as inflammatory bowel disease (IBD), a method is described herein and is optimized utilizing intestinal derived organoids, or “mini-guts”, aimed to simplify conditions through *ex vivo* techniques. Further, the method described is integrated for experiments conducted in Chapter 4 and is used to help elucidate the role of NF-κB inducing kinase (NIK), a protein that participates in the induction of the alternative NF-κB pathway. As such, this dissertation collectively investigated the role of a negative and positive regulator of the NF-κB pathway, being IRAK-M and NIK, respectively. Further, the method utilizing intestinal organoids was applied to study the epithelial cell specific contribution of these molecules to intestinal epithelial cell biology.

1. **Introduction to Innate immunity and adaptive immunity**

   From the perspective of a pathogenic microorganism, metazoans (i.e. multicellular organisms) represent a well-suited environment to infect and reside. In particular, mammalian physiology affords conditions that are favorable to foreign microbes, which include a stable temperature, abundant energy source, and interaction with species within a population to facilitate pathogenic transmission (Sund-Levander et al., 2002), (Speakman, 2005), (Engering et al., 2013). Therefore, in order to prevent such infections from occurring, evolution has selected elegant mechanisms for the host to prevent and limit the spread of disease through the action of the immune system (Schultz and Grieder, 1987). The mammalian immune system is a complex network of cells that orchestrate diverse functions, most of which are attributed to protecting the host from disease causing pathogens (Muller et al., 2008). This is accomplished by
what evolution has afforded, and what immunologists have classified as the two main branches of the immune system: the innate and adaptive immune system (Powers and Dean, 2016). Simplistically, innate immunity is known for its ability to act as a barrier from the external environment with internal tissues. Furthermore, innate immunity also involves the recognition of broad components of pathogens, and can rapidly induce an effector response once a pathogen is recognized (Akira et al., 2006). Adaptive immunity is known for being acquired following pathogenic insult, is highly specific to previously encountered pathogens through the action of T and B lymphocytes, and is long lasting (Bonilla and Oettgen, 2010). Tight regulation of both branches of the immune system are required for normal physiological process to occur, and pathologies ensue when signaling aberrations deviate from homeostatic norms. Hyperactive aberrations in both the innate and adaptive immune responses can result in severe tissue damage; whereas hypoactive aberrations can result from improper recognition of a pathogen, leading to opportunistic infection (Blach-Olszewska and Leszek, 2007), (Al Anazi, 2009).

1.1 Cells of the Innate Immune System

The importance of innate immunity cannot be understated. All metazoans have cells that compose an innate immune system, which protects from pathogens; whereas, the adaptive immune system is more specialized, and it is believed to have evolved stemming from a common ancestor with an innate immune system (Kimbrell and Beutler, 2001), (Beutler, 2004). The innate immune system is composed of a diverse range of cell types, which are categorically assigned based on the criteria of being a
foreground line of host defense (Alberts et al., 2008). It should be noted that clear cut definitions of innate immune cells are not always possible. Recently, cell types with both innate and adaptive immune cell characteristics have been discovered, and fittingly termed innate lymphoid cells. Innate immune cells include cell types that compose and maintain the anatomical barriers to the external environment; however, the main cell types that are pertinent to the innate immune response usually refer to the white blood cells including both mononuclear and polymorpho-nuclear phagocytes. Mononuclear phagocytes include the monocytes, macrophages, and dendritic cells, whereas polymorpho-nuclear phagocytes include neutrophils, basophils and eosinophils (Beutler, 2004). Monocytes are the circulating precursors to macrophages, and mature into macrophages once they have migrated from the circulation into tissues. It should be noted that not all macrophages are created equal. In particular, there are groups of tissue resident macrophages that are present in all organs and they display alternate epigenetic gene signatures. Currently, it is believed that the surrounding microenvironment is in constant communication with, and contributes to the diverse function of tissue resident macrophages (Lavin et al., 2014). Macrophages and dendritic cells are well known for their ability to engulf foreign microbes, as well as cellular debris, through a cellular process called phagocytosis, followed by presentation of foreign antigens to the adaptive immune system on class II MHC molecules (Metchnikov, 1884), (Banchereau and Steinman, 1998). Neutrophils are polymorphonuclear cells that are likely the first-responders to a pathogen. They undergo maturation in the bone marrow and enter into the circulation following their differentiation (Bainton et al., 1971). Ultimately, neutrophils have a limited lifespan following maturation, as they undergo
apoptosis as shortly as 6 hours after their entry into circulation (Cronkite and Fliedner, 1964), (Brinkmann and Zychlinsky, 2007). Neutrophils have the ability, similar to macrophages, to engulf foreign substances via phagocytosis (Cohn and Hirsch, 1960), and have recently attracted attention for the finding pertaining to their ability to exude their cellular contents, termed neutrophil extracellular traps (NETs), which combats pathogenic bacteria (Brinkmann et al., 2004). Basophils and eosinophils compose a small percentage of circulating leukocytes in the human body (Stone et al., 2010). There are aspects of basophil function that are unknown; however, basophils are currently understood to contribute to host defense against parasites (Min, 2008). They store preformed granules that contain histamine, and thus contribute to the allergic responses (Stone et al., 2010). Eosinophils are known for their response, mainly to helminth infection and allergies, but can also play a role in rare diseases (Stone et al., 2010). Once matured into fully differentiated eosinophils, they migrate into circulation, and will increase in abundance and locality due to the presence of Th2 associated cytokines (Rosenberg et al., 2007).

All of the cell types described above share a common feature, in that they all share a common lineage ancestor. All of these cells described above differentiate from a hematopoietic stem cell that normally resides in the bone marrow. These phenomena are highly relevant, because depending on which type of cell immunologists wish to investigate, isolated stem cells from the bone marrow can be differentiated ex vivo when the proper signals are provided that drive differentiation to a specific lineage. This
allows researchers to simplify complex conditions to solve specific questions, pertaining to a specific cell.

1.2 Interplay of innate immune system with GI system

Barrier surfaces, such as the GI tract, are a highly susceptible point of entry for pathogenic microorganisms when breached. Therefore, an adequate epithelial barrier, and immune response is necessary to prevent and contain any pathogenic insult. The GI system of humans, as well as other mammals, harbors a diverse array of microbes that share a commensal relationship with the host (Human Microbiome Project, 2012). This phenomenon poses a really fascinating question, one that currently perplexes researchers, and is related to immune activation versus immune tolerance. How is the body able to recognize and respond to a pathogen emanating from the GI system, while also subverting an active immune response against the commensal microbes that can share many similarities with the pathogen? The answer to this question is obviously not a simple one, but the answer partially lies in the several molecular mechanisms that regulate the interaction between the GI tract and immune system that allow homeostasis with the microbiota. This collectively starts with the physical barrier of epithelial cells that prevent translocation of bacteria from the intestinal lumen to the lamina propria. This intestinal epithelium is composed of polarized columnar epithelial cells that are remarkably only a single layer in thickness. (Abreu, 2010), (Mowat and Agace, 2014), (Williams et al., 2015). The turnover rate of epithelial cells in the GI tract is one of the fastest in the human body, occurring every 3-4 days, and is possibly related to the exposure of this tissue to hostile substances (e.g. bacterial products,
dietary components) (Creamer et al., 1961), (Cheng and Leblond, 1974). In the unfavorable event that a pathogen breaches the epithelial barrier, immune cells of both the innate and adaptive immune system strategically located beneath the layer of epithelial cells within the lamina propria will recognize and engage the pathogen (Figure 1.1). The lamina propria of the GI tract contains the highest abundance of macrophages, T-cells, and IgA secreting plasma cells; additionally, under normal conditions, macrophages constitute the most abundant leukocyte in the lamina propria of the GI tract (Mowat and Agace, 2014). Such a high abundance of phagocytic cells in the lamina propria is likely due to the high occurrence, or probability of foreign microbial encounter. Under homeostatic conditions, tissue resident macrophages found in the lamina propria are under consistent turnover, renewed by circulating monocytes that travel into the tissue from the blood stream (Bain et al., 2014). Once these cells migrate into the lamina propria, they can be distinguished by three major factors. These include the expression of the fractalkine receptor CX3CR1, the secretion of high amounts of IL-10 and TNF, and by being relatively inert to the presence of LPS (Bain et al., 2013). The low responsiveness of these macrophages to LPS suggests an important regulatory function, one that likely includes phagocytosis of debris without inducing an immune response. These cells contribute to several pleiotropic responses due to the secretion of the cytokines IL-10 and TNF. IL-10 is important for the regulation of immune cell function, and TNF can govern epithelial cell turnover (Shouval et al., 2014). Proper tissue homeostasis is partially maintained by constant crosstalk between the epithelium, immune cells, and the microbiota. It is fascinating that all the physiological processes work, in most cases, without any insult or abnormalities. As can be expected, these
Interactions between tissue and commensal microbes, as well as the molecular mechanisms that regulate immune tolerance versus immune activation are at the forefront for many scientific investigators. Aberrations in any of these interactions can tip
the homeostatic balance, and ultimately have the potential to contribute to tissue pathologies.

2. Introduction to the GI system

The GI system collectively refers to the hollow tube that begins at the mouth and terminates at the anus. The main functions of the GI system involve obtaining nutrients via digestion and absorption followed by the excretion of waste, while also protecting the host during these processes by forming a physical barrier with the external environment (Cheng et al., 2010). As chemoorganoheterotrophs, humans require energy sources from complex organic forms of carbon (i.e. carbohydrates, proteins and lipids) and must break these macromolecules down to simpler subunits in order for proper metabolism to occur. The GI system has evolved to mechanically and chemically break down ingested food, beginning in the mouth and ending at the colon, while also providing a large surface area of epithelial cells to maximize the absorption process, occurring from small intestine to colon. The colon aids in absorption of nutrients and water, and also harbors trillions of bacteria that form a commensal relationship with the host, and is referred to as the microbiome.

2.1 Anatomy of Small intestine and Colon

The GI tract is a hollow tube that is derived from the endoderm during gastrulation (Lewis and Tam, 2006). The small intestine is the segment of GI tissue that begins after the stomach, and ends at the cecum, which is followed by the large bowel (i.e. the colon). From its proximal to distal ends, the small intestine is further subdivided into
three segments: duodenum, jejunum, and ileum, respectively. The function of the duodenum pertains to acid neutralization, whereas the jejunum and ileum involve nutrient absorption. The major functions of the small intestine are to aid in digestion of ingested nutrients, and absorption of water, electrolytes and nutrients. The anatomical structures of the small intestine are quite striking; the surface epithelium is lined with fingerlike projections, termed villi (Figure 1.2 A, B). These fingerlike projections maximize the intestinal surface area, which enhances the efficiency in which nutrients can be absorbed into the blood stream. The intestinal epithelium is one of the most rapidly dividing tissues in the human body, with epithelial turnover being every 3-5 days (Cheng and Leblond, 1974), (Mayhew et al., 1999). Cell division is driven by intestinal stem cells that reside in the base of the intestinal crypts, which in a conveyor belt fashion, push the epithelial cells up towards the tip of the intestinal villus, where they eventually undergo apoptosis (Williams et al., 2015) (Figure 1.2 C). This results in a constant shedding of epithelial cells into the intestinal lumen to be excreted with the feces (Schuijers and Clevers, 2012). The colon shares similarities with the small intestine; however, there are distinct differences between the two tissues. Regarding epithelial anatomy, the colon does not contain villi, but rather has a flat surface epithelium (Colony, 1996), (Schuijers and Clevers, 2012). Similar to the small intestine, the colon contains crypts that harbor stem cells, and the epithelial cells of the colon are under constant renewal. The main epithelial cell types of the colon are columnar epithelial cells, goblet cells, and enteroendocrine cells (Colony, 1996). One key feature of the colon is that it is in constant contact with millions of bacteria, and thus contains
several goblet cells to produce mucus, which provides another physical barrier to GI bacteria.

**Figure 1.2.** A. Three dimensional graphic of the small intestine depicting the lumen lined with intestinal villi. B. Birdseye view of the fingerlike projections (villi). Intestinal crypts are at the base of the villi. C. Transverse section of intestinal villi. Intestinal crypts are the U-shaped structures at the base of the intestinal villi. *Figure from:* (Schuijers and Clevers, 2012). See copyright permissions.

### 2.2 Stem cells as contributors of epithelial cell fates and the Wnt signaling pathway

Intestinal stem cells reside at the crypt base, and in the case of the small intestine, reside next to the Paneth cells, which produce antimicrobial peptides and contribute to stem cell maintenance (Cheng and Leblond, 1974), (Sato et al., 2011). Intestinal stem cells give rise to all of the differentiated epithelial cells that compose the intestinal epithelium (Cheng and Leblond, 1974), (Bjerknes and Cheng, 2006). The past two decades of research has demonstrated the importance of the *Wnt* signaling pathway in the maintenance of the intestinal epithelium (**Figure 1.3**). WNT is a ligand that binds to Fizzled receptors that ultimately culminates in the stabilization and nuclear localization...
of the transcription factor β-catenin. The importance of canonical Wnt signaling was first demonstrated with transgenic mice lacking Tcf4, a key transcription factor that is activated in response Wnt ligands. Loss of Tcf4 in mice resulted in premature death, and interestingly, the intestinal epithelium of neonatal mice was entirely differentiated (Korinek et al., 1998). This provided the first clue that Wnt signaling contributed to the intestinal stem cell niche. A major breakthrough came recently, when it was discovered that both small intestine and colon intestinal stem cells express the cell surface receptor leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) (Barker et al., 2007). LGR5 was identified as a Wnt target gene, and further identified as the receptor for R-spondin. In the presence of WNT ligands, R-spondin amplifies and sustains Wnt signaling that is necessary to retain the stem cell niche (Kazanskaya et al., 2004), (Kim et al., 2008), (de Lau et al., 2011). With this knowledge, isolated intestinal crypts or single Lgr5+/GFP stem cells can be cultured ex vivo with the addition of several niche factors that
stimulate the Wnt signaling pathway (Sato et al., 2009). These “mini guts”, also termed organoids possess the characteristics of intestinal epithelium, and are proving to be a vital tool for GI research. Organoids are proving to be excellent tools to study GI epithelial cell proliferation, because the confounding factors of the immune system have been removed.

3. Pattern Recognition Receptors of the Innate Immune System

3.1 The Families of Pattern Recognition Receptors

Following pathogenic insult, the normal series of innate immune responses are to recognize, respond, and resolve the insult from the foreign invader (Beutler, 2004), but what mechanisms govern the ability of host cells to recognize a pathogen? It was Charles Janeway that first proposed the existence of so called “pattern recognition receptors” by innate immune cells, and rationalized that host innate immune cells likely recognize conserved molecules (i.e. patterns) that are unique to foreign microbes (Janeway, 1989). Time later proved that Janeway was ultimately correct in his prediction. The major breakthrough came when it was demonstrated that Toll-like receptor 4 (TLR4) was the bona fide receptor for the conserved bacterial molecule lipopolysaccharide (LPS) (Poltorak et al., 1998). This discovery, along with work conducted in the fruit fly by Jules Hoffman and works pertaining to dendritic cells linking innate and adaptive immunity by Ralph Steinman, was ultimately awarded the Nobel Prize in physiology and medicine in 2011. These findings were the first to demonstrate the importance of pattern recognition receptors (PRRs) to innate immunity. Broadly, it is now known that PRRs are located based on topology relative to the cell. Soluble
extracellular PRRs include members of the complement system, as well as the pentraxins, which bind to phosphocholine in a calcium dependent manner (Pepys and Hirschfield, 2003); cell surface receptors include the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs); and finally, Nod-like receptors (NLRs), Rig-I-like helicases (RLRs) and AIM2 receptors, which are located intracellularly in the cell cytosol.

PRRs recognize conserved molecules residing on or contained within foreign microbes that are fundamentally distinct from healthy host cells. These foreign patterns (i.e. molecules) can be of bacterial, viral, fungal, or protozoan origin; and certain PRRs can even recognize self-damage patterns (Thompson et al., 2011). The molecules recognized by PRRs are termed pathogen associated molecular patterns (PAMPs) and self-patterns are termed damage/danger associated molecular patterns (DAMPs) (Janeway, 1989). Following recognition of a PAMP/DAMP by a particular PRR leads to a rapid cellular response. This ensues via multiple coordinated signal transduction cascades that culminate in either the release of inflammatory molecules, an increase in the transcription of genes involved in cellular migration of immune cells, and when the signal is robust, activation of the adaptive immune system (Steinman and Witmer, 1978), (Medzhitov and Janeway, 1997), (Kawai et al., 2001), (Rahman et al., 2009).

Lastly, resolution of the immune response must ensue in order to prevent excessive inflammation that can result in destructive tissue damage (Beutler, 2004). The resolution phase occurs through negative feedback loops, which are a mechanism to restore homeostasis, and are paramount to proper physiological function. This can occur through a variety of means, for example, intracellular molecules that down-regulate the
signaling cascades that culminate in the activation of the inflammatory response (PTEN, SOCS-1, SHIP1, IRAK-M, CYLD, A20). Because both arms of the immune system are highly involved in the production of inflammatory mediators, many inflammatory related diseases can ensue when proper function is not maintained. Other mechanism to restore homeostasis can include the production of anti-inflammatory cytokines, such as interleukin-10 (IL-10), as well as the activation of regulatory cells that blunt the immune system, i.e. T-regulatory cells (Tregs) and myeloid derived suppressor cells (MDSCs). In summary, there are many ways the cell can restore balance once a response is generated.

3.2 Cell types of both GI and innate immune systems that express PRRs

The expression of PRRs on both intestinal epithelial cells (IEC) and innate immune cells has been shown to be crucial for proper intestinal and immune physiology (Vijay-Kumar et al., 2007). The cells that line the GI tract are composed of polarized epithelial cells, meaning they have an apical surface that borders the intestinal lumen, and a basolateral surface that borders the lamina propria (Abreu, 2010). The families of PRRs that have been most extensively studied pertaining to IEC are the TLRs, and their expression varies regarding localization on either the apical or basolateral membrane of the IEC. In the human colon, TLR5, which senses bacterial flagella, is expressed on the basolateral membrane of IECs and not the apical membrane on IECs (Gewirtz et al., 2001), (Rhee et al., 2005). This suggests that spatial separation of TLR5 from the intestinal lumen is important in preventing constitutive interaction of this receptor with the intestinal microbiota, and likely recognizes bacterial components once they have
breached beyond the apical membrane. Further, TLR1, TLR2, TLR4 and TLR9 are expressed in human small intestinal epithelial cells (Otte et al., 2004). The expression of these TLRs also display spatial polarization, with the highest expression on the basolateral surface of IECs; however, their expression is not limited to the basolateral membrane as they are found on the apical surface as well (Cario et al., 2002), (Lee et al., 2006). This begets the question, if TLRs are expressed on the apical membrane of IEC that are in contact with the luminal microbiota, why are these cells not constantly driving inflammation? This is likely due to the importance of proper TLR expression, and signaling for cellular maintenance of IECs. Indeed, TLR engagement of commensal PAMPs results in the production of protective factors, and ablation of the GI microbiota with antibiotics limits GI TLR activation, and results in profound susceptibility to dextran sulfate sodium (DSS), which is a chemical that leads to colitis in mice (Rakoff-Nahoum et al., 2004).

### 3.3 Inflammation and the NF-κB signaling pathway\(^1\) (Rothschild et al., 2018)

Inflammation, in certain contexts harbors a negative umbrella of pathologies; however, under proper physiological conditions, it serves a very important purpose. Broadly, inflammation functions to coordinate the repair of damaged tissue by informing the immune system that tissue damage is taking place. Inflammation results in cellular activation, increased blood flow to the affected area, and an influx of cells associated with the immune system. This coordinated effort from cells of the host immune system

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functions to clear the pathogenic insult, repair tissue damage and curb the inflammatory response in order to restore tissue homeostasis. At the cellular level, this is coordinated via an important regulator of transcription: the nuclear factor kappa light chain enhancer binding protein (NF-κB) transcription factor.

NF-κB is an evolutionarily conserved transcription factor found in species ranging from Drosophilia to humans, which underscores its critical role in the host immune response. (Ghosh et al., 1998). The last 3 decades of research have contributed greatly to our understanding of NF-κB. NF-κB functions as a prominent inducible transcription factor that regulates the immune system. Briefly, NF-κB it is known to regulate a vast array of genes ranging from the development of the embryo, to cell fate decisions, and is well known for its role as a prominent transcription factor that regulates the immune system (Beg et al., 1995), (Alcamo et al., 2001), (Boersma et al., 2011), (Zhang et al., 2017). Due to its diverse and broad biological functions, strict regulation of NF-κB signaling is paramount to proper tissue allostasis. When NF-κB signaling is aberrant, several maladies can ensue, such as susceptibility to infections, autoimmunity and cancer (Oeckinghaus and Ghosh, 2009), (Sun et al., 2013), (Greten et al., 2004). Thus, as with many major signal transduction pathways, several mechanisms tightly regulate NF-κB signaling and maintain the proper balance of activation and repression.

NF-κB in mammals consists of a total of five proteins that are predominantly present in an inactivated state in the cytosol as either homo- or hetero-dimers that include: RelA (p65), RelB, c-Rel, p105 and p100. The p105 and p100 proteins are unique because
they must undergo post-translational processing through the proteasome to form the active subunits, p50 and p52, respectively (Amir et al., 2004), (Ghosh et al., 1998). All isoforms contain a common Rel homology domain, which is responsible for DNA binding, as well as binding to the cytosolic inhibitory proteins, termed Inhibitor of –κB (IκB) (Ghosh et al., 1998). Several families of cellular receptors signal through NF-κB to initiate gene transcription and are known to coordinate signaling through either the canonical NF-κB pathway or the non-canonical NF-κB pathway (which is also termed the “alternative” NF-κB pathway). Molecules that signal via the canonical NF-κB pathway include cytokines, such as tumor necrosis factor (TNF), interleukin-1 beta (IL-1β), and the majority of PRRs (Ghosh et al., 1998). The non-canonical pathway is initiated by a much smaller repertoire of molecules that are members of TNF family, such as CD40, B-cell activating factor (BAFF), and lymphotoxin beta (LT-β) (Sun et al., 2013).

