# MUC1 in the relationship between inflammation and cancer in IBD

by

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University of Pittsburgh, 2006

Patients with inflammatory bowel disease (IBD), a chronic inflammatory disease of the colon, have an increased incidence of colon cancer. This has led to the hypothesis that chronic inflammation causes malignant transformation and promotes tumor progression. However, an alternative hypothesis can be made that everything starts with early malignant lesions, which activate innate but not adaptive immunity thus driving chronic inflammation. This imbalance between the innate and the adaptive immunity at the intestinal site may speed up colon cancer progression. To test this hypothesis we are examining development of colonic inflammation and associated colon cancer from the perspective of *de novo* expression of the tumor antigen MUC1 in both settings and innate and adaptive immune responses against it.

We have created an animal model that recapitulates *de novo* MUC1 expression in human IBD by crossing IL10<sup>-/-</sup> mice that develop IBD and colon cancer, with human MUC1 transgenic mice that express MUC1 under its own promoter, thereby maintaining human tissue specific expression of this molecule. Mice were sacrificed at various time points and colonic tissue sections assessed for inflammatory and malignant changes and MUC1 expression. We found that, like in humans, expression of normal MUC1 as well as hypoglycosylated (tumor) MUC1 increases with the severity of inflammation in IBD. In other experiments, MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice were vaccinated with TnMUC100mer, representing the hypoglycosylated (tumor) form of MUC1. MUC1-specific vaccination slows the progression to IBD as measured by rectal

prolapse. Vaccinated animals, that develop rectal prolapse, have fewer tumors than unvaccinated animals. We have developed an animal model of MUC1<sup>+</sup> IBD and colon cancer that mimics human disease. We show that MUC1-specific vaccination slows the progression to IBD and has a protective anti-tumor effect. We postulate that induction of MUC1-specific immunity, including effector and regulatory T-cells, restores the balance between adaptive and innate immunity, which resolves chronic inflammation and stops progression of premalignant lesions to cancer.

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# PREFACE

I want to start with a passage that has sustained me though my graduate studies and allowed me to appreciate the ups and downs of my graduate years as an important part of my journey.

Tucked away in our subconscious is an idyllic vision. We see ourselves on a long trip that spans the continent. We are traveling by train. Out the windows we drink in the passing scene of flatlands and valleys, of mountains and rolling hillsides, of city skylines and village halls.

But uppermost in our minds is the final destination. Once we get there our dreams will come true, and the pieces of our lives will fit together like a jigsaw puzzle. How restlessly we pace the aisles, damning the minutes for loitering – waiting, waiting, waiting for the station.

"When we reach the station, that will be it!" we cry.

"When I'm 18."

"When I am able to buy a new Mercedes-Benz!"

"When I put the last kid through college."

"When I have paid off the mortgage!"

"When I get a promotion."

"When I reach the age of retirement, I shall live happily ever after!"

Sooner or later we must realize there is no station, no one place to arrive at once and for all. The true joy of life is the trip. The station is only a dream. It constantly outdistances us.

"Relish the moment" is a good motto, especially when coupled with Psalm 118:24: "This is the day which the Lord hath made; we will rejoice and be glad in it."

It isn't the burdend of today that drive men mad. It is the regrets over yesterday and the fear of tomorrow. Regret and fear are twin thieves that rob us of today.

...So stop pacing the aisles and counting the miles. Instead climb more mountains, eat more icecream, go barefoot more often and swim more rivers, watch more sunsets, laugh more and cry less. Life must be lived as we go along. The station will come soon enough.

- Robert J. Hastings

Although to numerous to mention individually, I want to thank all who have shared in the various miles of my journey.

### **1.0 INTRODUCTION**

### 1.1 CHRONIC INFLAMMATORY DISEASE

The primary function of the immune system is to protect the host from potentially harmful invading pathogens as well as to protect against internal threats such as neoplastic disease. This is accomplished by recognition of host 'danger signals' followed by an acute inflammatory response, which is mediated by the integrated actions of the innate and adaptive immune systems. When this process goes awry, then the ensuing condition is the development of a chronic inflammatory disease.

Chronic inflammatory diseases can affect a variety of tissues/organs with some categorized as 1) autoimmune diseases, such as multiple sclerosis and systemic lupus erythematosus; 2) some having a viral etiology, such as chronic liver inflammation related to hepatitis virus infection; and 3) others of unknown origin, such as rheumatoid arthritis and inflammatory bowel disease. Each inflammatory process is different reflecting differences in the initial cause as well as the microenvironment of the affected tissue or organ. However, the striking similarity between the various chronic inflammatory diseases is the overproduction of certain proteins and the continued presence of immune cells at the affected site, ultimately resulting in profound tissue damage.

#### **1.1.1 Inflammatory bowel disease (IBD)**

Inflammatory bowel disease, which includes ulcerative colitis (UC) and Crohn's disease (CD), is clinically characterized as a chronic relapsing and remitting inflammatory condition affecting the gastrointestinal (GI) tract. UC affects the colon and is a superficial ulcerative disease; whereas CD is a transmural granulomatous disorder that affects any part of the GI tract (1, 2). Both CD and UC are fairly common in North America. CD is estimated at 150-200 cases per 100,000 population, while the prevalence of UC is estimated at 150-250 cases per 100,000 population. The incidence of CD appears to be increasing, while UC is relatively stable (3). This highlights the need for more effective therapies and prevention strategies.