3.4 Canonical NF-κB signaling pathway

Convergence on the canonical NF-κB signaling pathway occurs through the activation of several different families of receptors (i.e. TNFR, TLRs, NLRs, IL-1R) (Ghosh and Hayden, 2012). As a classical example of canonical activation, we will focus on MyD88-dependent TLR signaling (Figure 1.4), a process that occurs for the interleukin 1 receptor (IL-1R) and all TLRs with the exception of TLR3 (Yamamoto et al., 2003). IL-1/TLR signaling commences via binding of their respective ligands. Engagement of a PAMP to an extracellular TLR (i.e. TLR1, TLR2, TLR4, TLR5, TLR6) induces a conformational change and hetero- or homo-dimerization of the TLR. Dimerization leads
to a change in conformation of the receptor, followed by recruitment of adaptor proteins to the toll/interleukin receptor (TIR) domain of the TLR. The adaptor molecule MyD88 is recruited to extracellular dimers composed of TLR5 receptors, whereas Mal followed by MyD88 are recruited by TLR1/TLR2, TLR2/TLR6, TLR4 dimers (Kawai et al., 1999), (Fitzgerald et al., 2001), (Yamamoto et al., 2002), (Horng et al., 2002), (Didierlaurent et al., 2004), (Nishiya and DeFranco, 2004). MyD88 acts as a protein scaffold, as it contains both a TIR domain and a death domain (DD) in its protein structure. The DD of MyD88 recruits interleukin receptor associated kinase (IRAK) IRAK-4, followed by IRAK-1, all in a helical assembly to form a Myddosome complex (Wesche et al., 1997), (Lin et al., 2010). The close spatial proximity of IRAK-4 to IRAK-1 allows for IRAK-1 to

Figure 1.4. MyD88 Dependent Signaling Pathway. Engagement of a Toll-like receptor (TLR), with the exception of TLR3, induces a signal transduction cascade via the Myeloid Differentiation Factor 88 (MyD88) dependent pathway. MyD88 dependent signaling results in the activation of the canonical NF-κB pathway. Activation of NF-κB results in the transcription of multiple genes involved in inflammation, cell migration, cell survival, and negative feedback proteins to eventually restore homeostatic norms of the cell.
become rapidly phosphorylated by IRAK-4, and then undergo auto-phosphorylation (Wesche et al., 1997), (Li et al., 2002). Once phosphorylated, IRAK-1 leaves the receptor complex and interacts with TRAF-6. Upon activation, TRAF6 associates with Uev1A and Ubc13 and results in TRAF6 lysine-63 (Lys-63)-mediated poly-ubiquitination (Takaesu et al., 2000), (Deng et al., 2000). Lys63-mediated poly-ubiquitination of TRAF6 acts as an important scaffold resulting in the recruitment and docking of TAB2/3 and TGF-β activated kinase-1 (TAK1) (Wang et al., 2001), (Kanayama et al., 2004). Close association of the TAB2/3/TAK1 complex with poly-ubiquitinated TRAF-6 allows TAK1 to undergo auto-phosphorylation, resulting in TAK1 activation (Wang et al., 2001). Once TAK1 becomes phosphorylated, it can mediate downstream signaling by phosphorylating the inhibitor of κB kinase (IKK) complex on IKKβ subunits (Ninomiya-Tsujii et al., 1999), (Wang et al., 2001). IKKβ phosphorylation results in the IKK complex activation, composed of NEMO/IKKα/IKKβ subunits, to phosphorylate IκB (Mercurio et al., 1997). Phosphorylation of IκB results in its subsequent recognition by the SCF-βTrCP ubiquitin (Ub) ligase complex that covalently modifies IκB with Lys-48 poly-Ub chains, which ultimately leads to the degradation of IκB by the 26s proteasome (Chen et al., 1995). Once IκB is degraded, NF-κB dimers are liberated and predominantly shuttle into the nucleus and bind to -κB promoter and enhancer elements involved in the regulation of immunity, cell migration, cell adhesion, cell death and inflammation.

3.5 The Non-Canonical NF-κB Signaling Cascade

The activation of the non-canonical or alternative NF-κB signaling cascade is tightly regulated and has a much smaller group of ligands and receptors that can induce its
activation (Sun, 2012). Members of the TNF/TNFR superfamily, which include the lymphotoxin-β receptor (LTβR), CD40, B-cell activating factor receptor (BAFFR), receptor activator of NF-κB (RANK), TNFR2 and CD27 have all been shown to signal through the non-canonical pathway (Sun, 2012). Upon ligation of TNF family members to their receptors, the TRAF2-TRAF3-cIAP1/2 E3 complex is recruited to the receptor (Sun, 2012). This recruitment leads to accumulation of TRAF2 molecules, allowing TRAF2 to shift cIAP1/2’s Lys-48 Ub ligase activity towards TRAF3 via Lys-63 poly-ubiquitination of cIAP1/2 (Sun, 2012), (Hostager et al., 2003). When TRAF3 is modified via this mechanism, it ultimately leads to proteasomal degradation of TRAF3, allowing for accumulation of MAP3K14 (also commonly known as NF-κB Inducing Kinase (NIK)), resulting in increasing the levels of cytosolic NIK (Sun, 2012). Mechanistically, in an unstimulated state, TRAF3 acts as a binding partner for NIK bringing it close to the cIAP1/2 E3 ligase complex, which in this case leads to its degradation (Zarnegar et al., 2008, Liao et al., 2004). NIK is essential for the processing of p100 into active p52(Xiao et al., 2001), (Sun, 2012), which is a defining feature of the non-canonical NF-κB signaling pathway. However, NIK has also been shown to phosphorylate IKKα, which can act as a secondary kinase that mediates the activation of p100 (Senftleben et al., 2001). For this to occur, NIK must be present in relatively high concentrations inside the cell before interacting with IKKα. Thus, stabilization of NIK results in its accumulation and interactions with IKKα, culminating in phosphorylation and activation (Sun, 2012),(Senftleben et al., 2001).
It is thought that IKKα-mediated phosphorylation of p100 occurs on Ser-872 in vitro and Ser-866 and 870 in vivo (Sun, 2012). While the cause of this discrepancy is currently unclear, presumably, IKKα acts as a site-specific kinase for p100. This phosphorylation step targets the C-terminal inhibitory domains (the PID and the ankyrin repeat domain) of p100 for degradation via the proteasome (Sun, 2012). Ankyrin repeat domains (ARDs) have been shown to mask the nuclear localization sequence (NLS) of IκBα and interestingly, p100’s phosphorylation site contains a sequence similar to IκBα (Sun, 2012), (Oeckinghaus and Ghosh, 2009). Therefore, disruption of the ARD of p100 via proteasomal degradation may lead to unmasking of the NLS and subsequent translocation of p52/RelB to the nucleus (Oeckinghaus and Ghosh, 2009). Once in the nucleus, p52/RelB drives the transcription of a limited repertoire of genes, including CCL19, CCL21, CXCL12, and CXCL13. In contrast, when the TNFR family members are unstimulated, NIK is instead degraded via Lys-48 poly-ubiquitination (Sun, 2012). Consequently, IKKα will not become activated and phosphorylate p100. Thus, in this scenario, the NF-κB dimer cannot enter the nucleus and initiate transcription of target genes. Due to its essential function, NIK represents a “bottleneck” in the non-canonical NF-κB signaling cascade and is frequently targeted by mechanisms that have evolved to negatively regulate this alternative pathway.

4 Negative Regulation of Inflammation

4.1 Inducible Negative regulators of Inflammation

Well-controlled mechanisms to resolve NF-κB activation are required to prevent its potential destructive activities if this transcription factor if left unencumbered. These
include mechanisms that inhibit the action of NF-κB following its nuclear translocation. There are over 785 different molecules that counteract the activity of NF-κB that are either constitutively expressed in the cell, or induced by NF-κB following its activation; there are even exogenous molecules that inhibit its activity (i.e. from plants) (Gilmore and Herscovitch, 2006). The molecules (i.e. microRNA, proteins) that are induced are often referred to as negative regulators, because they act to restore homeostasis via negative feedback loops (Renner and Schmitz, 2009). Several, if not all of these molecules serve a very important function that act as the “brakes” to halt NF-κB activity. Certainly, these are not the only means utilized by the cell to reduce the activity of NF-κB, but proteins expressed following NF-κB activation will be the focus here. Analogous to an automobile losing its breaks, catastrophic outcomes can ensue if the cell loses these abilities to halt the actions of NF-κB. These include diseases resulting from abnormalities of the immune system, as well as certain types of cancers, mainly lymphomas as well as GI cancers (Clevers, 2004), (Karin, 2009). One can anticipate that loss of key components of a signal transduction cascade will result in severe maladies, if not lethality. The same is true for cellular systems; in fact, mutations in several of these proteins result in severe inflammatory disorders in both humans and mice, if and when such mutations are actually viable.

4.2 Inhibitor of κB (IκB)

There are 8 IκB proteins, known to negatively regulate NF-κB dimers, two of which are the inhibitory regions on both p100 and p105 prior to proteosomal processing. The mechanism of regulation functions, in the case of the canonical NF-κB pathway, by
binding to the Rel homology domain of NF-κB subunits via ankyrin repeats on the IκB proteins. IκBα is induced by NF-κB, and this mechanism of induction demonstrates an auto-regulatory feedback loop (Sun et al., 1993). The binding of IκBα to NF-κB results in the concealment of the nuclear localization sequence on NF-κB dimers, thus keeping NF-κB mainly contained in the cytosol under normal conditions (Ghosh and Hayden, 2012). This parallels the nuclear export signal on IκBα, which renders the flux of NF-κB dimer mainly in the cytosol. Once IκBα is degraded by the proteasome, the nuclear import signal on NF-κB is no longer blocked, and this allows for translocation to the nucleus to regulate gene transcription (Sun et al., 1993). One question arises regarding this feedback loop: if NF-κB upregulates IκBα, which contains a nuclear export signal, and NF-κB is in the nucleus with a nuclear import signal, how does IκB-α access NF-κB to halt its function? It turns out that both NF-κB and IκBα can shuttle into and out of the nucleus; thus, they are in constant flux between these cellular compartments. When the majority of IκBα is bound to NF-κB, the cytosolyic flux predominates and NF-κB is limited to the nucleus. When NF-κB is free of IκBα, the nuclear flux predominates and allows for NF-κB to bind enhancer regions on target DNA (Ghosh and Hayden, 2012).

4.3 Negative Regulation From The Interleukin Receptor Associated Kinase Family

There are four interleukin receptor associated kinases (IRAK) in mammals: three of which are known as IRAK-1, IRAK-2 and IRAK-4. These three family members act as positive regulators of NF-κB signal transduction (Thomas et al., 1999), (Muzio et al., 1997), (Suzuki et al., 2002), (Kawagoe et al., 2008). One family member, IRAK-3 or
IRAK-M, is unique from the other family members, because it acts as an inducible negative regulator of the canonical NF-κB signaling pathway (Wesche et al., 1999), (Kobayashi et al., 2002). All four family members share a N-terminal death domain that is important for homotypic protein-protein interactions with either MyD88, or with other IRAK members for signal transduction. More central in the protein sequence for all members is the kinase domain (Flannery and Bowie, 2010). IRAK-1 and IRAK-4 were first described as being the only functional kinases in the family, with IRAK-2 and IRAK-M being inactive or pseudo-kinases (Thomas et al., 1999), (Muzio et al., 1997), (Suzuki et al., 2002), (Kobayashi et al., 2002). This was determined by both biochemical and analytical methods. Analytically, the functional activity of a protein kinase can be predicted by its primary amino acid sequence (Hanks and Hunter, 1995). There are specific residues in the sequence of the kinase domain that are invariant for kinase function, and these include residues in the kinase subdomain VIb known as the HRD and DFG motifs (Hanks and Hunter, 1995), (Meylan and Tschopp, 2008). The invariant aspartic acid residues, that are essential for proper function, are mutated in IRAK-2 and IRAK-M to an asparagine and serine, respectively. This provides the rationale for the nonfunctional activity of these IRAK family members (Meylan and Tschopp, 2008). All IRAK proteins, apart from IRAK-4, contain a unique C-terminal domain (Flannery and Bowie, 2010). This domain contains TRAF6 binding motifs that, as the name implies, are important for interaction with TRAF6, and aid to propagate downstream signaling.

4.4 Molecular Mechanism of IRAK-M Function as a Negative Regulator
Early functional studies suggested that IRAK-M shared a redundant function with IRAK-1 and IRAK-2 (Wesch et al., 1999), despite the later prediction that IRAK-M was inactive or a pseudo-kinase. In overexpression systems and luciferase reporter assays, IRAK-1, IRAK-2, and IRAK-M were shown to function as positive regulators of NF-κB signaling. However, with \textit{in vitro} kinase assays, human IRAK-M was demonstrated to have very weak intrinsic kinase activity, especially compared to IRAK-1 (Wesch et al., 1999). Further, murine IRAK-M was shown to contain detectable kinase activity; albeit, at a lower level when compared to the robust auto-phosphorylation of human IRAK-1 (Rosati and Martin, 2002). These data suggest that the auto-phosphorylation, and therefore the kinase activity of IRAK-M is negligible for its function; however, it is tempting to speculate that IRAK-M may indeed function as an active kinase on a currently unknown cellular substrate following its induction. Despite these initial findings and speculation, the prevailing literature suggests that IRAK-M functions as a negative regulator of NF-κB signaling following TLR activation. These data are based on findings utilizing genetically modified mice with targeted deletions of the \textit{Irak-m} gene (Kobayashi et al., 2002). The \textit{Irak-m} gene contains 12 total exons and in order to define the role of IRAK-M, exons 9-11 were targeted for deletion (Kobayashi et al., 2002). These exons encode the amino acids predicted to constitute the putative kinase domain. Full length IRAK-M was not detected when a western blot was performed with an antibody specific for the C-terminus of IRAK-M (Kobayashi et al., 2002). Using these genetically modified animals, \textit{irak-m} was found to be induced by NF-κB, along with IκB and A20 following TLR stimulation, suggesting a negative feedback mechanism. Subsequent co-immunoprecipitation experiments demonstrated that IRAK-M has the capacity to bind to
TRAF6, leading to the current model that IRAK-M functions to inhibit IRAK-1 and TRAF6 interactions, which in turn inhibits downstream NF-κB activity (Kobayashi et al., 2002). The expression of IRAK-M can be induced by other transcription factors, such as C/EBP, Smad4, AP-1 and CREB (Lyroni et al., 2017), suggesting additional functions beyond the feedback mechanism currently described. Phenotypically, Irak-1/− mice do not display any abnormal fetal or postnatal development, but do develop osteoporosis later in life due to hyperactive osteoclast activity (Kobayashi et al., 2002), (Li et al., 2005).

These Irak-1/− mice were instrumental in defining IRAK-M as a negative regulator of NF-κB signaling and have been widely utilized to define its function. For example, consistent with increased NF-κB signaling, Irak-1/− bone marrow derived macrophages (BMDMs) display impaired endotoxin tolerance and hyper-production of inflammatory cytokines (i.e. IL-6, TNF and IL-12p40) following TLR stimulation with specific PAMPS, as well as, L. monocytogenes and S. typhimurium (Kobayashi et al., 2002). Consistent with the ex vivo BMDM studies, Irak-1/− mice were found to display enhanced small intestinal inflammation following in vivo exposure to S. typhimurium (Kobayashi et al., 2002). Interestingly, in these initial studies, Irak-1/− mice do not display enhanced morbidity or mortality following infection, despite the increased inflammation (Kobayashi et al., 2002). These findings are consistent with a more recent study that showed the Irak-1/− mice are protected in models of experimental colitis and colitis associated tumorigenesis (Rothschild et al., 2017). Mice lacking IRAK-M were found to have a robust immune response to bacteria translocating from the lumen following chemical
induced damage to the intestinal epithelial cell barrier (Rothschild et al., 2017). The attenuation in pathogenesis was associated with large expansions of gastrointestinal associated lymphoid tissue (GALT), increased neutrophil function, and enhanced T-cell recruitment (Rothschild et al., 2017). Complementary data revealed that the gastrointestinal (GI) tract of the \textit{Irak-m/} mice had a lower total colonic bacterial load compared to wild type counterparts, which could also contribute to attenuation of disease pathogenesis (Kesselring et al., 2016). Mechanistically, these data suggest the immune system in \textit{Irak-m/} animals is primed and more prone to a robust inflammatory response. In the GI tract, this improves the efficiency of the host response to pathogenic and commensal components of the host microbiome that drive disease processes.

It should be noted that while the consensus data identifies IRAK-M as a negative regulator of NF-\(\kappa\)B signaling, contrary data suggests a possible alternative mechanism. Recently, it was demonstrated that IRAK-M participates in TAK-1 independent NF-\(\kappa\)B activation downstream of TLR7 through MEKK3 (Zhou et al., 2013). With the use of IRAK-1/IRAK-2 double deficient mice and IRAK-1/-2/-M triple deficient mice, this study demonstrated that under highly specific conditions, IRAK-M functions in the absence of IRAK-1 and IRAK-2 and can actually activate NF-\(\kappa\)B signaling (Zhou et al., 2013). Mechanistically, IRAK-M interacts with IRAK-4 to form an IRAK-M myddosome complex in the absence of both IRAK-1 and IRAK-2 that modulates NF-\(\kappa\)B signaling through TAK-1 independent mechanisms (Zhou et al., 2013). While these data are certainly intriguing, the study ultimately concluded that IRAK-M exerts an inhibitory effect under normal conditions by indirectly inducing inhibitory proteins (A20, SHIP-1, SOCS1 and
IκB-α) (Zhou et al., 2013). Further clouding mechanistic insight, it has recently been revealed that the original \textit{Irak-m}^\text{-}\text{/} mice commonly used to characterize this protein may actually contain a truncated version of IRAK-M (Rothschild et al., 2017). Detailed sequencing analysis of the \textit{Irak-m} gene product was conducted following TLR stimulation of BMDM from genotype confirmed \textit{Irak-m}^\text{-}\text{/} mice (Rothschild et al., 2017). Under these conditions, a splice variant of the \textit{Irak-m} gene was identified that resulted from the splicing of exon 8 with exon 12, in essence splicing around the neo cassette (Rothschild et al., 2017). This type of splice variant is a common occurrence in genetically modified animals where functional domains are targeted, as opposed to the gene’s start site. Typically, these truncated proteins are dysfunctional and degraded by the cell, which preserves the knockout status of the animals. It is also important to note that the truncated IRAK-M protein has not yet been detected \textit{in situ} and may not exist \textit{in vivo} (Rothschild et al., 2017). However, overexpression of \textit{Irak-m}^\text{Δ9-11} and functional studies using the recombinant protein revealed that this truncation mutant is significantly more potent at activating NF-κB signaling than the wild type version (Rothschild et al., 2017).

Considering these data, we agree with the consensus findings that IRAK-M functions to negatively regulate NF-κB signaling under normal conditions. However, the possibility remains that IRAK-M may actually have a dual role under certain cell type or temporal specific conditions to also activate NF-κB signaling. This could be directly correlated with the cellular concentration of other IRAK family members or other critical cellular substrates. Resolution of the crystal structure of IRAK-M would provide valuable insight
into these questions. It should also be pointed out that the history of IRAK-M is similar to that of IRAK-2, which was first believed to function as an inactive pseudo-kinase (Meylan and Tschopp, 2008). However, it was later determined that IRAK-2 function was independent of IRAK-1 and plays a critical role in sustaining the late phase of NF-κB signaling through potentially functioning as an active kinase (Kawagoe et al., 2008). Future studies will indicate whether our interpretation into the function of IRAK-M will be modified, as we have previously seen with IRAK-2.

4.5 IRAK-M and mucosal tissues

IRAK-M was appropriately named upon its discovery in mammals based on its homology to the other IRAK family members and its tissue specific expression IRAK-M mRNA expression was first found to be limited to cells of the myeloid lineage, hence the “M” in IRAK-M. However, as scientific efforts intensified regarding the functional role of IRAK-M, evidence emerged that suggested IRAK-M expression is not limited to cells of myeloid origin. In fact, several reports have demonstrated the expression of IRAK-M in a wide array of tissues comprising the bone, liver, and cells being osteoclasts, epithelial cells, and neutrophils (Li et al., 2005), (Sumpter et al., 2011), (Kesselring et al., 2016), (Rothschild et al., 2017). The \textit{Irak-m -/-} mouse has greatly enhanced the current understanding regarding the functional role of IRAK-M. Originally, \textit{Irak-m -/-} mice displayed enhanced inflammation in the small intestine when challenged with \textit{S. typhimurium}, and due to the revelation that these mice did not succumb to potentially lethal effects due to endotoxic shock, it was concluded that these mice have enhanced innate immunity compared to wild-type mice (Kobayashi et al., 2002). The lung and GI
tract are mucosal surfaces that are constantly interacting with foreign substances, often
described as luminal antigens, which include commensal microorganisms. Thus, these
mucosal tissues represent a first line defense against exposure to foreign substances,
and serve, therefore, as a strategic defense site for cells of the innate immune system.
For this reason, many studies have focused on the role of IRAK-M in the GI tract, as
well as other mucosal surfaces including the lung (Berglund et al., 2010), (Biswas et al.,
2011),(Deng et al., 2006). It is interesting to note that innate immune cells, particularly
monocytes isolated from septic patients display so called enhanced endotoxin
tolerance, and reduced response to antimicrobial peptides (Deng et al., 2006).
Interestingly, in peritonitis-induced sepsis models, *Irak-m -/-* mice display enhanced
bacterial clearance, and had higher survival rates compared to wild-type mice following
secondary challenge with the bacteria *S. arengosa* (Deng et al., 2006). This enhanced
bacterial clearance, and ultimate protection from endotoxin tolerance was attributed to
increased MIP-2 chemokine production, resulting in an influx of neutrophils to the lung
to handle secondary pulmonary bacterial challenge (Deng et al., 2006).

Several mouse models exist that are utilized to mimic GI inflammatory diseases, such
as ulcerative colitis and colitis-associated cancer (Okayasu et al., 1990), (Neufert et al.,
2007). These include both chemically induced models of colitis, as well as genetic
models utilizing mice that are predisposed to developing colitis (Wirtz et al., 2007),
(MacDonald, 1994). In one chemically induced model conducted with the agent dextran
sulfate sodium (DSS), *Irak-m -/-* mice displayed robust GI inflammation, with an
increase in plasma concentrations of inflammatory cytokines, namely IL-6 and TNF
(Berglund et al., 2010). Furthermore, *Irak-m* -/- mice displayed reduced splenic and thymic weight, which normally increases in weight, and as these weights increase, a correlation is observed with respect to increased disease severity of colitis (Berglund et al., 2010). A genetic model of colitis is one that utilizes mice with a targeted disruption for the gene that encodes the cytokine IL-10 (Kuhn et al., 1993). Regarding IRAK-M, mice that are double deficient for IL-10/IRAK-M display increased inflammation in the colon leading to exacerbated colitis, as well as increased pro-inflammatory cytokine expression (Biswas et al., 2011). Evidence supports the claim that *Irak-m* -/- have an enhanced inflammatory phenotype. This phenotype, however, appears uniquely titrated at mucosal surfaces. Though these mice display robust inflammation following pathogenic encounter, they also display enhanced innate immunity to these pathogenic encounters, and mice are uniquely protected when monitoring their survival (Deng et al., 2006), (Rothschild et al., 2017).

Regarding GI colitis-associated tumorigenesis, *Irak-m* -/- mice were first characterized by robust tumorigenesis induction following completion of the AOM/DSS model (Klimesova et al., 2013). Klimesova et al. attributed this finding to an altered microbiota composition in *Irak-m* -/- mice, because wild-type mice were rescued from tumor formation when supplemented with antibiotics in their drinking water. Conversely, *Irak-m* -/- mice developed tumors both with and without antibiotics, and tumor formation in the GI tract of *Irak-m* -/- mice was attributed to increased inflammatory cytokine production (Klimesova et al., 2013). It is well recognized that inflammation is a growth promoting condition that contributes to the progression of cancer (Greten et al., 2004); therefore,
the authors concluded that this was the main mechanism attributed to tumorigenesis in the *Irak-m* -/- mice (Klimesova et al., 2013). It should be noted that microorganisms in the GI tract are important contributing factors in models that use DSS as an inducing agent for colitis. Laboratories often use different combinations of antibiotics to clear the microbiota, which should minimize the catastrophic inflammatory effects of DSS; however, this is not always the case. When a combination of ampicillin, vancomycin, neomycin and mitronidizole are used in combination with DSS, mice often display extreme morbidity and mortality, which is counter to what one would expect (Rakoff-Nahoum et al., 2004), (Hernandez-Chirlaque et al., 2016). Results with a combination of antibiotics should be interpreted with caution, because of heightened the variability of due to fluctuations in the GI microbiota.

Reports from other groups have demonstrated *Irak-m* -/- mice with decreased tumor progression in both xenograft models and AOM/DSS models (Xie et al., 2007), (Standiford et al., 2011), (Kesselring et al., 2016), (Rothschild et al., 2017). This has been attributed to enhanced innate immune functions in *Irak-m* -/- mice. Recently, Kesselring et al. demonstrated reduced tumor progression in *Irak-m* -/- mice following AOM/DSS colitis-associated tumorigensis (Kesselring et al., 2016). The authors of this study reported increased GI inflammation in *Irak-m* -/- mice during early phase colitis (i.e. acute colitis models); however, decreased inflammation driven tumorigenesis following AOM/DSS. This decrease in tumor burden was attributed to multiple factors, which include the absence of epithelial expressed IRAK-M, as demonstrated with bone marrow chimeric mice, and substantiated by a reduced overall absolute bacterial load in
the colon of \textit{Irak-m} -/- mice (Kesselring et al., 2016). Interestingly, co-housing studies, which aim to transfer GI bacteria of mice, due to their coprophagic nature, did not recapitulate tumor progression in wild-type mice with an inherited IRAK-M microbiome. Therefore, the mechanism attributed to decreased carcinogenesis was the induced expression of IRAK-M, leading to the stabilization of the transcription factor STAT-3, which has a prominent oncogenic role in colon cancer (Kesselring et al., 2016), (Jenkins, 2016), (Yu et al., 2014).

Evolution has provided unique and interesting mechanisms to tolerate commensal microbes, while also maintaining an adequate immune response following pathogenic encounter. There are multiple mechanisms, as well as negative regulatory proteins that restore NF-\textkappa B transcriptional activity to homeostatic norms. It is clear that IRAK-M participates in this process, and it is interesting that, at least in mice, tumor initiation and progression appears to be reduced. This suggests that IRAK-M represents a novel target for inhibition as a cancer therapeutic, with the intention of enhancing innate immunity to limit the progression of cancer.

4.6 A20: An inducible negative regulator of inflammation that works via post-translational modifications\textsuperscript{1}

A20 or Tumor necrosis factor alpha-induced protein 3 (TNFAIP3) is a protein that is rapidly induced by NF-\textkappa B, and functions through a feedback mechanism to halt the canonical signaling cascade. A20 is one of the most studied negative regulatory proteins targeting components of the NF-\textkappa B pathway and functions primarily by
modifying positive regulatory proteins in the cascade (Lee et al., 2000). The two mechanisms in which A20 functions is by modifying the posttranslational status of target proteins directly as a ubiquitin modifying enzyme (i.e. as both a ubiquitin ligase, and as a protein deubiquitinase (Wertz et al., 2004). How can a single protein blunt NF-κB, and other signaling pathways, by acting as both a ubiquitin ligase and de-ubiquitinase (DUB)? The answer lies in the type of linkage of poly-ubiquitin chains to proteins. Ubiquitin is a small 8.5 kDa peptide found in eukaryotic cells that can be covalently attached to proteins to regulate their function post-translationally. This is accomplished via the addition of poly-ubiquitin (poly-Ub) chains to amino acid residues on target proteins. Ubiquitin itself contains seven different lysine residues, and the particular lysine chain that is utilized to make the ubiquitin chain determines the regulatory status of the protein. The seven-lysine residues found on ubiquitin are K6, K11, K27, K29, K33, K48, and K63, with K48 and K63 linkages being the best studied, with currently known regulatory function (Tenno et al., 2004), (Varadan et al., 2004). Generally, when a target protein is modified by Lys-48-linked poly-ubiquitination, this serves as a molecular tag on the protein for recognition, and further destruction by the 26S proteasome (Wilkinson et al., 1980), (Voges et al., 1999). Additionally, Lys-63 linked poly-Ub is a posttranslational modification that generally results in the activation of tagged proteins (Tenno et al., 2004). In terms of A20, this describes the mechanism for its ubiquitin ligase domain; removing Lys-63 linked poly-Ub chains from a protein results in the attenuation of activity of the protein. By also functioning as a ubiquitin ligase, A20 has been shown to inhibit the canonical NF-κB pathway by the addition of Lys-48 linked poly-Ub chains to receptor interacting protein 1 (RIP1) (Wertz et al., 2004), (Bertrand et
RIP1 is an essential molecule that activates canonical NF-κB pathway downstream of the TNF receptor. Addition of Lys-48 linked poly-Ub chains to RIP1 targets this protein for degradation, thus removing a positive signal with the intent of restoring cellular homeostasis.

The importance of A20 as a key negative regulator of inflammation was demonstrated with the use of A20 knockout mice (Lee et al., 2000). In the absence of A20, mice display a robust inflammatory phenotype in the liver, intestine, bone joints, skin and kidney (Lee et al., 2000). Furthermore, severe cachexia occurs in several organs, and mortality commences postnatally within a few weeks of age; this can be perpetuated further when mice are given low doses of TNF or LPS (Lee et al., 2000). A20 does not appear to be crucial for fetal development, because mice display an appropriate Mendelian ratio when born. It was demonstrated in mouse embryonic fibroblasts (MEFs) that in the absence of A20, NF-κB transcriptional activity is sustained, and IκB-α mRNA is transcribed with similar, even possibly increased levels when compared to wild type mice. However, IκB-α protein levels do not rebound following TNF treatment. This was attributed to the continued activity of the IKK complex, because in the presence of a proteasome inhibitor named MG-132, IκB-α levels were restored. Furthermore, it was demonstrated that IκB-α was continually phosphorylated by the IKK complex following translation, which resulted in its constant degradation in A20 null cells (Lee et al., 2000). A20-Tnf and A20-Tnfr1 deficient mice also develop severe spontaneous inflammation, indicating a role for A20 in modulating either alternative innate immune pathways, or adaptive immune pathways (Boone et al., 2004). LPS stimulated A20-Tnf double
deficient BMDMs display a robust production of IL-6 and nitric oxide (NO) compared to WT, which was slightly attenuated compared to A20 deficiency alone. This, along with robust inflammation in A20-Rag1 double knockouts, suggested that A20 played a prominent role in negatively regulating innate immune pathways beyond those regulated by TNF (Boone et al., 2004).

Indeed, A20 was demonstrated to robustly regulate TLR signaling, as MyD88-A20 double deficient mice do not develop severe multi-organ inflammation and cachexia as seen in A20 knockout mice alone. Furthermore, components of the microbiota were found to drive spontaneous inflammation, as chimeric mice reconstituted with A20-/- bone marrow are rescued from severe spontaneous inflammation when treated orally with an antibiotic cocktail that diminishes the intestinal microbiota (Turer et al., 2008).

4.7 CYLD

CYLD is a constitutively expressed DUB that was originally described as a tumor suppressor gene (Bignell et al., 2000). Loss of function of CYLD in humans is associated with familial cylindromatosis, which are multiple benign skin cancers usually localized to the face and neck (Bignell et al., 2000). Additional mechanistic studies revealed that loss of CYLD contributes to an overall cellular resistance in apoptosis (Brummelkamp et al., 2003), which likely contributes to cancer pathogenesis. CYLD is a member of the Ub-specific protease family (USP), which represents the largest family of DUBs in the human genome (Nijman et al., 2005). As a bona fide cysteine protease, CYLD cleaves Lys-63 linked chains of poly-Ub on target proteins (Komander et al.,
This serves as a repressive function because addition of poly-Ub on Lys-63 is generally a post-translational modification that leads to increased activity of modified proteins. Interestingly, in addition to Lys-63 mediated de-ubiquitination, CYLD has the ability to de-ubiquitinate via linear Ub chains (Sato et al., 2015). It is interesting to note that CYLD and A20 cleave Ub chains on several of the same substrates that participate in the signal transduction cascade in the NF-κB pathway, including but not limited to RIP1, IKΚγ, TRAF2 and TRAF6 (Kovalenko et al., 2003, McDaniel et al., 2016, Trompouki et al., 2003, Brummelkamp et al., 2003). This overlap in negative regulatory function demonstrates the importance of signaling molecules, such as TRAF6, as primary nodes in the induction of the inflammatory cascade. The post-translational modification of several of the same molecular targets between A20 and CYLD could be due to steady-state and inducible negative regulation. Further kinetics and substrate avidity studies are necessary to explain the similar molecular targets between these two molecules.

Studies utilizing independently generated Cyld<sup>−/−</sup> mice have yielded highly insightful findings, albeit somewhat conflicting. In one study, the Cyld<sup>−/−</sup> animals demonstrated significant defects in NF-κB and JNK signaling in macrophages following TLR and CD40 stimulation (Zhang et al., 2006). However, this effect appeared to be stimuli specific, as TNF stimulation did not appear to be impacted by CYLD deficiency (Zhang et al., 2006). Importantly, this study observed normal B and T-cell development and function (Zhang et al., 2006). While there are several differences in the proposed mechanistic actions of CYLD, these findings are in general agreement with other groups using these and other
independently generated *Cyld*−/− animals (Massoumi et al., 2006), (Lim et al., 2007). However, at least one additional group has not observed these phenotypes using another independently generated *Clyd*−/− mouse line (Reiley et al., 2004). These animals did not recapitulate the defects in NF-κB, JNK, ERK, or p38 signaling (Reiley et al., 2004). Rather, these animals showed significant attenuation of T cell receptor (TCR) signaling in the absence of CYLD (Reiley et al., 2004).

5. Inflammatory maladies of the GI tract

5.1 Inflammatory Bowel Diseases

Inflammatory bowel disease (IBD) is an inclusive term for two similar, yet distinct diseases, being ulcerative colitis (UC) and Crohn’s disease (CD); both diseases are characterized by aberrant inflammation resulting in the pathogenesis of tissues localized to the gastrointestinal (GI) system. The distinguishing features between these two diseases are based on the localization of the GI inflammation, gross pathology, and microscopic pathological characteristics (Ahmad et al., 2002), (Satsangi et al., 2006). Based on locality, ulcerative colitis is generally limited to inflammation of the large bowel, also termed the colon; whereas Crohn’s disease can cause inflammation-driven pathologies, usually in an intermittent pattern, affecting any portion of the GI tract, even the mouth. The most common GI area affected in Crohn’s disease is the colon and terminal ileum of the small intestine (Abraham and Cho, 2009). Crohn’s disease patients can have inflammatory lesions that affect all GI tissue layers, defined as transmural inflammation; whereas, inflammation in ulcerative colitis usually only affects the mucosal layer of the colon (Abraham and Cho, 2009). Ulcerative colitis, as the name
implies, display distinct ulcers present in the colon that may contribute to blood in the stool. Crohn’s disease patients may have similar ulcerative lesions, but a diagnosis can be distinguished over ulcerative colitis if these lesions are found in other interspersed portions of the GI tract.

Gross pathologies are a method utilized to confirm a diagnosis between ulcerative colitis and Crohn’s disease; however, because IBD is a multifactorial disease of differing severities in the human population, diagnosis is not always simple (Yantiss and Odze, 2006). Gastroenterologists have built upon two models, aimed to guide clinicians to determine if a patient suffers from ulcerative colitis or Crohn’s disease, which include methods of identification termed the Vienna and Montreal models, which were conveniently named after the cities where gastroenterologists convened when the guidelines were established (Satsangi et al., 2006). The Vienna model, first proposed in 1998, described features to distinguish Crohn’s disease based on three predominant elements. These include A, B and L classifications, which designated for age of onset (A), disease behavior, and location of the disease (L), respectively (Satsangi et al., 2006). In 2005, gastroenterologists reassessed the Vienna model and came up with new guidelines that describe the Montreal model, which expanded on the guidelines to identify Crohn’s disease, as well as the distinguishing features of ulcerative colitis (Satsangi et al., 2006). Thus, the more comprehensive Montreal model is the preferred system used among clinicians (Spekhorst et al., 2014).

5.2 Factors that influence IBD pathogenesis
There are several genetic mutations that may predispose individuals to IBD. Genome wide association studies (GWAS) have demonstrated that \textit{NOD2} and \textit{IL23r} mutations are associated with the predisposition of individuals to Crohn’s disease; however, there is currently no single gene mutation solely responsible for IBD pathogenesis (Ma et al., 1999), (Hugot et al., 2001), (Satsangi et al., 1996), (Kanaan et al., 2012). Interestingly, more genetic mutations have been found that are associated with predisposing individuals to Crohn’s disease compared to ulcerative colitis, as demonstrated via monozygotic twins, but different genetic alterations are seen in both diseases (Thompson et al., 1996), (Orholm et al., 2000). A definitive cause of IBD onset and pathogenesis is currently unknown, yet environmental and genetic components have been described to play a significant role (Podolsky, 1991). Patients that have the highest predisposition for IBD are Caucasians, usually of European decent, and disease onset usually takes place in the fertile years of a patient’s life (Probert et al., 1993). Interestingly, males and females are equally likely to develop IBD, thus, there are no gender biases seen in human patients (Rosenblatt and Kane, 2015). Furthermore, because there are several components that contribute to the development of IBD, treatment regimens are generally aimed to improve quality of life for patients, as no cure currently exists for both Crohn’s disease and ulcerative colitis (Braus and Elliott, 2009).

\textbf{5.3 The Interrelationship Between Inflammation and Cancer}

Chronic inflammation, which is common in IBD patients, is an enabling characteristic of cancer (Hanahan and Weinberg, 2011). Notably, IBD patients, especially ulcerative colitis patients, are at a heightened risk for the development of GI related cancers,
termed colitis-associated cancer (Thorsteinsdottir et al., 2011). Empirical evidence was provided in mice that ultimately linked inflammation and cancer when components of the NF-κB signaling cascade were mutated (Greten et al., 2004). Components of the immune system are important players in this context, because they normally contribute to the inflammatory milieu, especially in response to pathogens. As was seen earlier, aberrations in NF-κB signaling can result in hyperactive inflammatory signaling, and production of these cytokines can have a profound impact on the surrounding tissue. In terms of chronic inflammation, this can result in a pro-growth environment that leads to cancer progression. The revelation that inflammation is highly regulated by components of the immune system, it is vital to understand the contribution of the immune system to inflammatory related diseases, such as IBD.

**Concluding Remarks**

Several factors contribute to proper physiology and maintenance of the GI system. Having a comprehensive understanding at the cellular level is a good starting point for advancing therapeutic potential to treat maladies, particularly IBD and colon cancer. For this reason, it is important to investigate specific components of cellular signaling pathways, such as negative regulatory proteins, to determine their contribution pertaining to these particular diseases. Further, broadening our perspectives to encompass instances that alter the interactions of host cells and endogenous microbes shows great potential to uncover unknown aspects that contribute to disease. There will always be a need to understand these complex phenomena, and hopefully recent
advances, as well as ones to come, will not only impact our understanding, but also strengthen treatment options for patients in the near future.

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Chapter 2:
Enhanced Mucosal Defense and Reduced Tumor Burden in Mice with the Compromised Negative Regulator IRAK-M

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A. Abstract
Aberrant inflammation is a hallmark of Inflammatory Bowel Disease (IBD) and colorectal cancer. As discussed in the previous chapter, the inflammatory signaling pathway has many regulatory mechanisms, both through positive and negative regulation. IRAK-M is a critical negative regulator of TLR signaling and overzealous inflammation. Here we utilize data from human studies and Irak-/- mice to elucidate the role of IRAK-M in the modulation of gastrointestinal immune system homeostasis. In human patients, IRAK-M expression is up-regulated during IBD and colorectal cancer. Further functional studies in mice revealed that Irak-/- animals are protected against colitis and colitis associated tumorigenesis. Mechanistically, our data revealed that the gastrointestinal immune system of Irak-/- mice is highly efficient at eliminating microbial translocation following epithelial barrier damage. This attenuation of pathogenesis is associated with expanded areas of gastrointestinal associated lymphoid tissue (GALT), increased neutrophil migration, and enhanced T-cell recruitment. Further evaluation of Irak-/- mice revealed
a splice variant that robustly activates NF-κB signaling. Together, these data identify IRAK-M as a potential target for future therapeutic intervention.

B. Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) represent the clinical manifestations of inflammatory bowel disease (IBD). In humans, there is a strong association in patients afflicted with IBD to further develop colitis associated cancer (CAC) (Greten et al., 2004). Gastrointestinal (GI) immune homeostasis is, in part, maintained by pattern recognition receptors (PRRs); including, but not limited to the Toll-like receptors (TLRs). There are 10 known TLRs in humans that recognize pathogen associated molecular patterns (PAMPs) associated with invading microbes. (Kawai and Akira, 2011). In the gut, TLRs sense and respond to commensal flora translocation, resulting from damage to the epithelial cell barrier during the course of disease progression (Saleh and Elson, 2011). Following TLR engagement to its pathogenic ligand, a highly coordinated signaling cascade leads to the activation of specific transcription factors including nuclear factor-κB (NF-κB), activating protein-1 (AP-1), or in the case of viral recognition, interferon regulatory factor-3 (IRF-3). The dominant signaling pathway utilized depends upon which TLR is stimulated in response to the pathogenic ligand; however, all TLRs, with the exception of TLR-3, signal through the myeloid differentiation factor 88 (MyD88) adaptor molecule (Kawai et al., 1999), (Takeuchi et al., 2000), (Jiang et al., 2006). A MyD88 independent pathway exists for both TLR-3 and TLR-4 that has been shown to signal through the adaptor molecule TIR-domain-containing-adaptor-inducing interferon-β (TRIF) (Yamamoto et al., 2003), (Hoebe et al., 2003), (Kawai et al., 2001). In healthy
individuals, these TLR signaling cascades are responsible for rapid activation of innate host defenses and further activation of the adaptive immune response (Medzhitov and Janeway, 1997). However, uncontrolled activation of TLR signaling promotes chronic inflammation and is associated with a variety of autoimmune diseases, including IBD (Cook et al., 2004).

The TLRs that utilize the MyD88 dependent pathway have been reported to signal through a family of Interleukin Receptor Associated Kinases (IRAKs), which contain four known family members being: IRAK-1, IRAK-2, IRAK-M, and IRAK-4 (Wesche et al., 1997), (Muzio et al., 1997), (Wesche et al., 1999), (Li et al., 2002). IRAK-1, IRAK-2, and IRAK-4 have been described to play a role in the transduction of downstream signaling from MyD88; conversely, IRAK-M is rather unique and has been shown to be a negative regulator of TLR signaling (Kobayashi et al., 2002). The initial reports pertaining to IRAK-M described it as a member of the Pelle/IRAK family, and when overexpressed in mammalian cells, had the capacity to activate NF-κB through interactions with TNF receptor associated factor-6 (TRAF-6) (Wesche et al., 1999). Following the generation of an *Irak-m<sup>-/-</sup>* mouse, IRAK-M was confirmed to function as a negative regulator of TLR signaling (Kobayashi et al., 2002).

Here we report that *IRAK-M* is significantly up-regulated in human patients with IBD and colitis associated neoplasia. We also show that *IRAK-M* is up-regulated in advanced stages of colorectal cancer (CRC). Prior studies have evaluated IRAK-M in models of experimental colitis and colitis associated tumorigenesis utilizing *Irak-m<sup>-/-</sup>* mice and
shown that these animals are highly sensitive to both inflammation and neoplasia.
However, contrary to these prior observations, more recent studies have revealed that 
\textit{Irak-m}\textsuperscript{−/−} mice are actually protected from inflammation driven tumorigenesis in the colon
(Kesselring et al., 2016). IRAK-M was found to support colorectal cancer progression
through the reduction of antimicrobial defenses and the stabilization of STAT-3
(Kesselring et al., 2016). Due to these conflicting reports, we sought to better elucidate
the contribution of IRAK-M during IBD and colitis associated tumorigenesis. Our studies
are, in general, complementary to the more recent findings that support a role for IRAK-
M in disease pathogenesis. Here we report that \textit{Irak-m}\textsuperscript{−/−} mice were significantly
protected against dextran sulfate sodium (DSS)-mediated GI inflammation and
azoxymethane (AOM)/DSS mediated tumor formation. We further show that the
immune system in the GI tract of \textit{Irak-m}\textsuperscript{−/−} mice is primed and highly efficient at
eliminating components of the microbiome translocating from the GI lumen following
damage to the epithelial barrier. This priming and attenuation of disease appears to be
associated with expanded areas of gastrointestinal associated lymphoid tissue (GALT),
increased and efficient neutrophil migration, and enhanced T-cell recruitment.

Upon further evaluation of the \textit{Irak-m}\textsuperscript{−/−} mice, we discovered the formation of a \textit{Irak-m}
splice variant. The splicing event joins exon 8 with exon 12, splicing around the
neomycin resistance cassette (Kobayashi et al., 2002), forming a \textit{Irak-m}\textsuperscript{Δ9-11} truncation
mutant. This truncation mutant functions to robustly induce NF-κB signaling when
overexpressed in HEK293T cells. Together, our data confirms previous findings that
IRAK-M functions as a critical mediator of inflammatory signaling pathways and is essential in maintaining immune system homeostasis in the GI system.

C. Materials and Methods:

Human Metadata Analysis

*IRAK-M* expression was assessed using a publicly accessible microarray metadata analysis search engine (http://www.nextbio.com/b/nextbioCorp.nb), as previously described (Kupershmidt et al., 2010). The following array data series were analyzed to generate the human patient and cell expression data: GSE10714; GSE59071; GSE9686; GSE13367; GSE36807; GSE16879; GSE10191; GSE52746; GSE9452; GSE38713; GSE4183; GSE37283; GSE37364; GSE10715.

Reagents

Ultra-pure LPS from *E.coli*, PGN-SA, Pam3CSK4, Poly (I:C) H.M.W, Flagellin-BS, ODN-1668, Imiquimod were purchased from Invivogen. Mouse TNF-α was purchased from Biolegend. Mouse IL-6, IL-10, TNF-α, IL-1β and IFNγ ELISA kits were purchased from BD Biosciences. Rabbit polyclonal IRAK-M antibody was purchased from Millipore (Millipore 07-1481). Rabbit monoclonal β-Actin (Cell Signaling 13E5), Rabbit monoclonal-HA (Cell Signaling C29F4) and anti-rabbit-HRP were from Cell Signaling. Antibodies for Ly6G, CD11b, CXCR2, CD14, Ly6C, IAE, and CD62L were from Biolegend. PCR primers were generated against exons of murine *Irak-m* utilizing Primer3 software and purchased from Integrated DNA Technologies. Dextran sulfate sodium DSS salt (36,000-50,000 M.Wt.) was purchased from MP Biomedicals.
Azoxymethane (AOM) was from Sigma. RNA isolation Miniprep kit was from Zymo Research. Espresso mammalian expression CMV cloning kit was purchased from Lucigen. Plasmid pGL4.32[luc2P/NF-κB-RE/Hygro] and pRL-null were from Promega.

**Cell Culture**

HEK293T cells (ATCC) were grown in Eagle’s Minimum Essential Medium (EMEM) + 10% Fetal bovine serum (FBS) (Atlanta Biologicals). THP-1 cells (ATCC) were grown in RPMI + 10% FBS + 0.05 mM 2-mercaptoethanol.

**Bone Marrow Derived Macrophage Assessments**

Mouse bone marrow was harvested from both tibias and femurs for individual mice and plated in non-tissue culture treated sterile petri dishes containing 1x DMEM supplemented with 10% FBS + 20% L929 conditioned media for 7 days. Bone marrow derived macrophages (BMDMs) were re-plated on day 7 by aspirating L929 growth media and incubated with 10 ml ice cold sterile 1x PBS containing 5mM EDTA. Cells were incubated at 4 °C for 30 minutes and gently scraped following incubation. Cell containing supernatants were transferred to 50 ml conical tubes and centrifuged for 10 minutes at 300 x g. BMDMs were resuspended in 20 ml warm growth media and replated in tissue culture treated sterile plastic ware at: 2x10^5 cells per well in triplicate for 24 well plates; 2x10^6 cells per 60mm² dish; 5x10^6 cells per 10mm² dish. Wild-type and *Irak*<sup>-/-</sup> BMDMs were re-plated in separate 24 well plates and allowed to adhere overnight. Cell culture media was aspirated the following day and BMDMs were washed with 1 ml 1x PBS. Following PBS aspiration the cells were treated with 500 μl total
volume per well of 1x DMEM +10% FBS containing specific pathogen associated molecular patterns (PAMPs) for 24 hours at a concentration of: Poly I:C (6.25 μg/ml), PGN-SA (10 μg/ml), LPS (10 ng/ml), Pam3CSK4 (300 ng/ml), TNF-α (75 ng/ml).

**Colon organ culture supernatants**

Following the acute colitis model or AOM/DSS model, colon sections were also collected to establish organ cultures to determine local cytokine levels as previously described (Greten et al., 2004), (Allen et al., 2010), (Williams et al., 2015). Briefly, approximately 1-cm² strip of the distal colon was removed and washed with 200 U/ml Penicillin and 200 μg/ml streptomycin in 1x PBS. Following the wash, each respective colon section had the total mass measured and recorded for downstream normalization. Each individual distal colon section was then transferred to an individual well of a 24 well plate and supplemented with 1ml of DMEM media containing 200 U/ml Penicillin and 200 μg/ml streptomycin with no additional supplements. Following overnight incubation, the supernatants were collected and centrifuged at 300 x g for 10 minutes to collect cell free supernatants. ELISAs were then conducted on the supernatants to determine the respective cytokine level and each sample was normalized to their respective colon section mass.

**Bacterial Counts.**

Bacterial counts were determined from mouse whole blood collected via cardiac puncture. 25 μl of whole blood was plated, as well as two 10 fold serial dilutions were
prepared in sterile 1x PBS and 25 µl aliquots were plated on LB agar plates. Following overnight incubation at 37 °C, bacterial colonies were counted.

**Western Blot**

Experiments utilizing WT and *Irak-m⁻/⁻* BMDMs were seeded in 60mm² dishes and allowed to adhere overnight. Experiments with HEK293T cells were seeded in 6 well plates at 3x10⁵ cells per well. BMDMs were treated with control media or stimulated with 10 ng/ml LPS, 10 µg/ml PGN-SA or 300 ng/ml Pam3CSK4 for 24 hr. Cells and BMDMs were washed with ice cold 1x PBS and were subsequently lysed in cell lysis buffer containing: 2% SDS (Fisher Sci), 100 mM NaCl (Fisher Sci), 10 mM Tris-HCl pH 7.4 (Fisher Sci), 1x Halt Protease inhibitor (Thermo). Whole cell lysates were sonicated to shear endogenous nucleic acids. Protein concentration was quantified by BCA (Pierce) and 15 - 20 µg total protein was boiled in 1x Blue Loading Buffer + 1x DTT (Cell Signaling) at 95 °C for 5 min and electrophoresed with either Bolt 4-12% Bis-Tris gels (Thermo) or Biorad 4-12% Criterion XT Bis-Tris Gel in 1x MOPs buffer (Thermo). Gels were transferred onto 0.2 µm PVDF membranes (Millipore) in 1x Tris-glycine + 20% methanol. Membranes were blocked for 30 minutes with 5% non-fat dry milk (Carnation Brand) dissolved in TBS+0.05% Tween 20 (TBST). Primary antibodies were used 1:1000 and incubated at 4 °C overnight with gentle shaking. Primary antibodies were either: anti-IRAK-M (Millipore 07-1481), anti-β-Actin (Cell Signaling 13E5) and anti-HA (Cell Signaling C29F4). IRAK-M, β-Actin and HA were visualized using anti-rabbit IgG linked to HRP, detected with ECL western blotting substrate Pico (Pierce) or Dura (Pierce) by x-ray radiography. Densitometry was analyzed using ImageJ software.
RT-PCR

BMDM were seeded in 10mm² dishes and allowed to adhere overnight. Cells were then treated with 1x DMEM +10% FBS without PAMP stimulation or 300 ng/ml Pam3CSK4 (Invivogen) for 24 hr. RNA was isolated using Zymo Research miniprep kit. Total RNA was quantified and 2 μg was converted to cDNA with High Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacture’s instructions. 5-10 ng of cDNA was amplified for different exons of murine Irak-m by RT-PCR. The primer sequences used for Irak-m exons are:

**Irak-m Exon Primers**

<table>
<thead>
<tr>
<th>Exon:</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-8</td>
<td>GGACATTTCGAAACCAAGCAT</td>
<td>TGTGCCATTTGTGCACCTGTA</td>
</tr>
<tr>
<td>9-10</td>
<td>CCAGCTCCAACCCAAACTAA</td>
<td>TGTTTCGGGTCATCCAGCAC</td>
</tr>
<tr>
<td>10-11</td>
<td>GGACCTCTCTCATGGAACTGA</td>
<td>CCAGAGAGGACAGGACTTTGC</td>
</tr>
<tr>
<td>12</td>
<td>TCCTTCAGGTGTCCTTCCACTG</td>
<td>CCTCTTCTCCATTGGCTTGCTC</td>
</tr>
<tr>
<td>5-12</td>
<td>GTGCAGAGAAACGACCCTG</td>
<td>TGGGAGGGTCTTCTGCAAAA</td>
</tr>
</tbody>
</table>

RT-PCR products were electrophoresed in 1x TBE and visualized on 1% agarose gel stained with ethidium bromide.

**Molecular Cloning, Overexpression and Dual Luciferase Assay.**
Molecular cloning of the *Irak-m* gene of both wild-type (*Irak-m*) and mutant (*Irak-m<sup>Δ9-11</sup]*) was performed from BMDMs stimulated for 24 hr with 300 ng/ml Pam3CSK4. RT-PCR was conducted utilizing Phusion DNA high fidelity polymerase (Thermo) with primer sequences generated with homology to either the WT *Irak-m* gene, or the *Irak-m<sup>Δ9-11</sup>*) gene, and the espresso CMV cloning and expression system (Lucigen) according to the manufacture's instructions. Primer sequences are:

**CMV-Irak-m-HA Cloning Primers**

<table>
<thead>
<tr>
<th></th>
<th>Forward 5′→3′</th>
<th>Reverse 5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT Irak-m-HA</strong> (Forward) 5′→3′</td>
<td>GAAGGAGATACCACCATGGCCGGCCGCTGGTGCGGGGCCCGT</td>
<td>GGGCACGTCATACGGGATACTGACTTTTGGACTGTTCATG</td>
</tr>
<tr>
<td><strong>WT Irak-m-HA</strong> (Reverse) 5′→3′</td>
<td>GAAGGAGATACCACCATGGCCGGCCGCTGGTGCGGGGCCCGT</td>
<td>GGGCACGTCATACGGGATAAGGACGTGGGAGGGTCTT</td>
</tr>
<tr>
<td><strong>Irak-m&lt;sup&gt;Δ9-11&lt;/sup&gt;-HA</strong> (Forward) 5′→3′</td>
<td>GAAGGAGATACCACCATGGCCGGCCGCTGGTGCGGGGCCCGT</td>
<td>GGGCACGTCATACGGGATAAGGACGTGGGAGGGTCTT</td>
</tr>
<tr>
<td><strong>Irak-m&lt;sup&gt;Δ9-11&lt;/sup&gt;-HA</strong> (Reverse) 5′→3′</td>
<td>GGGCACGTCATACGGGATAAGGACGTGGGAGGGTCTT</td>
<td>GGGCACGTCATACGGGATAAGGACGTGGGAGGGTCTT</td>
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6-well plates (Corning) were seeded with 3x10<sup>5</sup> HEK293T cells (ATCC) per well and allowed to adhere overnight. Cells were transfected with a total of 2.7 μg of DNA using Lipofectamine P3000 reagent (Thermo) according to the manufacturer's instructions. Transfection included: 100 ng pGL4.32[ luc2P/NF-κB-RE/Hygro] (promega), 100 ng pRL-null (promega), and decreasing amounts of either pME-CMV-*Irak-m*-HA tagged (625ng-10ng); pME-CMV-*Irak-m<sup>Δ9-11</sup>-HA* tagged (2500ng-40ng); 500 ng pME-CMV-β
galactosidase-HA; or 2500 ng pME-CMV-Empty Vector. The total amount of transfected plasmid DNA remained constant for each treatment by co-transfecting with the proper amounts pME-CMV-Empty Vector. 500 ng pME-CMV-\(\text{galactosidase-HA}\) served as a positive control for transfection efficiency. Following 18 hr of transfection, a pME-CMV-\(\text{galactosidase-HA}\) transfected well was treated with 10 ng/mL human IL-1\(\alpha\) (invivogen) for 6 hr as a positive control for the dual luciferase assay. Following 24 hr transfection, luminescence catalyzed by firefly luciferase and renilla luciferase of the cell lysates was determined using Dual-Luciferase Reporter Assay System (promega) according to the manufacturer’s instructions.

Experimental Animals
All mouse studies were approved by the Institute for Animal Care and Use Committee (IACUC) at Virginia Tech and in accordance with the Federal NIH Guide for the Care and Use of Laboratory Animals. The \(\text{Irak}\)-\(\text{m}^{-}\) mice were generated as previously described (Kobayashi et al., 2002) and purchased from The Jackson Laboratory. All studies were controlled with either littermate and/or co-housed WT animals that were maintained under specific pathogen-free conditions and received standard chow (LabDiet) and water \textit{ad libitum}. All experiments described utilized age matched male mice.

Experimental Colitis and Colitis Associated Tumorigenesis
In order to assess acute experimental colitis, mice were given either 3 or 5\% DSS dissolved in drinking water available \textit{ad libitum} for 5 days as previously described
(Williams et al., 2015b), (Schneider, 2013). On day 5, mice were withdrawn from DSS and given regular drinking water *ad libitum* until euthanasia was performed on day 7. Cumulative semi quantitative clinical scores for acute experimental colitis were assessed as previously described (Williams et al., 2015b), (Schneider, 2013). Tumorigenesis was induced via a single intraperitoneal (i.p.) injection of AOM (10 mg/kg of total body weight) and supplemented with three cycles of 2.5% DSS in drinking water available *ad libitum* for 5 days with 2 weeks of recovery between cycles, as previously described (Neufert et al., 2007), (Allen et al., 2010). While subjected to DSS, mice were monitored for weight loss, physical body condition, stool consistency, and rectal bleeding. Upon completion of each model, whole blood was collected by cardiac puncture for bacterial counts, flow cytometry assessments of leukocyte populations, and serum isolation. Colon sections were collected for H&E staining. Blinded to treatment and mouse genotype, examination of histopathology was conducted by a board-certified veterinary pathologist (T.L.). Colon H&E sections were evaluated and scored as previously described (Williams et al., 2015b). Additional colon sections were further prepared for immunohistochemistry and stained with anti-β-catenin and DAPI to determine β-catenin levels in the AOM+DSS studies.

**FACS**

Leukocytes and lymphocytes were evaluated from either: bone marrow, spleen, or whole blood utilizing flow cytometry. Cells were immunostained with antibodies for the respective cell surface markers prior to FACS analysis. Sorted cells were further evaluated for CXCR2, CD14, CD11b+, Ly6G+, CD4+, CD8+, Ly6C+, IAE, and CD62L.
**ELISA**

Cell culture supernatants or colon organ culture supernatants were collected from each individual well in 1.7 ml tubes and centrifuged at 300 x g for 10 minutes to remove residual cells. Cell-free supernatants were then assayed for mouse IL-6 and/or IL-10 (BD Biosciences) according to the manufacturer’s instructions.

**Statistical Analysis**

Data are represented as mean ± standard error of mean (S.E.M.) unless otherwise indicated. Graphs and statistical analysis were conducted via GraphPad PRISM software. Complex data sets were analyzed by 1 way analysis of variance (ANOVA) and followed by either Tukey-Kramer HSD or Newman-Keuls method. The Kaplan-Meier test was conducted to determine group survival. A value of p<0.05 were considered statistically significant.

**D. Results**

*IRAK-M Expression is Significantly Increased in Human Patients with IBD and CRC*

Previous studies have shown that *IRAK-M* expression is increased in IBD patients (Fernandes et al., 2016), (Gunaltay et al., 2014). Thus, we initially sought to evaluate these findings and expand our analysis to further evaluate *IRAK-M* expression in the context of colitis associated neoplasia and CRC using a retrospective metadata analysis of publicly available gene expression data (Kupershmidt et al., 2010). Our analysis revealed that the relative expression of *IRAK-M* is significantly increased in human
patients with active forms of IBD (Figure 2.1A). Patients that suffer from IBD have a higher predisposition to CAC (Karlen et al., 1999); thus, we also included CAC patients in our data analysis and found that IRAK-M is also significantly increased in patients with active UC with inclusive areas of neoplasia (Figure 2.1A). Independent of CAC, we were also interested in evaluating IRAK-M expression in the context of CRC. Thus, we also analyzed expression levels of IRAK-M in patients diagnosed with both low and high-grade CRC. CRC patients were stratified based on the Dukes’ staging system (A/B) and (C/D). From these data, it is apparent that IRAK-M expression is significantly increased in patients with more advanced CRC (grades C/D) compared to the patients with less advanced CRC (grades A/B) and patients not diagnosed with CRC (Figure 2.1B). To gain greater insight into IRAK-M function, we next sought to evaluate IRAK-M expression in different human cell types of relevance to IBD, CAC, and CRC, with particular emphasis on specific immune cell populations and colon epithelial cells.

Figure 2.1. IRAK-M Expression is Increased During Inflammatory Bowel Disease Exacerbation and in Advanced Colorectal Cancer Patients.
Figure 1. IRAK-M Expression is Increased During Inflammatory Bowel Disease Exacerbation and in Advanced Colorectal...
(Figure 2.1C). Prior literature documents the expression of IRAK-M in cells of the myeloid lineage (Wesche et al., 1999). This is supported by our metadata analysis (Figure 2.1C). However, our findings further revealed that IRAK-M is also highly expressed in eosinophils and neutrophils (Figure 2.1C). We also found IRAK-M expressed in all of the other cell types assessed, albeit at lower levels than the macrophages, myeloid progenitor cells and granulocytes (Figure 2.1C). Together, these data suggest that IRAK-M plays a significant role in modulating the immune response in the GI system and is up-regulated in the context of IBD and CRC. This up-regulation is likely in response to increased TLR and interleukin 1 receptor (IL-1R1) signaling in these disease states.

Irak-/- Mice Are Protected Against DSS Induced Colitis
We next sought to better characterize the contribution of IRAK-M in the context of IBD. Prior studies have evaluated \textit{Irak-m}⁻/⁻ mice in both dextran sulfate sodium (DSS) and \textit{Il10}⁻/⁻ colitis models (Berglund et al., 2010), (Biswas et al., 2011). These prior studies revealed that IRAK-M functions to attenuate the progression of experimental colitis (Berglund et al., 2010), (Biswas et al., 2011). Here we utilized \textit{Irak-m}⁻/⁻ mice in an acute colitis model induced with 5% DSS (Okayasu et al., 1990). This is a standard model utilized previously by our laboratory in similar studies evaluating mediators of innate immunity in IBD (Williams et al., 2015a), (Williams et al., 2015b), (Allen et al., 2012), (Allen et al., 2010). We found that \textit{Irak-m}⁻/⁻ mice are protected in this experimental colitis model (Figure 2.2). Clinically, the \textit{Irak-m}⁻/⁻ mice demonstrate significant improvements in weight change and clinical parameters associated with disease progression compared to the wild-type (WT) animals (Figure 2.2A and 2.2B). Consistent with our clinical observations, \textit{Irak-m}⁻/⁻ mice also displayed significantly increased colon length and less severe tissue damage as evident by histopathology evaluation of H&E stained colon sections compared to the WT counterparts (Figure 2.2C and 2.2D). Interestingly, when viewing the histopathology of the colon from \textit{Irak-m}⁻/⁻ mice we observed localized and highly structured areas of lymphoid cells throughout the colon. These structures were identified and confirmed to be expanded areas gut associated lymphoid tissue (GALT) by a board certified veterinary pathologist (T.L.) (Figure 2.2E). We hypothesized that the protective phenotype observed in the \textit{Irak-m}⁻/⁻ mice following acute DSS exposure was due, in part, to the significant increase in GALT in the colon of the \textit{Irak-m}⁻/⁻ mice, which were present irrespective of DSS treatment (Figure 2.2E).

\textbf{Figure 2.2. Attenuated Experimental Colitis Pathogenesis in \textit{Irak-m}⁻/⁻ Mice.}
Figure 2. Attenuated Experimental Colitis Pathogenesis in Irak-m-/- Mice

A. % Weight Change over time following DSS treatment.

B. Clinical Score analysis showing significant differences between groups.

C. Colon Length comparison showing extended length in Irak-m-/- mice.

D. Histological images comparing Mock, Irak-m-/+ DSS, and Wild Type + DSS groups.

E. Irak-m-/- Mock GALT images.

F. Bacteria Counts in Blood showing reduced counts in Irak-m-/- compared to Wild Type.

G. Colon IL-6 (pg/ml) comparison showing increased levels in Irak-m-/- DSS group.

* †
To test this hypothesis, we measured the systemic bacteremia in whole blood of both WT and Irak-m\(^+/\)- mice following the acute DSS model. Our data lend support to this hypothesis as bacterial counts were significantly reduced in Irak-m\(^+/\)- mice (Figure 2.2F).

Further, we observed increased levels of IL-6 from Irak-m\(^+/\)- colon organ culture supernatant following DSS treatment, which is consistent with the increased GALT (Figure 2.2G). While increased IL-6 is typically associated with detrimental inflammation, our histopathology assessments revealed that the inflammation was highly localized to these areas of GALT, with minimal damage to the epithelial cell barrier (Figure 2.2D). Collectively, our data suggest that Irak-m\(^+/\)- mice are protected against DSS induced colitis by displaying increased resistance to bacterial translocation and bacteremia.

**Attenuation of Experimental Colitis in the Irak-m\(^+/\)- Mice is Associated with Enhanced Neutrophil and T-Cell Responses.**
We hypothesized that the increased GALT observed in the *Irak-m* mice was a significant contributing factor in protecting these animals during experimental colitis. To test whether *Irak-m* protection from acute DSS was associated with increased leukocyte recruitment and function, we evaluated the leukocyte composition in whole blood using flow cytometry (Figure 2.3A). Under basal conditions, we found the neutrophil count was significantly higher in the blood from naïve *Irak-m* mice compared to WT animals (Figure 2.3A). Interestingly, following exposure to DSS the number of neutrophils significantly increased in WT mice; however, the number of neutrophils in the *Irak-m* mice maintained elevated levels with or without exposure to DSS (Figure 2.3A). We further measured the levels of CXCR2 and CD14 from both naïve animals and mice in the experimental colitis model (Figure 2.3B and 2.3C). Under both naïve and DSS treated conditions we observed increased levels of CXCR2 and CD14 from *Irak-m* Ly6G⁺/CD11b⁺ leukocytes, suggesting *Irak-m* neutrophils are primed prior to tissue insult, which likely improves the efficiency in recruitment to a site of infection, such as the colon when bacterial translocation occurs. To further support this hypothesis we performed a neutrophil chemotaxis assay in response to macrophage inflammatory protein 2 (MIP-2). When bone marrow from WT and *Irak-m* mice was primed with doses of LPS and subjected to the chemotaxis assay, we observed increases in neutrophils in a dose dependent manner for *Irak-m* mice (Figure 2.3D). Further, we also observed increased numbers of CD4⁺, CD8⁺ T-cells and monocytes.
isolated from the spleen of *Irak-m*/*- mice following exposure to DSS (Figure 2.3E). This finding is consistent with prior studies that indicated increased T-cell recruitment in *Irak-m*/*- mice in the experimental colitis model (Berglund et al., 2010), (Klimesova et al., 2013). Monocytes isolated from the spleen of *Irak-m*/*- mice following DSS exposure displayed increased cell surface expression of IAE and decreased expression of CD62L (Figure 2.3F). Further, we hypothesized that the differences in neutrophil and T-cell recruitment in *Irak-m*/*- mice was the result of differences associated with the GI microbiota composition. When mice were treated with antibiotics for two weeks prior to DSS administration, similar susceptibilities to pathogenesis were observed between WT and *Irak-m*/*- mice (Figure 2.4A-C). Collectively, our data suggests that *Irak-m*/*- mice are protected from experimental colitis due to efficient recruitment of neutrophils and T-cells following microbial translocation. These data provide a mechanism of acute colitis protection and lend support to the phenotype that GALT in *Irak-m*/*- mice contributes to the overall protection observed in the experimental colitis model.

*Irak-m*/*- Mice Are Protected Against Inflammation Driven Colon Tumorigenesis.
Our retrospective metadata analysis revealed that IRAK-M likely modulates IBD, CAC, and CRC in human patients (Figure 1A and 1B). Based on our findings in the IBD model, we next sought to evaluate the *Irak-m<sup>-/-</sup>* mice in a model of colitis associated tumorigenesis. Here we utilized the AOM+DSS model (Williams et al., 2015a).

**Figure 2.4. Increased Morbidity and Mortality in Antibiotic + DSS Treated Mice**
Irak-/-mice displayed improved morbidity and mortality throughout the course of the model compared to the WT animals, suggesting attenuation of disease progression compared to the WT counterparts (Figure 2.5A-C). When colon organ culture supernatants were assayed for IL-6 and IL-10, Irak-m/- mice displayed significant increases in both cytokines (Figure 2.5D-E). Beyond the significant attenuation of clinical features and enhanced cytokine responses, the Irak-m/- mice displayed dramatic resistance to tumorigenesis (Figure 2.6). The overall gross polyp formation was significantly higher in WT mice; conversely, no polyps were detected in Irak-m/- mice over the course of the study (Figure 2.6A-C). Decreased tumor burden was further supported by histopathology assessments of H&E stained colon sections (Figure 2.6D). As described for the experimental colitis model, GALT formation was prominent in Irak-m/- mice (Figure 2.6E). Consistent with the macroscopic colon evaluation, histopathology scoring revealed significant reductions in areas of hyperplasia and dysplasia in Irak-m/- mice (Figure 2.6F-G). Likewise, we found that
Figure 2.5. Colitis Associated Tumorigenesis Progression is Significantly Reduced in *Irak*-m<sup>-/-</sup> Mice

A. % Survival

B. % Weight Change

C. Clinical Score

D. IL-6 (pg/ml/mg)

E. IL-10 (pg/ml/mg)
the levels of β-catenin are also markedly reduced in \textit{Irak-m}⁻/⁻ mice following AOM+DSS (Figure 2.6H). Collectively, our data indicates that the \textit{Irak-m}⁻/⁻ mice are resistant to both experimental colitis and inflammation driven tumorigenesis.

**Identification of a Splice Variant of the \textit{Irak-m} Gene in \textit{Irak-m}⁻/⁻ Mice**

Expression levels of \textit{IRA-K-M} are highest in macrophages and previous studies have utilized both human and mouse macrophages to study IRAK-M function (Wesche et al., 1999), (Kobayashi et al., 2002). Thus, we were interested in determining the response of IRAK-M in murine bone marrow derived macrophages (BMDM) when challenged with diverse PAMPs. In our hands, BMDM from our \textit{Irak-m}⁻/⁻ mice display significantly increased levels of IL-6 compared to WT BMDMs when stimulated for 24 hours with different TLR ligands, specifically the TLR2 ligand Pam3CSK4 (Figure 2.7A). Further, we assessed a broad panel of secreted cytokines following 24 hour treatment with TLR 1-9 agonists. Specifically, \textit{Irak-m}⁻/⁻ BMDMs display increased levels of TNF with TLR1/2
Figure 2.6. *Irak-m*<sup>−/−</sup> Mice Display Attenuated Polyp Formation in the Colitis Associated Tumorigenesis Model.

A. B. C. D. E. F. G. H.
agonists and decreased IL-10 with TLR 1/2, 7, and 9 agonists (Figure 2.8A-E). This is consistent with prior reports pertaining to IRAK-M being a negative regulator of TLR signaling (Kobayashi et al., 2002). We also observed differences in IL-6 secretion between male and female Irak-m⁻/⁻ BMDMs when stimulated with Pam3CSK4 (Figure 2.9). However, during routine follow-up studies, we observed IRAK-M protein induction in both the WT and Irak-m⁻/⁻ strains when stimulated with various TLR ligands using an antibody specific for the C-terminus of IRAK-M (Figure 2.10). This was unexpected, as our Irak-m⁻/⁻ mice were acquired from a commercial vendor, albeit reconstituted from frozen embryos, and previously characterized (Kobayashi et al., 2002). It was previously reported that generation of the Irak-m⁻/⁻ mouse targeted exons 9-11 for deletion by homologous recombination inserting the neomycin resistance cassette in place of these
Figure 2.7. Disruption of the Murine Irak-m Locus Results in the Formation of an Irak-mΔ9-11 Splice Variant.
three exons (Kobayashi et al., 2002). In order to test the hypothesis that IRAK-M was present in our mutant mice, we proceeded by further investigating the phenomena at the
2.8 Differences in TLR-2, TLR-7 and TLR-9 Stimulation in Irak-m Mutant Mice.
Figure 2.8. Differences in TLR-2, TLR-7 and TLR-9 Stimulation in irak-m Mutant Mice. Bone marrow was harvested from 8-week age matched males. BMDMs were derived and seeded in 24 well plates as described in the Materials and Methods. Cells were stimulated with either: Control media, 6.25μg/ml Poly(I:C), 10 ng/ml LPS, 10 μg/ml PGN-SA, 300 ng/ml Pam3CSK4, 50 ng/ml standard Flagellin-BS, 5 μM CpG DNA ODN1668, 1 μg/ml Imiquimod (R837), 20 ng/ml m-IL-1β, 75 ng/ml m-TNFα. Cell free supernatants were collected and assayed by ELISA for extracellular: A. IL-6. B. IL-10. C. TNF (75ng/ml TNFα treatment indicates the upper limit/saturation range of the ELISA). D. IL-1β (Data plotted is extrapolated, as the values were below the detection limit of the ELISA). E. IFN-γ. Data indicates the mean from n=3 WT, n=3 irak-m/- mice. Error bars indicate SEM. Data was analyzed by 1-way ANOVA followed by Tukey’s HSD. *p<0.05, **p<0.01, ***p<0.001.

Figure 2.9. Comparison of Male and Female Bone Marrow Derived Macrophages
mRNA level. When BMDMs were stimulated for 24 hours with the TLR1/2 ligand Pam3CSK4 to induce Irak-m transcription, we observed an amplification band corresponding specifically to exon 12 of Irak-m by RT-PCR (Figure 2.7B). PCR primers were further designed to amplify the region spanning the length of exons 5 and 12 (Figure 2.7C). Interestingly, a shift of approximately 400bp was observed in the BMDMs from our Irak-m/- animals, which suggested a splice variant had been generated. This was indeed confirmed by Sanger sequencing, which revealed a splice occurred after exon 8 and connecting it with exon 12 in the amplification band (Figure 2.7D). Loss of the neo cassette only occurred at the mRNA level, likely after exon splicing, and not at the DNA level. This is evident because the genotyping primers are targeted against a portion of the neo cassette, which is present in the genotyping for the Irak-m/- mice. Based on this revelation, we have defined this splice variant as Irak-mΔ9-11. In order to test the functionality of this truncated transcript, we cloned both the WT and mutant Irak-mΔ9-11 transcripts. Overexpression in HEK293T cells of both the WT and truncated Irak-mΔ9-11 transcripts both had the capacity to activate an NF-kB dependent luciferase reporter. However, NF-kB activation was robustly increased following overexpression of Irak-mΔ9-11 compared to the WT (Figure 2.7E), suggesting a functional and potent role for this mutant protein. We further validated the specificity of the commercially available

**Figure 2.9. Comparison of Male and Female Bone Marrow Derived Macrophages.** IL-6 ELISA of BMDM stimulated for 24 hrs with respective Pamps. Concentrations of Pamps are described in the Materials and Methods. n=3 WT males, n=3 Irak-m/- males, n=3 WT females, n=3 Irak-m/- females. Data was analyzed by 1-way ANOVA followed by Tukey’s HSD. ***p<0.001.
Figure 2.10. Western Blot Evaluation of Protein Expression of IRAK-M.

Western blot evaluation revealed that IRAK-M protein is present in both the WT and *Irak-/-* treatment groups following 24hr stimulation with specific PAMPs. Western blot is normalized to β-actin and human THP-1 cells are used as a positive control. Densitometry is quantified with numerical values below β-actin using ImageJ. Further, independent validation of the IRAK-M antibody used displayed no specificity for the murine WT-IRAK-M-HA or *Irak-Δ9-11*-HA overexpression constructs (data not shown).

IRAK-M antibody used in Figure S1, which displayed no specificity for the WT-IRAK-M or *Irak-Δ9-11* overexpression constructs (data not shown).

E. Discussion

Overzealous inflammation associated with dysregulated innate immune signaling is a significant component of IBD pathogenesis. This hyper-inflammation is often associated with aberrant TLR signaling. There is significant interest in better characterizing the contribution of proteins, like IRAK-M, that regulate PRR signaling in IBD, CAC, and CRC. This interest is due to the revelation that these regulatory proteins significantly
attenuate disease pathogenesis in IBD mouse models. For example, prior studies by our group and others have shown that a diverse group of negative regulatory proteins, including NLRP12, NLRX1, TOLLIP, A20, and GIT2, also function to maintain immune system homeostasis in the gut through the attenuation of hyper-responsive inflammation (Mukherjee and Biswas, 2014), (Vereecke et al., 2014), (Hammer et al., 2011), (Boj et al., 2015), (Allen et al., 2012), (Zaki et al., 2011), (Soares et al., 2014), (Singh et al., 2015). Many of these negative regulatory proteins have been found dysregulated in IBD and CRC patients. For example, previous reports have indicated that IRAK-M expression is significantly increased in IBD patients with both UC and CD (Fernandes et al., 2016), (Gunaltay et al., 2014). These findings are consistent with our analysis of retrospective metadata that revealed increased IRAK-M expression, not only in active UC and CD patients, but also in the context of colitis associated neoplasia (Figure 2.1). Our retrospective metadata analysis revealed that IRAK-M expression increases with severity of CRC progression (Figure 2.1). Together these data likely reflect an increase in the innate immune response during both IBD and CRC that is driven by PRR activation. These data are consistent with recent findings that revealed increased IRAK-M induction in colon tumor cells associated with the combined effects of Wnt and TLR activation (Kesselring et al., 2016). The increase in IRAK-M expression, as well as the expression of other genes that encode negative regulators of PRRs, is likely an attempt to reign in overzealous inflammation and maintain some level of immune system homeostasis during IBD and CRC.
The \textit{Irak-m\textsuperscript{-/-}} mouse model has been an essential tool to determine the negative regulatory mechanisms underlying TLR signaling. \textit{Irak-m\textsuperscript{-/-}} mice have proven to be more sensitive to TLR stimulation and display impaired endotoxin tolerance, likely due to the hyper-activation of NF-\(\kappa\)B signaling (Kobayashi et al., 2002). In addition to negatively regulating canonical NF-\(\kappa\)B signaling, IRAK-M inhibits the non-canonical NF-\(\kappa\)B cascade, in part, through modulating the degradation of NF-\(\kappa\)B inducing kinase (NIK) (Su et al., 2009). In prior tumor injection models, loss of \textit{Irak-m\textsuperscript{-/-}} has been shown to result in enhanced innate immune responses and the attenuation of tumor growth (Xie et al., 2007), (Standiford et al., 2011). These studies were based on tumor injection models with \textit{Irak-m\textsuperscript{-/-}} mice. However, the overall conclusions of each study are consistent with the observations reported here. In both prior studies, the \textit{Irak-m\textsuperscript{-/-}} animals demonstrated significant resistance to tumor growth and pathogenesis following inoculation. Mechanistically, the attenuation of tumorigenesis was associated with increased activation and proliferation of B cells and T cells, specifically CD4\(^+\) and CD8\(^+\) T cells (Xie et al., 2007). Our group has previously shown that \textit{Irak-m\textsuperscript{-/-}} macrophages display enhanced uptake of acLDL, as well as increased percentages of macrophages that uptake apoptotic thymocytes (Xie et al., 2007). Reports have demonstrated IL-10R signaling plays a dramatic role in macrophage function (Shouval et al., 2014); additionally, IL-10 is produced from macrophages following the uptake of apoptotic cells (Chung et al., 2007), (Zhang et al., 2010). Therefore, though we have not explicitly tested, we speculate that increased phagocytosis of apoptotic cells by \textit{Irak-m} mutant macrophages results in increased IL-10 leading towards a tolerant, and potentially protective phenotype.
Our findings in the experimental colitis and colitis associated tumorigenesis models were initially surprising, as Irak-/- mice appear to have increased inflammation. However, upon further investigation we discovered that the inflammation is actually highly compartmentalized and isolated to regions of enhanced GALT, with little damage or effect to the epithelial cell barrier. As we detail in the current manuscript, we believe that these expanded areas of GALT are actually beneficial to these mice and function to improve the efficiency of the immune response to translocating microbes from the GI lumen. This is further supported as (Kesselring et al., 2016) described increased GI epithelial barrier permeability in Irak-/- mice using FITC labeled dextran.

IRAK-M modulation of experimental colitis and colitis associated tumorigenesis has been previously evaluated in Irak-/- mice (Berglund et al., 2010), (Biswas et al., 2011, (Klimesova et al., 2013). In the initial study that evaluated IRAK-M function during DSS-induced colitis, Irak-/- mice were treated with 3% DSS for 5 days and allowed 2 days to recover prior to harvest (Berglund et al., 2010). In this model, Irak-/- mice were found to be sensitive to DSS and presented with significantly increased clinical and histopathological features associated with disease progression (Berglund et al., 2010). This study also found elevated cytokine, chemokine, and T-cell transcription factor mRNA expression in Irak-/- colon tissue and increased systemic IL-6 and TNF levels in the plasma following DSS administration (Berglund et al., 2010). Subsequent studies by a different group utilizing the AOM+DSS colitis associated tumorigenesis model in both conventional and germfree conditions also reported increased sensitivity and
tumorigenesis in the Irak-/- mice (Klimesova et al., 2013). Similar to the study by Berglund et al, the Irak-/- mice were shown to have enhanced pro-inflammatory responses and increased T-cell accumulation in the tumor tissue and local lymph nodes (Klimesova et al., 2013). The mechanism associated with the increased sensitivity was correlated with altered commensal microbe metabolic activity in Irak-/- mice, suggesting a difference in the microbiome between the WT and Irak-/- animals (Klimesova et al., 2013). Beyond DSS based models, IRAK-M has also been evaluated in the Il-10-/- model of spontaneous colitis (Biswas et al., 2011). Similar to the findings from the DSS models, loss of IRAK-M resulted in increased TLR signaling, resulting in increased inflammation and expression of pro-inflammatory signaling pathways (Biswas et al., 2011). The Il-10-/- model is highly dependent on the intestinal commensal flora and subsequent germfree studies evaluating Irak-m expression further suggest that Irak-m-/- sensitivity in this experimental colitis model is dependent on the composition of the resident GI microbiota (Biswas et al., 2011). The microbiota composition cannot be underestimated for DSS based models as the severity to DSS colitis can drastically change based on the microbiota composition (Hernandez-Chirlaque et al., 2016). We postulate that differences observed between labs utilizing Irak-m-/- mice in DSS models could be due to altered microbiome compositions or differing percentages and lots of DSS used among labs.

Though our data provides contradictory evidence compared to the previous findings pertaining to IRAK-M, (Berglund et al., 2010), (Biswas et al., 2011), (Klimesova et al., 2013) our data is consistent with a more recent study that also showed Irak-m-/- mice
display reduced tumor burden compared to their WT counterparts in the AOM/DSS model (Kesselring et al., 2016). This was attributed to enhanced epithelial cell barrier function, specifically localized to tumor sites in the GI tract of Irak-\textit{m/}\textendash mice (Kesselring et al., 2016). This was shown to be associated with reduced activity of the oncogene STAT-3. Our findings described in the current manuscript lend support to this conclusion pertaining to Irak-\textit{m/}\textendash mice challenged in the AOM/DSS model (Figure 2.5 and Figure 2.6). We further confirm the findings pertaining to decreased Wnt signaling (Kesselring et al., 2016) as demonstrated by the reduced intracellular β-catenin in our study (Figure 2.6H). Finally, the analysis of human biopsies from this prior study revealed that human patients with increased IRAK-M expression have worse cancer survival (Kesselring et al., 2016), which corroborates our metadata analysis described (Figure 2.1). Collectively, our data pertaining to IRAK-M is complementary to the major findings by Kesserlring et al. Together, these studies extend the mechanistic insight associated with IRAK-M modulation of IBD and colitis associated tumorigenesis.

IRAK-M functions as a negative regulator of TLR and IL-1R1 signaling by either attenuating the IRAK-1/IRAK-4 phosphorylation event or stabilizing the TLR/MyD88/IRAK-4 complex (Kobayashi et al., 2002). Originally, we sought to better define the mechanisms associated with IRAK-M attenuation of inflammation in the context of experimental colitis and colitis associated tumorigenesis. Through the course of our experiments, our \textit{in vitro} data suggested that BMDMs from Irak-\textit{m/}\textendash mice displayed an augmented inflammatory response (Figure 2.7A), which is consistent with the initial reports characterizing IRAK-M and the Irak-\textit{m/}\textendash mice (Kobayashi et al., 2002).
However, we were intrigued after discovering a protein band pertaining to IRAK-M in BMDMs from the *Irak-m<sup>−/−</sup>* mice after treating with specific pathogenic ligands (Figure 2.4). As previously described in the original manuscript reporting the generation of *Irak-m<sup>−/−</sup>* mice, exons 9-11 were targeted for deletion by homologous recombination and the insertion of a neo cassette (Kobayashi et al., 2002). Our genotyping confirms the successful targeting of *Irak-m*. However, as we show in our current studies, a splice variant of the *Irak-m* gene is formed following BMDM stimulation in the targeted *Irak-m<sup>−/−</sup>* animals. The splicing event circumvents the neo cassette and joins exon 8 with exon 12, defined here as *Irak-m<sup>Δ9-11</sup>*. Together, our data suggests that the inclusion of exon 12 in the mRNA effectively stabilizes the transcript. Further functional studies using overexpression systems revealed that this truncation has the potential to robustly activate NF-κB signaling (Figure 2.7E). If this splice variant is present in the *Irak-m<sup>−/−</sup>* mice, then it is possible that the *Irak-m<sup>Δ9-11</sup>* variant could result in potential phenotypes not characteristic of a true knockout mouse. These could include reduced or hyperactive activity for IRAK-M functioning as either a dominant negative or potentially even a dominant positive.

In conclusion, our data strongly suggests that IRAK-M functions to modulate inflammatory signaling pathways and is critical in maintaining immune system homeostasis in the gut. However, increased IRAK-M is associated with increased disease pathogenesis and increased cancer severity in human patients. Our findings in mice revealed that the immune system in *Irak-m<sup>−/−</sup>* animals is primed and highly efficient at eliminating microbes translocating from the GI lumen. This increased microbial
clearance is associated with reduced experimental colitis and colitis associated
tumorigenesis. Together, our data identify IRAK-M as an essential regulator of
inflammation and is critical in the maintenance of mucosal immune system homeostasis
in health and disease.

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Chapter 3:
The Ex Vivo Culture and Pattern Recognition Receptor Stimulation of
Mouse Intestinal Organoids

Published as:

A. Abstract:
While the previous chapter employed conventional in vivo and in vitro techniques to study the role of IRAK-M, the present chapter discusses the utility and potential of optimizing primary intestinal organoids as a model system in mucosal immunology. The complexities of the organoid growth characteristics carry significant caveats for the investigator. Specifically, the growth patterns of each individual organoid are highly variable and create a heterogeneous population of epithelial cells in culture. With such caveats, common tissue culture practices cannot be simply applied to the organoid system due to the complexity of the cellular structure. Counting and plating based solely on cell number, which is common for individually separated cells, such as cell lines, is not a reliable method for organoids unless some normalization technique is applied. Normalizing to total protein content is made complex due to the resident protein matrix. These characteristics in terms of cell number, shape and cell type should be taken into consideration when evaluating secreted contents from the organoid mass. This protocol has been generated to outline a simple procedure to culture and treat small intestinal organoids with microbial pathogens and pathogen
associated molecular patterns (PAMPs). It also emphasizes the normalization techniques that should be applied when gene expression and protein analysis are conducted after such a challenge.

**B. Introduction:**

The ability to harvest and culture primary organoids have been described for small intestine, colon, pancreas, liver and brain and are exciting advances germane to understanding a more physiologically representative phenomena for tissue biology (Sato et al., 2009) (Lancaster et al., 2013). The first methods describing the culture and maintenance of small intestinal organoids was reported by Sato et al. out of the lab of Hans Clevers (Sato et al., 2009). Prior to this method, harvesting and culture of primary intestinal epithelial cells proved to be limited and ineffective in sustaining epithelial cell growth. Methods included dissociation of tissue via incubation with enzymes, such as collagenase and dispase, which would ultimately lead to the outgrowth of intermixed primary fibroblast cells (Freshney and Freshney, 2002). These conditions would also be time restricted in sustaining the epithelial cell culture. Minimal to no epithelial cell niche would form, as the epithelial cells would enter apoptosis due to the lack of appropriate growth factors or loss of contact integrity, termed anokis (Vachon et al., 2002). The advent of the 3D-organoid culture system has provided a method to culture primary intestinal cells containing a spectrum of intestinal cell types in sustained culture (Sato et al., 2009). These epithelial organoids have advantages over cell lines being that they are composed of several differentiated cells, and better mimic the organ they are derived from *in vivo* (Hynds and Giangreco, 2013). The process to ultimately “grow a
“mini gut in a dish” has proven to be a valuable tool for assessing the response of intestinal epithelium under different stimuli. Investigating the interaction of primary intestinal cells with microbial PAMPs is relevant to the field of immunology as these molecular patterns can regulate diverse responses from both host and microbe (Kaiko and Stappenbeck, 2014). Not only can investigators now explore these interactions with mouse organoids, but they can be cultured from humans as well (Sato et al., 2011). This technology has the potential to dramatically alter personalized medicine and it is tempting to speculate about advances that this technique will make possible in the near future.

The overall goal of this method is to provide a protocol for the culture, expansion, and treatment of intestinal organoids with a variety of stimuli. Such stimuli can ultimately range from vaccines, bacterial pathogen associated molecular patterns (PAMPs), live pathogens, gastrointestinal (GI) and cancer therapeutics. This method is focused on describing an adequate technique for proper normalization when working with non-homogenous cell structures, which must be taken into consideration when conducting an assay based on cell number.

C. Materials and Methods:

All research was approved and conducted under Virginia Tech IACUC guidelines

1. Prepare R-Spondin1 Conditioned Media From HEK293T-Rspo1 Cell Line
1.1) Seed HEK293T-Rspondin1 secreting cells at 5-10% confluency in a T-175 flask with 40 ml of growth media and incubate at 37 °C + 5% CO2. Rspondin1 can be alternatively purchased as a recombinant growth factor.

1.2) Grow the HEK293T-Rspondin1 cells for seven days or until reaching 95% confluency determined by bright-field microscopy. Harvest the 40 ml of conditioned media and transfer to a 50 ml conical tube. The T-175 flask of HEK293T-Rspondin1 secreting cells can be discarded.

1.3) Centrifuge at 300 x g for 10 min at 4 °C to pellet cellular debris. Collect the supernatant, termed Rspondin1 conditioned media, and aliquot into 15 ml tubes. Store aliquots at -20 °C for short term or -80 °C for long term storage.

2. Preparation of Organoid Growth Media and Reagents for Harvesting Small Intestinal Crypts

2.1) One-day prior to harvesting, place the frozen protein matrix on ice and thaw overnight at 4 °C. Autoclave dissecting scissors, forceps and glass slides that will be used for crypt harvest.

2.2) The following day, begin by preparing 40 ml of organoid growth media containing supplements and growth factors by adding stock concentrations to 40 ml of 1x Advanced DMEM/F-12. Media supplements contain: 10 mM HEPES - 400 μl, 1x glutamine supplement - 400 μl, 1x Vitamin B27 without vitamin A - 400 μl, 1x N2 - 200
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□ l, 1 mM N-acetyl-cysteine - 40 □ l, 100 ng/ml m-Noggin - 40 □ l, 50 ng/ml m-EGF - 4 □ l
and place in a 37 °C water bath until usage. Warm a 15 ml aliquot of R-spondin1 to 37
°C in a water bath.

2.3) Warm three sterile 24 well plates to 37 °C by placing in an incubator. Prepare 50 ml
per mouse 1x PBS + 2 mM EDTA and cool to 4 °C. Prepare 100 ml per mouse 1x PBS
containing 10% FBS and cool to 4 °C.

3. Harvesting *Mus musculus* Small Intestinal Crypts for Organoid Culture (*Sato et
al., 2009*)

3.1) Sacrifice a male C57B6/J mouse age 6-12 weeks of age raised on standard rodent
chow and water available *ad libitum* according to institutional guidelines. Euthanize via
CO₂ asphyxiation followed by cervical dislocation. Note: Keep carcass on ice until
tissue harvest and perform the tissue harvest in a biological safety cabinet to minimize
the risk of contamination.

3.2) Note: It is optional shave the mouse to remove the fur before submersing in 70%
ethanol.

Submerse euthanized mouse in 70% ethanol for 2 min and pin limbs to pinning board
with the dorsal side of the mouse touching the board.

3.3) Use pre-sterilized dissecting scissors and forceps to make a mid-line incision on
the ventral portion of the mouse. Make the incision at the genitalia proceeding cranial to
the base of the neck. Open the skin incision laterally with tweezers and pin the skin to the pinning board to expose the peritoneum.

3.4) Make a mid-line incision in the peritoneum of the abdomen with dissecting scissors and fold the peritoneum with forceps laterally and pin to the pinning board in order to expose the abdominal organs. Note: take care to avoid cutting abdominal organs.

3.5) Remove the small intestine from the abdominal cavity with dissecting forceps and scissors by cutting the proximal junction of the small intestine connected to the stomach and the distal junction connected to the caecum. Place the small intestine a sterile petri dish containing 10 ml of ice cold 1x PBS.

3.6) Flush the contents contained within the lumen of the small intestine with ice cold 1x PBS using a 1 ml pipette. Repeat this step until the debris within the lumen of the small intestine is removed.

3.7) Cut the small intestine with dissecting scissors into 3-4 approximately equal length strips and lay the strips longitudinally on a new sterile petri dish. For each strip, insert the cutting edge of the dissecting scissors inside the lumen of the small intestine to make an incision the entire length of the strip. Use forceps to laterally fold open the incision in order to expose the lumen of the small intestine.
3.8) Use a sterile glass slide to gently scrape away the villi on the luminal surface of the small intestine. Cut the small intestine into 1-2 cm length strips with dissecting scissors and transfer these strips to a 50 ml conical tube containing 10 ml ice cold 1x PBS.

3.9) Mix the contents gently and allow the tissue contents to settle to the bottom of the 50 ml conical tube. Aspirate the 1x PBS and repeat this three times by washing the tissue with 10 ml of ice cold 1x PBS. On the final wash leave the conical tube on ice for 10 min containing 10 ml 1x PBS and the small intestine tissue segments.

3.10) Aspirate the 1x PBS and add 25 ml of 1x PBS + 2 mM EDTA. Place on a rocking platform at 4 °C or in an ice bucket for 45 min. While the tissue is incubating, label six 15 ml conical tubes “fraction #1-6.”

3.11) After incubation, allow the tissue contents to settle to the bottom of the tube and aspirate 1x PBS + 2 mM EDTA. Add 10 ml of 1x PBS +10% FBS and shake the tube vigorously by hand 10 times.

3.12) Allow the tissue contents to settle to the bottom of the tube. Remove and transfer the supernatant to the tube labeled “fraction 1”. Repeat the 1x PBS + 10% FBS shaking step five times and transfer the supernatant contents in order to the appropriately labeled fraction tube.
3.13) Centrifuge at 125 x g for 5 min. Aspirate the supernatant and resuspend pellets in 5 ml pre-warmed 1x Advanced DMEM/F-12, with no growth factors added.

3.14) Centrifuge at 78 x g for 2 min. Aspirate 4 ml of the supernatant and resuspend each pellet in 1 ml of remaining media.

3.15) Remove 0 µl from each fraction and add to a glass slide. Visualize crypts and debris under a light microscope and determine which fractions to combine and pool accordingly to achieve the greatest percentage of crypts to debris ratio. The authors find that fractions 2-6 yield the greatest percentage of crypts to be plated. The fractions to pool are most often fraction 3 with fraction 4, and fraction 5 with fraction 6.

3.16) Centrifuge fractions to be plated at 125 x g for 5 min. Aspirate supernatant leaving 50-100 µl remaining above the pellet.

3.17) Keep tubes on ice and add 1 ml of protein matrix to the pooled fractions. Pipet up and down slowly to prevent addition of air bubbles.

3.18) Remove pre-warmed 24 well plates from 37 °C incubator and add 50 µl of protein matrix/crypt suspension in the middle of each well. Transfer the seeded plate to a 37 °C incubator for 10 min to allow the protein matrix drop to solidify.
3.19) Add 450 $\mu l$ of previously prepared organoid growth media + 50 $\mu l$ of R-spondin1 conditioned media per well. Incubate plates at 37 °C + 5% CO$_2$ overnight.

3.20) Add 50-100 $\mu l$ of R-spondin1 conditioned media per well daily. Every 3rd day replace entire organoid growth media. The protein matrix drop maintains its integrity for one week, and after 7 days proceed to passaging organoids.

**4. Passaging Organoids Every 7th Day**

4.1) Thaw the protein matrix on ice overnight at 4 °C the day before passaging. Warm one sterile 24 well plate to 37 °C for at least 30 min. Prepare organoid growth media described in step 2.2 and keep at 37 °C until usage.

4.2) Remove organoid plate from incubator and commence passaging of plate on ice.

Aspirate growth media with a 10 ml pipette from 3-4 wells to start.

4.3) In the aspirated wells, pipette up and down in order to dislodge the protein matrix drop from the plate. Gentle scraping with the tip of a 10 ml pipette is helpful in dislodging the protein matrix drop. Transfer the contents to a 15 ml conical tube.

4.4) Incubate the 15 ml conical tubes on ice for 10 min and centrifuge at 125 x g for 5 min at 4 °C. Observe a white pellet at the bottom of the conical tube. Gently
aspirate the majority of the supernatant above the organoid pellet leaving 100-200 μl above the pellet.

4.5) Break up the organoid crypts by using a 23 gauge needle attached to a 1 ml syringe. Aspirate and eject 10-15 times in order to dissociate the crypts. Minimize air bubble formation due to excessive pipetting.

4.6) Resuspend the organoids in 500 μl of protein matrix and add 50 μl per well in a new pre-warmed 24 well plate. Add 450 μl of previously prepared organoid growth media + 50 μl of R-spondin1 conditioned media per well. Incubate plates at 37 °C overnight.

5. Plating Organoids on Day 14 for Pattern Recognition Receptor Stimulation with PAMPs and Listeria monocytogenes for Gene Expression Analysis

5.1) Two days prior to challenge, streak out L. monocytogenes on BHI agar plate.

5.2) The following day pick a L. monocytogenes colony and grow in 10 ml of BHI broth at 37 °C overnight shaking at 200 rpm.

5.3) Thaw the protein matrix on ice overnight at 4 °C the day before organoid challenge. Warm one sterile 24 well plate to 37 °C for at least 30 min. Prepare organoid growth media described in step 2.2 and keep at 37 °C until usage.
5.4) Remove organoid culture from incubator and commence passaging of organoids.

5.5) Aspirate media from 3-4 wells and pipette up and down in order to dislodge the protein matrix drop from the plate. Gentle scraping with the tip of a 10 ml pipette is helpful in dislodging the protein matrix drop. Transfer the contents to a 15 ml conical tube.

5.6) Incubate the 15 ml conical tubes on ice for 10 min. Centrifuge at 125 x g for 10 min at 4 °C. Observe a white pellet at the bottom of the conical tube. Gently aspirate the supernatant leaving approximately 100 μl of residual supernatant behind.

5.7) Resuspend the organoid pellet in 5 ml of ice cold 1x PBS and remove a 10 μl aliquot for counting. Add the 10 μl aliquot to the middle of a hemocytometer without the glass cover slip. Gently overlay the glass coverslip on top of the drop. The authors find that the hemocytometer will clog with organoids if the glass slide is not first removed.

5.8) Count the total number of organoids via a light microscope in every grid/ the entire chamber of the hemocytometer and determine the organoid concentration: number of total organoids counted/10 μl.
5.9) Remove an appropriate volume from the 15 ml conical tube that will allow adequate numbers of organoids to be seeded for the assay and centrifuge this suspension at 125 x g for 10 min. **Note: The authors find that a range between 40-100 organoids per well of a 24 well plate is sufficient for RNA analysis.**

5.10) Aspirate the supernatant and resuspend the pellet in 500 μl of protein matrix without generating air bubbles. Add 50 μl of organoid/protein matrix suspension per well in a 24 well plate. **Note: The amount of protein matrix used to resuspend organoids will vary for the number of treatment conditions and technical replicates.**

5.11) Add 500 μl of organoid growth media (without N-acetyl-cysteine) to each well and incubate at 37°C + 5% CO₂ for 1 hr.

5.12) Prepare 2x concentrations of PAMPs and live *L. monocytogenes*. Measure the OD of live *L. monocytogenes* and streak on a BHI plate for counting. Treat organoids at 1 x 10⁶ CFU/ml. For example, the authors find that an OD of 0.6 ≈ 1 x 10⁹ CFU/ml under typical conditions in their laboratory. The OD to CFU determination will vary by bacteria type and should be assessed prior to organoid stimulation.

5.13) Streak out a serial dilution from the treatment stock of *L. monocytogenes* to accurately determine the treatment CFU/ml. The authors find that a 1 x 10⁸ dilution
of 100 μl on a BHI agar plate is sufficient to count colonies to determine an accurate CFU/ml.

5.14) Prepare a heat killed solution of *L. monocytogenes* by taking a 1 ml aliquot of the live *L. monocytogenes* solution and centrifuge at 5,000 x g for 10 min. Aspirate the supernatant and wash the bacterial pellet with 1 ml of 1x PBS.

5.15) Centrifuge the *L. monocytogenes* at 5,000 x g for 10 min and resuspend the pellet in 1 ml of 1x PBS. Heat kill the *L. monocytogenes* in a 1.7 ml polypropylene tube by heating at 80-90 °C for 1hr. Culture a 100 μl aliquot of the heat killed *L. monocytogenes* on a BHI agar plate to confirm no live bacteria remain.

5.16) Add 500 μl of the appropriate 2x PAMP and/or microbe treatment to 500 μl resident media in designated wells determined by the user. **Adding a 500 μl aliquot of a 2x PAMP/microbe treatment to 500 μl of resident media will bring the treatment concentration to 1x.** Incubate at 37 °C + 5% CO₂ for 24 hr.

5.17) The following day count *L. monocytogenes* colonies for an accurate determination of treatment CFU/ml.

5.18) Aspirate media from the plate of treated organoids and wash wells three times with 1x PBS.
5.19) Proceed to RNA extraction via phenol/chloroform (Chomczynski and Sacchi, 2006) or a RNA extraction kit according to manufacturers instructions. Evaluate gene expression using standard qRT-PCR (Allen et al., 2011).

6. Plating Organoids on day 14 for PAMP and L. monocytogenes Challenge for Protein Analysis in Supernatant

6.1) Two days prior to challenge start a cell culture of Caco-2 cells in a T-75 flask with 1x DMEM + 10% FBS and incubate the Caco-2 cells at 37 °C + 5% CO₂. Begin a bacterial culture of L. monocytogenes on BHI plate and incubate plate in a bacterial incubator at 37 °C.

6.2) The following day pick a L. monocytogenes colony and grow in 10 ml of BHI broth at 37 °C overnight. Thaw the protein matrix on ice overnight at 4 °C the day before organoid challenge.

6.3) The following day warm one sterile 96 well black-walled plate to 37 °C for at least 30 min. Prepare organoid growth media without N-acetyl-cysteine described in step 2.2 and keep at 37 °C until usage. Remove organoid culture from incubator and commence passaging of organoids.

6.4) Aspirate media from 3-4 wells and resuspend with a pipette in order to dislodge the protein matrix drop from plate. Gentle scraping with a 10 ml pipette is helpful in dislodging the protein matrix drop. Transfer the contents to a 15 ml conical tube.
6.5) Incubate the 15 ml conical tubes on ice for 10 min. Centrifuge at 125 x g for 10 min at 4 °C. Observe a white pellet at the bottom of the conical tube. Gently aspirate the supernatant leaving 100 μl of residual supernatant behind.

6.6) Resuspend the organoid pellet in 5 ml of ice cold 1x PBS and remove a 10 μl aliquot for counting. Add the 10 μl aliquot to the middle of a hemocytometer and gently overlay the glass coverslip. The authors find that the hemocytometer will clog with organoids if the glass slide is not first removed.

6.7) Count the total number of organoids via a light microscope in every grid of the hemocytometer/the entire chamber and determine the organoid concentration:

\[
\text{number of total organoids counted/10 } \mu \text{l.}
\]

6.8) Remove a appropriate volume from the 15 ml conical tube that will allow adequate numbers of organoids to be seeded for the assay and centrifuge this suspension at 125 x g for 10 min. Note: The amount of protein matrix used to resuspend organoids will vary for the amount of treatment conditions and technical replicates. The authors find that 40 - 100 organoids is sufficient for secreted protein analysis.

6.9) Aspirate the supernatant and resuspend the pellet in 500 μl of protein matrix/well without generating air bubbles. The amount of protein matrix used to
resuspend organoids will vary for the amount of treatment conditions and technical replicates.

6.10) Leave at least two columns empty on the 96 well black-walled tissue culture plate as these will serve as wells seeded with Caco-2 cells for the generation of a standard curve. Add 50 μl of protein matrix + organoid suspension per well to a pre-warmed 96 well black-walled tissue culture plate. Store the plate at 37 °C for 10 min to allow protein matrix to solidify.

6.11) Add 100 μl of organoid growth media (without N-acetyl-cysteine) to each well and incubate at 37 °C + 5% CO₂ for 1 hr.

6.12) Prepare 2x concentrations of PAMPs and live *L. monocytogenes*. Add 100 μl of each given treatment condition per well and incubate for 24 hr. **Incubation and Treatment times to be determined by the investigator.**

6.13) The following day plate Caco-2 cells in the standard curve wells. Begin by warming sterile 1x PBS, 0.25% trypsin and 1x DMEM + 10% FBS to 37 °C.

6.14) Remove the T-75 flask containing Caco-2 cells and aspirate the cell culture media. Wash the cells with 10 ml 1x PBS. Aspirate the 1x PBS, add 2 ml 0.25% Trypsin and place T-75 flask in 37 °C incubator for 15 min.
6.15) Visualize the flask under a light microscope to ensure the cells have detached then add 8 ml of 1x DMEM + 10% FBS to the detached Caco-2 cells and transfer into a 15 ml conical tube. Remove a 10 μl aliquot and count the cells by light microscopy using a hemocytometer. Note: The authors find that a upper cell number of 60,000 cells/well work well and dilutions can be conducted for generation of the standard curve down to 2500 cells/well.

6.16) Resuspend the Caco-2 cells in protein matrix and add 50 μl per well to appropriate wells. Allow the protein matrix to solidify at 37 °C for Caco-2 cells.

6.17) Following the treatment time for the organoid assay, aspirate and save the organoid cellular supernatant media and keep on ice. Wash the wells three times with 1x PBS. Add 100 μl of 100% methanol and incubate at 4 °C or on ice for 20-30 min to fix the organoids.

6.18) Following the methanol incubation, wash the wells three times with ice cold 1x PBS. The plate can now be stored at 4 °C for 1-2 weeks.

6.19) Add nuclear staining dye at a concentration of 1 μg/ml per well, cover the plate with aluminum foil and incubate at 4 °C overnight.
6.20) Use a 96 well plate reader to measure nuclear staining dye excitation/emission (350nm/461nm respectively) and normalize each organoid well to the standard curve of Caco-2 cells.

6.21) Proceed to downstream assays, such as ELISA (Allen et al., 2011), of cell culture supernatant normalizing to cell number.

D. Results:
When following this protocol to cultivate intestinal organoids, characteristic sphere shaped organoids will be present after harvesting. The addition of R-spondin1 conditioned media daily will initiate the growth and budding of the organoids. The growth of organoids is shown in Figure 1 (A-F), and is representative of intestinal organoids on days 1, 2, 4, 5, 6 and day 14. Figure 1F represents the non-homogeneous growth characteristics of organoids on day 14 of PAMP challenge. Once the organoids are grown to an adequate number they can be replated and challenged with various PAMPs and/or microbes. Expression analysis can be performed via standard techniques. This is represented in Figure 2 (A-C) which shows the mRNA expression of inflammatory cytokines IL-18, IL-6, and TNF-α which were analyzed following a 24 hr challenge of organoids with heat killed L. monocytogenes and the PAMP flagellin. Figure 3 (A-C) demonstrates the relative nuclear staining of intestinal organoids with nuclear staining dye following fixation with minimal background staining of debris in the resident protein matrix.
This method of fixation and staining can be applied to normalize assays, which will account for differing cell numbers in each well. This is apparent in Figure 4 when generating a standard curve of serial dilutions of Caco-2 cells plated in protein matrix, then fixed and stained with nuclear staining dye. The $R^2$ value of 0.89 indicates a linear relationship between cell number and mean fluorescent intensity, and the linear equation can be used to normalize organoids to cell number.

Figure 3.1
**Figure 3.1. Small Intestinal Organoid Growth**

Time course of growth for murine small intestine derived organoids following isolation. 


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**Figure 3.2**

A. WT Organoids IL-18 Expression

B. WT Organoids IL-6 Expression

C. WT Organoids TNFα Expression
Figure 3.2. mRNA Expression of Inflammatory Cytokines Following 24 hr PAMP Challenge

Relative mRNA expression of wild-type intestinal organoids challenged for 24 hr with PAMPs and heat killed *Listeria monocytogenes*. A-C) represent fold change of the inflammatory cytokines IL-18, IL-6 and TNF-α respectively.

Figure 3.3
Figure 3.3. Relative Fluorescent Staining of Organoids for Normalization

Nuclear staining of organoids with following fixation. **A)** Bright field of organoids. **B)** Fluorescent staining of organoids with nuclear staining dye. **C)** Merged image of bright field and fluorescent staining.

Figure 3.4

**Standard Curve of Caco-2 Cells**

Standard curve generated from triplicate wells. Cell seeding had a range starting at 60,000 cells per well with dilutions down to 2,500 cells per well.
Figure 3.5

1. Harvesting R-spondin conditioned media. 2. Harvesting small intestinal crypts, and growing organoids. 3. Plating organoids, followed by PAMP treatment, and normalization.
E. Discussion:

The culture and maintenance of intestinal organoids is a procedure that can be mastered by any individual with adequate tissue culture technique. There are subtleties in passaging when compared to growing cells in a more conventional monolayer, but these subtleties are not difficult to overcome. The critical steps of this method involve being able to grow the organoids to a high enough density for optimal seeding. Experiments must be scaled down with organoids as large seeding densities that can commonly be achieved with cell lines are not practical. This becomes especially apparent when there are multiple treatment groups.

This protocol is intended to provide a step-by-step method to study host-pathogen interactions of the intestinal epithelium with a variety of different bacterial, viral, and fungal pathogens, as well as address difficulties using this system with respect to normalization. Reports are available that describe the interactions of intestinal organoids with the bacterial pathogen *Salmonella*, yet do not address normalization methods when measuring secreted cytokines (Zhang et al., 2014). There are several difficulties that are encountered when normalizing organoid cultures by different methods. Normalizing secreted protein via BCA is an option; however, the growth components required for organoid culture (N2 and/or Vitamin B27) interfere with the BCA assay (data not shown). Normalizing via cellular viability, such as a modified MTT assay has been described (Grabinger et al., 2014); however, a treatment that will alter the mitochondrial metabolic activity of the organoids will introduce an inaccurate method for normalization via this technique as MTT is based on reduction by the action of
mitochondrial dehydrogenases (Slater et al., 1963). It is also necessary to remove N-acetyl-cysteine from the media if normalization via the MTT technique is desired.

The authors find that fixing with methanol and staining nuclei with nuclear staining dye is an effective normalization technique against a standard curve of Caco-2 cells. Although the growth characteristics of this colon cancer cell line do not exactly mimic the growth characteristics of intestinal organoids, using a nuclear stain and measuring mean fluorescence intensity is a good strategy to normalize against total cell number. Taken together, the technique described here provides a good starting point to mimic host-pathogen interactions with adequate normalization that is essential for making accurate interpretations using this model system. The significance of this technique with respect to alternative methods is that proper normalization must be performed when conducting any evaluation of secreted protein, such as ELISA based assays.

F. References:


Chapter 4:

NF-κB inducing kinase (NIK) is essential for adequate expression of the stem cell marker LGR5 and results in enhanced proliferation of intestinal epithelial cells.

A. Abstract

In the previous chapter, we discussed an optimized method for the culture and stimulation of intestinal organoids. Here, we utilized this method further to study intestinal epithelial cell proliferation. Intestinal epithelial cell (IEC) proliferation is required to maintain homeostasis throughout the gastrointestinal (GI) tract. IECs act as a protective barrier from the external environment within the GI lumen, exhibiting rapid turnover and thus maintaining the integrity of the barrier. Cellular renewal relies on several signal transduction pathways working in synchronization; and aberrancies in this regenerative process contribute to cancer initiation within the intestine. The non-canonical NF-κB signaling pathway is emerging as an important pathway that aids in adequate proliferation of intestinal epithelial cells. Here, we investigated the contribution of the non-canonical NF-κB pathway to IEC proliferation in a mouse model deficient for NF-κB inducing kinase (NIK). In the absence of NIK, IEC organoids demonstrated defects in proliferation, consistent with limited expression of the intestinal stem cell marker, LGR5. Our results demonstrate the essential role of NIK to proper IEC proliferation, and ultimately GI maintenance.
B. Introduction

Epithelial cells lining the small intestine and colon within the GI tract undergo rapid turnover compared to those residing in other tissues. For example, epithelial cells composing the skin turnover on a 47-48 day basis while, in contrast, intestinal epithelial cells turnover every 4-5 days (Iizuka, 1994), (Creamer et al., 1961). This rapid renewal process is associated with protection from various diseases, such as colon cancer, since increased turnover on a bi-weekly basis prevents cells from accumulating mutations due to toxic substances encountered within the GI tract. Intestinal epithelial cells differentiate from intestinal stem cells (ISC) at the base of the intestinal crypt and migrate up the villus in single-file manner. Upon reaching the apex of the villus, IECs undergo apoptosis and are subsequently shed into the intestinal lumen to be excreted. This consistent and rapid renewal is important in protecting the GI tract from the hostile environment within the lumen of the intestine. In the absence of constant IEC renewal, any breach in the intestinal epithelial barrier may lead to deleterious effects. Such consequences may include endotoxic shock due to the plethora of bacterial pathogen associated molecular patterns (PAMPS) that stimulate surrounding immune cells. Furthermore, neutrophils responding to local invaders produce vast quantities of reactive oxygen species (ROS), which can be genotoxic to surrounding cells.

The non-canonical, or alternative NF-κB signaling pathway has been shown to be vital for the proper development of secondary lymphoid organs, as well as proper B-cell maturation (Claudio et al., 2002). This alternative NF-κB pathway is activated
downstream of TNF-family receptors, including BAFF, LTβR, and CD40; thus, absence of this pathway results in immunodeficiency of the adaptive immune system. One crucial molecule that propagates downstream signaling when this pathway is stimulated is NF-κB inducing kinase (NIK). In quiescent cells, NIK is constantly poly-ubiquinated and degraded, thus maintaining the level of NIK within the cell at low levels. Following a stimulus, NIK is stabilized, and levels rise within this cell, allowing NIK to act as a functional kinase on its substrate IKKα, which ultimately leads to NF-κB activation, primarily through RelB-p52 heterodimers. Recently, it has been demonstrated that the silencing of NIK in the surrounding stroma of the intestine results in increased metastasis of colon cancer cells to distant sites in the body (Maracle et al., 2018). The purpose of this study was to investigate the role of NIK in relation to the GI system. In vivo, NIK maintains GI homeostasis by preventing aberrant cellular hyperplasia and inflammation. When cultured ex vivo, NIK is required for proper IEC proliferation. Our results demonstrate that loss of Nik results in decreased IEC turnover due to reduced expression of the stem cell receptor LGR5.

C. Materials and Methods

Reagents

Advanced DMEM F12 media, N2 supplement, B27 without Vitamin A, HEPES, Glutamax supplement were purchased from ThermoFisher Scientific. HEK-293T-Rspondin1 cells were cultured as previously described (Kim et al., 2005), and utilized to generated R-spondin 1 conditioned media previously described (Rothschild et al., 2016). L- cells were purchased from ATCC and utilized to generate Wnt3a conditioned
media as described by ATCC. qRT-PCR primers for mouse LGR5 and KRT20 were purchased from TheromFisher Scientific. Antibodies for EphB2, EpCAM4, anti-mouse APC, anti-donkey Alexaflur488 were purchased from ThermoFisher.

**Colonic Organoid Isolation and Culture.**

Colons from age matched 6-12 week old C57BL6J and Nik-/- mice were dissected separately and colonic crypts were isolated as previously described (Sato et al., 2011). For each genotype, approximately 1000 crypts were plated in each well of a 6-well tissue culture (TC)-treated plate, containing in 100ul matrigel per well. Matrigel was allowed to solidify for 10 minutes in a 37°C TC incubator. Organoids were cultured 900ul basal media containing: 1x N2, 1x B27 without vitamin A, 10mM HEPES, 1x Glutamax supplement, 100ng/ml m-Noggin, 50ng/ml m-EGF, 1mM N-acetyl cysteine, 25nM Y27632, and 1x Penicillin/Streptomycin. An additional 100 ul R-spondin1 conditioned media and 100 ul Wnt-3a media was added to each well during seeding (day 0) for initial culturing. After 24 hours, the culture media was aspirated, each well was washed with warm 1x PBS without Calcium/Magnesium; fresh medium without Y27632 was then added to each well. Following day 2, 100ul R-spondin 1 and 100ul Wnt-3a media was added to each well daily. The medium was completely replaced every 3 days.

**Growth tracking of organoids**

Following 7 days of culture, organoids for each respective genotype were passaged and single cell suspensions were made. This was accomplished by scraping and
transferring matrigel drops to a 50ml conical tube. Tubes were allowed to incubate on ice for 15 min, and were subsequently centrifuged at 300g for 10 min. PBS was aspirated and matrigel pellets were manually disrupted using a syringe with a 22G needle. Organoids were centrifuged again and pellets were incubated for 10 min at 37°C with 1x TrypLE, centrifuged and TrypLE was aspirated. Cells were then passed through a 70 um cell strainer to make single cell suspensions. Cells were counted, assessed for viability with Trypan blue, and plated at 20,000 cells per well in a 6 well dish. Single cells were tracked for growth over the next 6 days. Media was changed each day, and each genotype received the same media, media volume, and concentration of organoid growth factors.

**qRT-PCR**
Colon crypts were isolated as previously described (Sato et al., 2011). Crypts were pelleted by centrifugation and harvested for RNA using Zymogen quick RNA isolation kit. 2μg RNA was converted to cDNA using high-capacity cDNA reverse transcription kit according to the manufacturer’s instructions. 50ng cDNA was plated in each well as a template, and amplified using TaqMan gene expression master mix according to the manufacturer’s instructions.

**FACS**
Single cell suspensions of colonic crypts were made by first isolating colonic crypts. Crypts were incubated for 20 min at 37°C in TrypLE (ThermoFisher). Crypt digests were centrifuged and resuspended in 1x DMEM + 10 % FBS and passed through a 70 μm
cell strainer. Single cell suspensions were stained with respective antibodies for 30 min at 4°C, and washed with 1x PBS before FACS sorting.

**Statistical Analysis**

Graphs are represented as the mean ± standard error of mean (S.E.M.). Graphs and statistical analysis were conducted via GraphPad PRISM software. Data sets were analyzed by Mann-Whitney U test, and statistical significance was determined using a P value < 0.01 unless otherwise indicated.

**D. Results**

**NIK is needed for proper proliferation of colonic organoids.**

Previous studies have demonstrated that the Wnt signaling pathway is critical when culturing intestinal organoids *ex vivo* (Barker et al., 2007). Furthermore, a correlation has been observed between inflammation driven by the classical NF-κB pathway and colon cancer (Greten et al., 2004). Here we tested the alternative NF-κB pathway in relation to colonic organoid proliferation by utilizing *Nik/-* mice. In order to assess whether NIK influences colonic proliferation, we cultured colonic crypts from *Nik/-* mice *ex vivo* for one week using both wild-type and *ApcMin* crypts as negative and positive controls, respectively (Figure 4.1A). As anticipated, *ApcMin* crypts proliferated at an accelerated rate compared to those derived from wild-type mice. To our surprise, *Nik/-* crypts proliferated, however the proliferation rate was drastically reduced when compared to both wild-type and *ApcMin* crypts. This was observed both qualitatively
using microscopy (Figure 4.1A), and quantitatively when organoid diameter was measured after one week (Figure 4.1B).

**Figure 4.1** A. Growth tracking of organoids from single cell suspensions of wild-type, Apc<sup>Min</sup>, and Nik<sup>−/−</sup>. Images are representative for day 1-6. Scale bar = 100 µm B. Quantification of organoid diameter following one week of growth. 30 organoids were measured in a similar manner for both wild-type and Nik<sup>−/−</sup> from randomly selected tissue culture wells. Apc<sup>Min</sup> quantification was omitted to not obscure the graph. Experiments were repeated a minimum of two times with similar results.

Colonic organoids from Nik<sup>−/−</sup> mice have impaired proliferation due to reduced Lgr5 expression and enhance Krt20 expression.

In order to determine the cause of the reduced proliferation of Nik<sup>−/−</sup> colonic organoids, stem cells derived from each of the different strains were characterized. This was accomplished by evaluating the expression of Lgr5, a crucial receptor and marker of intestinal stem cells (ISC), from freshly isolated colonic crypts, and evaluating gene
expression by qRT-PCR. Interestingly, we found \textit{Lgr5} levels of \textit{Nik}/- crypts to be significantly reduced compared to the wild-type crypts (Figure 4.2A).

![Figure 4.2 A. Relative expression of \textit{Lgr5} from wild-type and \textit{Nik}/- colonic crypts. B. Relative expression of \textit{Krt20} from wild-type and \textit{Nik}/- colonic crypts. For both figures: wild-type n=3 mice, \textit{Nik}/- n=3 mice. \textit{Gapdh} was used as the housekeeping gene and experiments were repeated a minimum of two times with similar results.]

A reduction in the expression of \textit{Lgr5} suggests either that the number of stem cells from \textit{Nik}/- is lower compared to the wild-type, or the amount of receptor on a per stem cell basis is drastically reduced. We also analyzed the expression of \textit{Krt20}, which has been shown to increase in expression to correlate with differentiated IEC, as opposed to ISC (Merlos-Suarez et al., 2011). A greater expression of \textit{Krt20} was observed from \textit{Nik}/- crypts (Figure 4.2B). Collectively, these expression results suggest that in the absence of NIK, not only are stem cell pools reduced, but the differentiated intestinal
epithelial cell lineages are greatly increased. These data provide a possible mechanistic explanation for the reduced proliferation of the *Nik*−/− colonic organoids.

**Single cell suspensions of individual ISC from *Nik*−/− crypts yields reduced organoid colonies**

In order to determine whether NIK plays a role in not only stem cell number, but in proliferation as well, we tested single cell suspensions from harvested colonic crypts. As was consistent with our previous findings, this experiment yielded a reduction in the total number of colonic organoid colonies from *Nik*−/− crypts (**Figure 4.3A**). This suggests that the stem cell pool from *Nik*−/− crypts are reduced, thus leading to a reduction in the number of individual organoid colonies that were derived from single stem cells. To further evaluate the difference in stem cell number between wild-type and *Nik*−/− stem cells, we performed FACS analysis on the ISC surface marker EphB2 and the IEC marker EpCAM1 as shown by Merlos-Suárez *et al.* (**Figure 4.3B**). The results, though subtle, do display altered populations in the EphB2-high pool and EpCAM1-high pool between wild-type and *Nik*−/− crypts. Separate gating strategies are indicated to assess the differences between these two groups.
Figure 4.3 A. Total organoid colonies from single cell suspension made after one week of culturing colonic organoids. B. FACS analysis of single cell suspensions made from both wild-type and Nik−/− colonic crypts. Cells were stained for EphB2 and EpCAM. A separate gating strategy is depicted as previously described by Merlos-Suárez et al. to emphasize the ISC pools between the genotypes. Experiments were performed once.
E. Discussion

One major role of the NF-κB pathway is to enhance cellular survival by driving anti-apoptotic mechanisms (Karin, 2009). The ability of the NF-κB pathway to act as an oncogene is possible, yet rare, and limited to certain types of leukemia (Karin, 2009). Evidence suggests that the classical and alternative NF-κB pathways are capable of crosstalk between each other, with NIK having the ability to stimulate the classical pathway (O’Mahony et al., 2000), (Ramakrishnan et al., 2004). Based on our ex vivo culture of Nik-deficient colonic organoids, reduced growth and proliferation may result from globally reduced stimulation of the classical NF-κB pathway as a direct consequence of the loss of NIK. Reduced cellular survival, and increased apoptosis would be expected with reduced classical NF-κB activation, similar to the phenotype observed in Nik-deficient organoids. However, reduced Lgr5 expression in Nik-deficient organoids suggests a mechanism that may involve the collaboration between the alternative NF-κB pathway and the Wnt signaling pathway, or some component of the alternative pathway that directly contributes to the reduced expression of LGR5.

The discovery of LGR5 as the receptor responsible for contributing to ISC maintenance has greatly advanced our understanding of intestinal stem cell biology (Barker et al., 2007). Not only is Lgr5 a Wnt responsive gene, but changes in Lgr5 expression suggest the Wnt signaling pathway has been altered in the intestine (Huels and Sansom, 2017). It was recently shown that both R-spondins and Wnt ligands are both necessary to maintain ISCs and allow them to differentiate into IECs that compose the crypt-villus
architecture (Yan et al., 2017). Yan et al. demonstrated that when Wnt ligands are absent, R-spondins are unable to compensate for the loss of Wnt signaling, resulting in crypt death. Conversely, if R-spondins are lost, Wnt signaling is temporarily sufficient for crypt maintenance; however, the LGR5 stem cell pool is lost, and the crypt eventually dies. A similar phenomenon may explain the results observed in Nik deficient organoids; one possible explanation is that there may be alternative sources of secreted R-spondins other than epithelial cells in vivo, thereby promoting crypt survival.

Previous studies have shown NIK to be an essential protein kinase that participates in the activation of NF-κB downstream of TNF, CD95, and IL-1 receptors in the alternative NF-κB pathway, (Malinin et al., 1997). Furthermore, Nik deficiency in mice leads to reduced B-cells numbers in the lymph nodes and spleen, which also contributes to impaired IgA production (Brightbill et al., 2015). Since IgA is the predominant class of antibody to be produced in mucosal tissues, one possibility for the alternative responses observed in vivo and ex vivo could be explained by reduced IgA production in NIK deficient mice. An impaired IgA response may ultimately contribute to a disrupted intestinal microbiome, which may perpetuate an enhanced inflammatory state within the intestinal system. Since IgA coating has been shown to predict the bacterial populations that contribute to colitis in mice (Palm et al., 2014), it is possible that loss of IgA coating, and ultimately immune defenses, may perpetuate a hypersensitive state in the GI tract. Our studies demonstrate NIK to be a pleiotropic molecule that is of critical importance to IEC homeostasis; therapeutic targeting of NIK may be eventually used for treatment of GI-related maladies.
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Chapter 5
Discussion and Future Directions

5.1 The Contribution of IRAK-M to GI Immunology

Our findings pertaining to the contribution of IRAK-M were slightly unexpected. One would anticipate that absence of a negative regulatory protein of the inflammatory cascade would lead to a runaway inflammatory effect in the host, as well as contribute to increased carcinogenesis, because increased inflammation can lead to increased cancer, especially in the colon (Greten et al., 2004). However, in DSS-induced colitis and AOM/DSS-induced colitis-associated tumorigenesis studies, it was apparent that absence of IRAK-M was actually protective in both of these respective models. In our hands, \textit{Irak-m/-} mice display decreased morbidity and mortality when challenged in the acute DSS colitis model. Our mechanistic data explaining this discrepancy suggests that \textit{Irak-m/-} mice have increased inflammation following DSS challenge, but also enhanced innate immune functions, particularly with neutrophils. Furthermore, the innate immune function of these neutrophils greatly contributes to and counteracts the destructive tissue damage that occurs from bacteria, which breach the intestinal barrier following DSS challenge. We propose that this enhanced state of immunity present in \textit{Irak-m/-} mice also signals the adaptive immune system, mainly the T-lymphocytes, to be in a heightened activation state. Following this logic, it is likely that cells of the adaptive immune system from \textit{Irak-m/-} mice contribute to the anti-tumorigenic state displayed in these animals following AOM/DSS challenge, as T cells are known to destroy cancerous cells (Martinez-Lostao et al., 2015). An alternative explanation is that it is likely that difference in the microbiome in the GI tract between \textit{Irak-m/-} mice and
the wild-type mice plays a role in the differences observed in the acute colitis model. In fact, when an antibiotic cocktail of ampicillin, vancomycin, neomycin, and metronidazole were given to mice for two weeks prior to DSS challenge, both wild-type and \textit{Irak-m-/-} mice displayed similar morbidity and mortality following DSS administration. This suggests that specific components of the microbiota contribute to the protective phenotype displayed in \textit{Irak-m-/-} animals. Metabolomics of specific bacterial products produced in the GI tract could help explain the discrepancies observed between our laboratory and others that have utilized these mice.

5.1.1 The Significance of the \textit{Irak-m}^{Δ9-11} Truncation Mutant

The central dogma of biology is that cells contain DNA, and the genes that are expressed from DNA are transcribed into mRNA; further, mRNA is then translated into proteins that carry out the functional roles in the cell. At the transcription level, specifically in eukaryotes, mRNA is processed in a “cut and paste” fashion in the nucleus by a small nuclear RNA (snRNA)-protein complex called the spliceosome (Berget et al., 1977). The major function of the spliceosome is to process newly transcribed mRNA into fully mature mRNA that then leaves the nucleus and encodes for proteins in the cytosol. In eukaryotes, not all of the nascent mRNA that is transcribed in the nucleus is encoded into proteins. Exons of the mRNA template, which only make up a small fraction of the encoded mRNA, are spliced together to form much smaller mature mRNA, while introns are removed from the mRNA template and degraded (Berget et al., 1977). As such, a single gene can encode for many altered versions of a protein based upon which exons are spliced together, and are termed splice variants.
One unexpected and interesting finding was that the commercially available \textit{Irak-m/-} animals form a spliced \textit{Irak-m} mRNA that joins exons 8 with exon 12. Murine \textit{Irak-m} is located on chromosome 10, and contains 12 total exons that form the coding region for the \textit{Irak-m} gene. There are 5 naturally occurring \textit{Irak-m} splice variants, and only two form fully encoded proteins, using 1-12 and exons 2-12 to encode for splice variant proteins, respectively (ensemble.org). The truncation mutant that is present in \textit{Irak-m} mutant animals is strictly a result of the knockout strategy used by the scientists that generated these animals, and is not a normal splice variant that is known to occur in wild-type mice. This was unexpected, because the first reports describing this knockout mouse did not describe exon 12 being present in the mRNA transcript that was seen via northern blot (Kobayashi et al., 2002). The relevance to having exon 12 present in this transcript lies in the potential to encode a possibly stable truncated IRAK-M protein.

There are several factors that contribute to the stability of mRNA inside the cell, and one factor for stability that contributes to mRNA translation is the addition of a polyadenylation (poly-A) tail to the 3’ end of the mRNA. Since exon 12 is the last naturally occurring exon for the \textit{Irak-m} gene, the poly-A tail is likely contained in the mRNA transcript, and it is therefore equally likely that the addition of this exon contributes to the stability of this spliced transcript for protein translation. Therein lies the problem for a truncated protein to be encoded from this transcript, which may function in an unknown mechanism inside the cells. As it happens, the exons that were targeted for deletion, being exons 9 through 11, encode a portion of the kinase domain of IRAK-M. Interestingly, the current understanding for the function of IRAK-M describes
this molecule as a non-functional kinase, so a mutation was targeted in this gene in an
already non-functional domain. Further, even more relevance is apparent because the
mechanism for IRAK-M has been described that demonstrates this protein’s function is
mediated through protein-protein interactions with other molecules, namely IRAK-1 and
IRAK-4 (Wesche et al., 1999), (Zhou et al., 2013). This mainly occurs through death
domain- death domain interactions with other proteins, and the death domain for IRAK-
M is encoded at the 5’end (N-terminal) region of this protein, which would be present in
a protein encoded from the resulting *Irak-mΔ9-11* splice variant.

Though this finding is novel and poses interesting questions into the functional role for
IRAK-M, it still remains highly speculative as to whether a protein is present and
functional. The fact that these *Irak-m* mutant mice display an alternative phenotype
compared to wild-type animals demonstrates the successful mutation of this gene. It is
highly likely that the mutation generated to make this mouse was successful in creating
a null *Irak-m* allele; however, research is still required to fully validate whether or not this
is the case. A strategy that could be used to validate whether a truncated IRAK-M
protein is present in these animals, would be to generate an antibody that is specific for
the N-terminal domain of IRAK-M, as most of the current commercially available
antibodies target the C-terminal region of this protein. Then, a simple western blot could
be conducted to validate if a protein is or is not present in these mice. Of the five
commercially available antibodies specific for murine IRAK-M, only one of these
antibodies were successfully validated in our laboratory. This was conducted by cloning
wild-type IRAK-M, along with a C-terminal HA tag, into a plasmid containing the strong
mammalian CMV promoter. In this fashion, wild-type IRAK-M will be highly expressed when transfected into HEK293T cells. Unfortunately, the antibody that was successfully validated will not provide answers as to whether a IRAK-M\(^{\Delta 9-11}\) protein is present in the mutant mouse strain, because the epitope that this antibody recognizes is encoded in exons 9 and 10.

### 5.1.2 Studies to Overcome the Truncation Mutant Conundrum

An alternative strategy, without the use of antibodies and western blot would be a genetic approach. An option would be to test whether mice that are heterozygous for this mutation have similar inflammatory phenotype as wild-type mice or the Irak-m/−mice. Assuming a truncated protein is present, the heterozygous mouse will display similar responses to the knockout mouse if the mutation created is in fact a dominant mutation. Alternatively, if the mutation generated is a null mutation, then the resulting wild-type allele from the heterozygous mice will function in a similar manner to the homozygous wild-type mouse.

Overall, our results, along with other laboratories demonstrate that IRAK-M is an important intracellular signaling molecule with complex functions. Our research appears to demonstrate that the function of IRAK-M following TLR ligation is TLR specific. Our results suggest that some, but not all of the murine TLRs, when stimulated with respective ligands displayed significant differences in IL-6, TNF, and IL-10. These included TLR-2, TLR-7 and TLR-9, and all of these respective TLRs utilize the MyD88 dependent pathway exclusively. One possibility is that that these particular TLRs signal
through similar NF-κB dimers, recall that NF-κB can form up to 15 combinations of dimers, and each dimer can have slightly alternating functions (Zhang et al., 2017). This also could be due to the concentration and treatment time of the particular PAMPs utilized to stimulate the cells. This poses an interesting question related to dose response and receptor threshold limits. The complexity of cellular signaling is highlighted in many aspects of dosage, timing and kinetics, because stimulation of a cell can result in early activation, late activation, and repressive responses at the gene level (Wang et al., 2012). An interesting avenue to potentially pursue would be to determine the precise mechanistic role for IRAK-M when specific TLRs are activated. This has already been done in the case of TLR-7 (Zhou et al., 2013); however, investigation of TLR-9 and TLR-2 would provide additional interesting insight into the mechanistic functions of IRAK-M.

5.1.3 Therapeutic Targeting of IRAK-M and the Clinical Implications

Our findings implicate IRAK-M to be an candidate molecular target for patients afflicted with inflammatory maladies, such as IBD and inflammation driven colorectal cancer. IRAK-M appears to be a molecule that shifts the balance toward host protection in both acute colitis and inflammation driven tumorigenesis models. Though more mechanistic research is required to fully elucidate the functions of IRAK-M, it is tempting to envision a therapeutic strategy targeting IRAK-M in patients that currently suffer from, or are predisposed to IBD and inflammation driven colorectal cancer. Based on our findings, acute inhibition of IRAK-M would be an optimal therapeutic approach to treat patients
with such maladies. Though no molecular inhibitors for IRAK-M currently exist, compounds that inhibit the interaction of IRAK-M with other death domain containing proteins would be a potential strategy. Another interesting approach could be to inhibit IRAK-M and the mRNA level with a DNAzyme approach. DNAzymes are catalytically active antisense strands of DNA that will bind to sense mRNA targets, and cleave them for degradation (Burgess, 2012). Designing a DNAzyme specific for IRAK-M mRNA could be utilized as a strategy to block the function of IRAK-M with minimal off target effects, due to the rationale that IRAK-M is expressed in a cell type specific manner, and is induced following cellular activation by transcription factors like NF-κB and AP-1, as opposed to being constitutively transcribed (Lyroni et al., 2017). From our results, one would anticipate that utilizing this approach would lead to a reduction in colitis, and even prevention of colitis associated tumorigenesis in patients with active tumors.

IRAK-M is an interesting target for modulating the immune system, specifically via inhibition, because it does not appear to be as essential of an inhibitory molecule when compared to other negative regulatory molecules of inflammation like A20. Absence of IRAK-M, at least acutely, does not result in severe multi-organ inflammation and tissue damage, as is the case in mice with the loss of A20 (Lee et al., 2000). Therefore, if this molecule is nonfunctional in mutant Irak-/- animals, complete inhibition of IRAK-M may result in similar therapeutic benefits, as our results have demonstrated in the Irak-m mutant mice. Further, IRAK-M may be a key molecular target for patients suffering from life-threatening infections. One can envision a therapeutic strategy that would aim to inhibit the function of IRAK-M, which would result in heightened immunological functions
of the immune system. This could be a novel strategy to treat patients with a suppressed immune system, as is common under conditions of sepsis, as well as elderly patients with infectious pneumonia. IRAK-M appears to be a novel molecule for this type of approach, because its inhibition results in enhanced bacterial clearance, without negative consequences, such as destructive tissue damage.

5.1.4 IRAK-M Targeted Therapies

Recent technologies have emerged that are very promising for gene therapy; specifically, with enzymes that can be manipulated to efficiently edit DNA, via the use of CRISPR-Cas9 technology (Brouns et al., 2008). Utilizing a gene editing approach would be an option for creating a similar IRAK-M mutant in humans. One would anticipate that targeting exons 9 through 11 of human IRAK-M for deletion, with CRISPR-Cas9, would result in a similar outcome that has been seen in the mutant irak-m-/- mice. Further, it would be expected that this type of edit of IRAK-M would lead to diminished colitis, and resistance to colorectal cancer in patients that carried a mutation in the kinase domains of IRAK-M. This type of application could be conducted on the precursor cells to macrophages, many of which are the hematopoietic stem cells found in the bone marrow. In a manner that similar to generating chimeric antigen receptor (CAR) T-cells, bone marrow could be extracted from human patients, hematopoietic stem cells could then be individually isolated and transfected in the laboratory with the specific CRISPR-Cas9 guide RNAs targeting exons 9-11 of IRAK-M for deletion. The successfully transfected stem cells could then be returned to the patient that carry the desired IRAK-M mutation, and would ideally result in a permanent therapeutic benefit.
for the patient. One of the promising options of this technique is that the therapy is essentially permanent; therefore, the need for additional medications in the future should be reduced. Additionally, one can envision an option in which expression or repression of a gene, following the CRISPR-Cas9 strategy, is induced with a chemical compound; analogous to the inducible mouse models that utilize estrogen receptor-CRE-lox system. In this way, a mutation could be induced, or a gene repressed when an exogenous molecule, such as tamoxifen is administered.

5.1.5 Future Directions with IRAK-M

In terms of future research regarding IRAK-M, it is necessary to develop and validate a functional antibody to the murine protein, preferably a monoclonal antibody that recognizes the N-terminus of IRAK-M. This would help solve the question of what is occurring in the knockout mouse. In addition to this, one would anticipate the actual mechanistic role of human IRAK-M to be reexamined. Though this molecule is currently believed to act as a non-functional kinase, intensive biochemical analysis would help validate whether IRAK-M is completely devoid of kinase activity. First and foremost, currently IRAK-M has not been crystalized, and it would be highly relevant to determine the full 3-D structure of this molecule. In addition, when IRAK-M was first discovered, it was shown to have weak autophosphorylation capacity (Wesche et al., 1999), yet it is believed that this molecule is completely devoid of kinase activity. One hypothesis is that this molecule actually behaves as a functional kinase, but doesn’t have as strong phosphorylation capacity when compared to other IRAK family members. It would be very interesting to determine if IRAK-M acts on an unknown substrate that it modulates.
via post-translational modification to affect cellular signaling. An approach to solve this perplexing question would be to make an overactive kinase mutant in kinase subdomain VIb. Would changing the endogenous serine in the HRD motif to an aspartic acid restore kinase function, and if so, what would be the substrate that IRAK-M phosphorylates? If indeed IRAK-M acts as a functional kinase on a currently unknown substrate, it is essential to determine what this substrate would be, as it is difficult to decipher the exact cellular signaling events that IRAK-M would modulate without fully understanding the kinase-substrate relationship (Berwick and Tavare, 2004).

If indeed IRAK-M phosphorylates a particular substrate, a “chemical genetic” approach could be used experimentally to answer this question (Shah and Shokat, 2003). This would involve making a mutation in the ATP binding pocket of the kinase of interest, in this case IRAK-M, and converting a hydrophobic amino acid to one with a small side chain. In this manner, the kinase would still be catalytically active, but has now gained the capacity to use a form of ATP that can be labeled at the γ-phosphate with a covalently attached traceable substituent, like biotin (Shah and Shokat, 2003), (Berwick and Tavare, 2004). Even cell permeable ATP analogs can be used, so this technique would not be limited to use with only cell lysates (Fouda and Pflum, 2015). Furthermore, because this mutated kinase is the only kinase able to use this analog of ATP as a substrate, the phosphorylated species, i.e. the substrate of the kinase, will become uniquely labeled. Regarding IRAK-M, this could entail making a mutation that converts the tyrosine gatekeeper of the ATP binding pocket of IRAK-M to a glycine or alanine.
Then, this type of assay could be conducted to determine whether IRAK-M functions as an active kinase on a particular substrate.

Another interesting method would be to use a Reverse In Gel Kinase Assay (RIKA) (Li et al., 2007). This method is optimal because of its ingenuity and the relative ease in its practical application; it requires common sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents to perform this experimental procedure. To summarize, a catalytically active kinase of interest is mixed with the reagents to make a SDS-PAGE gel, and in this fashion, the kinase will be embedded into the gel matrix once the gel has polymerized. Then, cell lysates can be run by electrophoresis, and following its completion, a series of buffer exchange steps can be performed that cause the proteins within the gel to refold back into their native conformations. Further, radiolabeled ATP at the γ-phosphate can be incubated with this gel, and because of the close proximity of the embedded kinase with potential substrates, the kinase will add radioactive or “hot” ATP to the potential substrates. Now, any radioactive molecules can be visualized with radiographic methods, and excised from the gel to be processed via mass spectrometry. Thus, this provides a relatively inexpensive method to determine previously unidentified substrates of the protein kinase.

4.2 Impact of Recent Methodologies to Study GI Immunology.

4.2.1 Elucidation of the GI Microbiota

Significant progress within the last decade has been made with respect to GI research. The use of transgenic technology, particularly the use of genetically modified mice, has
been at the forefront of many of these advances (Thomas and Capecchi, 1987), and additional creativity from the scientific community continues to fuel progress within the field. With respect to GI immunology and pathology, momentum has been building with research pertaining to the symbiotic interactions between the GI systems and the intestinal microbiota, and how the composition of the microbiota can influence health and disease of the host. Studying these interactions are complex due to several factors, but are partially due to the overwhelming diversity of bacterial genera present in the GI tract, particularly in the colon (Human Microbiome Project, 2012). In fact, the reproducibility between laboratories with respect to DSS colitis studies can be confounded, and attributed in part, to the variability in the microbiota composition within mice from lab to lab (Hufeldt et al., 2010). In order to minimize confounding factors associated with the GI microbiota, researchers should make every effort to understand and validate the composition of the microbiome present in the GI tract of mice, which will help limit discrepancies among investigators. In addition, many laboratories have adopted the practice of utilizing germ-free animal facilities, in which animals are derived under sterile conditions, and are thus unable to be colonized by the microorganisms that constitute the microbiome. This provides an excellent tool for researchers as specific bacterial species can be added back on an individual microbe basis to these sterile mice, allowing their microbiome to be colonized by a single microbial species. This allows the interaction between host and microbe to be studied without other confounding microbial communities. Of course, this system is not perfect: because humans have many diverse types of microbes in the GI tract, it is likely that a shift in composition of the whole microbial community contributes to health and disease.
Though germ-free animals are a very useful and powerful scientific tool, interpretations should be made with this caveat in mind.

4.2.2 Bioinformatics and the Impact of Intestinal Organoids on Future Research

A strategy to accomplish such a thorough undertaking for determining the microbiome associated between laboratories would be to obtain microbiome profiles of the mouse colony; specifically, with 16s ribosomal DNA sequencing technology, as well as conducting metabolomics of bacterial products produced in the GI tract. This would greatly advance our understanding of specific bacterial communities’ contribution regarding colitis and cancer models, as well as how the composition may vary between differing genotypes of mice. Additionally, ex vivo tissue culture, specifically with intestinal organoids derived from the intestinal stem cells is an excellent strategy to reduce complex confounding effects that occur when using live animal models. This is one significant advantage of culturing ex vivo organoids from specific tissues, such as the gut. Specifically, gastric, small intestinal and colonic organoids can be cultured ex vivo and screened with substances such as specific drugs, microbial species, and PAMPs to assess, for example, proliferative and anti-proliferative effects on epithelial cells. A great example that has demonstrated the usefulness of this strategy was conducted by Kaiko et. al., 2016. With the use of a Cdc25a-luciferase reporter mouse, Kaiko et. al. screened colonic organoids with a broad panel of microbiome derived metabolites with the aim of determining if any metabolites enhanced or inhibited cellular proliferation. Kaiko et. al. determined that the microbial metabolite butyrate inhibited stem cell proliferation under physiologic concentrations that are normally present in the
GI tract. Further, convincing evidence supported their hypothesis that the production of butyrate contributed to the invaginations that form the intestinal crypt (Kaiko et al., 2016). The rationale was that differentiated colonic epithelial cells toward the top of the crypt metabolize butyrate rapidly, which prevents its availability and interaction with the colonic stem cells at the base of the crypt. Therefore, it is likely that the relationship of microbial metabolites, namely butyrate, contributes to the U-shaped invaginations that form the colonic crypt. The work by Kaiko et. al. highlight the powerful importance of the ex vivo organoid culture, along with screening technology to the scientific method to advance our current understanding of intestinal anatomy and physiology.

4.2.3 Research Applications Utilizing Intestinal Organoids
One advantage of the organoid system over traditional immortalized cell lines is due to the inclusion of many intestinal cell types in the organoid (Sato et al., 2009). Mimicking the intestinal stem cell niche allows for maintenance of the intestinal stem cells that give rise to the fully differentiated epithelial cells present in the GI system; thus, this closely models the physiology of the GI tract without the complex confounding interactions with other organs that are present in a live animal, such as the immune system and others. The importance of this methodology is demonstrated by the ability to culture intestinal organoids from human patients with a minimally invasive biopsy taken from a patient. For example, ex vivo organoids derived from patients with genetic mutations that cause cystic fibrosis have been useful for devising and evaluating therapeutic strategies. Patients with cystic fibrosis have a mutated gene that encodes for a chloride ion channel, which results in the inability to make surfactant that moistens the lungs.
(Dekkers et al., 2013). Because this global mutation occurs throughout the genome, intestinal organoids derived from cystic fibrosis patients can be cultured and monitored over time, screened for compounds that restore proper activity of the intestinal organoid, and advance chemotherapeutic options for patients with genetic diseases (Dekkers et al., 2013).

An exciting application with ex vivo organoids is the concept of microinjection of individual bacterial strains directly within the organoid lumen. This application is a novel concept, and one of relevance to help simplify complex variables that occur between the intestinal microbiota with the intestinal epithelial cells. A method has been described that utilizes this very concept with gastric organoids, and the bacterium H. pylori (Bartfeld et al., 2015), but can also be applied to colon derived organoids. Specific species of bacteria have been associated with the induction of colorectal cancer (Castellarin et al., 2012), (Abed et al., 2016), and introduction of these pro-carcinogenic bacterial strains with intestinal organoids may have utility as a primary model to determine the full contribution of these carcinogenic bacteria. Additionally, organoids from genetically modified mice can be used as a model to study growth and proliferation of intestinal stem cells ex vivo.

5.2.4 Future Directions with Organoids: a model system to study both genetic and host-microbiome interactions

The alternative NF-kB pathway and principally NF-kB inducing kinase (NIK) is known for its role in immune cell function and B-cell maturation (Eden et al., 2017), (Claudio et al.,
2002), yet its role in intestinal epithelial cells is currently under investigation. The results in the present dissertation suggest NIK to be critical for proper proliferation of intestinal organoids and maintenance of epithelial cells. Since NIK has been shown to contribute to cellular proliferation in the adaptive immune system (Claudio et al., 2002), (Li et al., 2016), the results shown herein are consistent with these prior studies. Importantly, however, our research findings are novel, as we have demonstrated NIK to be important in intestinal stem cell (ISC) proliferation. Moreover, it was recently shown that Nik-/- mice develop spontaneous eosinophilic esophagitis (Eden et al., 2017); this association in combination with the results of the present dissertation further bridges the importance of NIK to epithelial cell barrier function. It will be an interesting line of further investigation to determine whether the alternative NF-κB pathway and the Wnt signaling pathway collaborate in regulating the expression of LGR5. Indeed, future mechanistic studies will be critical in uncovering the role of NIK with proper stem cell proliferation.

Intestinal organoids are a great model system to further study host-pathogen interactions, or host-commensal interactions between GI epithelial cells with respective microorganisms. One particular bacterium that our group has worked with, in collaboration with Dr. Dan Slade (Virginia Tech, Biochemistry Department), is *Fusobacterium nucleatum*. *F. nucleatum* has received attention in recent years because of its association and presence in tumors of human patients with colon cancer. Thus, this bacterium is implicated with contributing to the oncogenic transformation of intestinal epithelial cells. Our group has been interested in determining the functional role that *F. nucleatum* plays in colorectal cancer with the use of mouse colonic
organoids. The interesting phenomenon of *F. nucleatum* is that it is a normal portion of the microflora found in the human mouth, and is not normally found in the GI tract; which provokes several intriguing questions: how is it that this bacterium is localized to colonic tumors? Does it actually cause colon cancer or does it localize to a colon tumor after a tumor has been initiated by alternative mechanisms? One hypothesis is that *F. nucleatum* is able to migrate through the bloodstream, entering from the gums and can localize to the colon through this route, ultimately resulting in its tumorigenic potential. A likely reason is that *F. nucleatum* levels increase in the blood after the brushing of one’s teeth, thus, it is possible that humans receive a substantial septic *F. nucleatum* dose twice a day, depending on hygienic habits. The use of colonic organoids is a strategic model to test this hypothesis, because it is relatively straightforward to treat colonic organoids in vitro with this bacterium. One practical use of this technique is that colonic epithelial cells that compose the organoid demonstrate cellular polarity when grown in matrigel, similar to that of the actual organ under *in vivo* conditions (Sato et al., 2009). Thus, the basolateral portion of the epithelial organoid would be the point of entry the bacteria would exploit when traveling from the bloodstream to the tissue.

### 5.2.5 Gene Therapy Targeting Multi-potent Stem Cells

One can envision stem cell based therapeutics, in combination with gene therapy, to advance treatments for human patients that suffer from genetic mutations, specifically point mutations. One major hurdle with gene therapy is the ability to correct a mutation that becomes retained throughout the genome of future daughter cells. This can be overcome if the mutation is corrected in the multi-potent stem cells themselves,
because it is these cells that are retained in adult tissue, and further give rise to fully differentiated cell types that populate the organ. In this respect, the corrected mutation will be retained in future daughter cells, and essentially cure that aberrant genetic disease. This is one of the very promising aspects of CRISPR-Cas9 based gene therapy; however, if this strategy is to be used in adult humans, not all multipotent stem cells have been able to be isolated, so currently, only a limited number of diseases could be addressed with this approach.

5.3 Conclusion
It will be exciting to view what the future holds regarding research advances in IBD, mucosal immunology, and cancer fields. With the use of intestinal organoids, many novel compounds may be discovered that improve therapeutic options for patients with inflammatory related diseases. Furthermore, stem cell based approaches in combination with gene therapy offer a promising methodology to treat or potentially cure diseases caused by point mutations. Though the cost of this type of treatment may be expensive, this is the type of personalized medical approach that could be utilized to treat an individual’s particular type of malady.

There remain many unanswered question regarding cellular signaling and how modulation of one single molecule can affect so many aspects related to the cell. In conjunction with our current findings, continued research efforts focused on IRAK-M will be essential and contribute to the global understanding of how this particular molecule modulates aspects of the immune system. In the larger clinical context, the elucidation
of the mechanistic role of IRAK-M offers significant promise towards the development of novel therapeutic strategies for the treatment of GI pathologies, such as IBD and colorectal cancer.

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Nature Reviews Immunology

Regional specialization within the intestinal immune system Allan M. Mowat, William W. Agace, Sep 19, 2014

Negative Regulation of Inflammation: Implications for Inflammatory Bowel Disease and Colitis Associated Cancer

Daniel Rothschild, Virginia-Maryland College of Veterinary Medicine Sep 2018

Figure 1: Anatomy of the intestinal mucosa and its immune apparatus.

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Sep 2018 1

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