Although the etiology of IBD is unknown, it is perpetuated by a loss of immune balance in the GI tract, which includes cellular components such as massive infiltration of lymphocytes and macrophages, and protein mediators such as cytokines, chemokines, growth factors, eicosanoids and reactive oxygen metabolites. The fundamental question is whether the persistent inflammation represents a primary defect in the mucosal immune system or a secondary consequence of a driving stimulus.

#### 1.1.1.1 Toll-like receptors in IBD

Toll-like receptors (TLRs) comprise a family of 11 individual transmembrane pattern recognition receptors (PRRs), which are differentially expressed in a variety of cell types. PRRs recognize conserved pathogen associated molecular patterns (PAMPs) that are unique to microbial organisms. After ligand binding, TLRs dimerize and undergo conformational changes allowing them to bind adaptor molecules. There are four adaptor molecules; myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL),

TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1 (TICAM1) and TRIF-related adaptor molecule (TRAM) (4) and the variety of responses mediated by distinct TLR ligands can be attributed to the selective usage of these adaptor molecules. Although their primary function is to recognize pathogens, inappropriate TLR signaling contributes to the pathogenesis of many diseases including severe sepsis, meningitis, atherosclerosis, multiple sclerosis, systemic lupus erythematosus, and IBD (5). TLRs represent the first point in an inflammatory response where the immune system tailors its response to specific pathogens and this functional specialization occurs by differential distribution of TLRs and further specialization exits at the level of the receptor itself. The consequence of TLR signaling, mostly through nuclear factor kappa B (NFkb), is the upregulation of different sets of genes that modulate different functional events. Their functional specificity is highlighted by TLR4, which is expressed on myeloid dendritic cells (DCs) and monocytes, and recognizes lipopolysaccharide (LPS) a cell-wall component of bacteria. LPS derived from *E. coli* induces interleukin-12 (IL-12) in DCs promoting a CD4<sup>+</sup> T helper-1 (Th1) adaptive immune response, however, LPS derived from P. gingivalis fail to induce IL-12 from DCs instead stimulates a CD4<sup>+</sup> T helper-2 (Th2) adaptive immune response (6). Differences are also seen with TLR2 signaling where binding of different ligands can stimulate either Th2 or T regulatory immune responses (7).

In the absence of pathogens, the normal colonic epithelium is exposed to greater than  $10^{12}$  colony-forming units of commensal bacteria, and the gut is said to be in a state of 'controlled inflammation'. The ability of the immune system to maintain control is due mostly to the relative paucity of TLR on intestinal epithelial cells (8), however, this profile is altered in IBD patients. TLR4 is strongly up-regulated in intestinal epithelial cells and lamina propria

mononuclear cells in the lower GI tract in patients with IBD (9), and Hausmann *et al* found significant increases in TLR2 and TLR4 in inflamed mucosa compared to normal mucosa and this expression was localized in macrophages. These macrophages responded with a 16-fold increase of cellular interleukin-1 $\beta$  (IL-1 $\beta$ ) mRNA when stimulated with LPS in contrast to macrophages from normal mucosa which had no increase in IL-1 $\beta$  mRNA (10). This has important implication in disease manifestation as TLRs influence the nature of the immune response by controlling the production of cytokines. The majority of DCs in healthy colonic mucosa produce interleukin-10 (IL-10) with very few producing interleukin-6 (IL-6) or IL-12. In contrast, in Crohn's disease the majority of colonic DCs produce IL-6 or IL-12 (11). Defects at this phase of the inflammatory response have been proposed as an underlying cause of IBD, however it is difficult to determine if these changes represent a causal factor in IBD or if they occur as a result of an ongoing inflammation, as inflammatory cytokines interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) have both been shown to up-regulate intestinal epithelial TLR4 expression *in vitro* (12, 13).

#### 1.1.1.2 Nucleotide-binding oligomerization domain proteins in IBD

The nucleotide-binding oligomerization domain (NOD) family of proteins comprises more than 20 different mammalian proteins that are intracellular pattern recognition receptors. Members of this family share a tripartite domain structure consisting of a C-terminal leucinerich-repeat (LRR) domain, which is involved in ligand recognition, a control NOD domain, which facilitates oligomerization and has ATPase activity, and an N-terminal domain comprised of protein-protein interaction cassettes such as caspase recruitment domain (CARD). NOD1 has an N-terminal domain that contains a single CARD, in contrast NOD2 has two CARDs (14). NOD1 and NOD2 are cytoplasmic proteins mainly expressed by two cell types, antigen presenting cells (APCs) and epithelial cells, and play a role in intestinal regulation of proinflammatory signaling through NF $\kappa$ B in response to bacterial ligands. NOD1 is encoded by the caspase-recruitment domain 4 gene (*CARD4*) and NOD2 is encoded by *CARD15*. Both proteins recognize peptides that are derived from the degradation of peptidoglycan, a component of bacterial cell-walls. More specifically, NOD1 ligand is  $\gamma$ -D-glutamyl-meso-diaminopimelic acid (iE-DAP), which is not present in Gram-positive bacteria (with a few exceptions), and NOD2 ligand is muramyl-dipeptide (MDP) a component of all bacteria (15-17). Therefore, these proteins have complementary and nonoverlapping functions.

Genome-wide scans of patients with IBD identified mutations in NOD2 gene as a risk factor in CD (18, 19). Sequencing of this gene indicated a cytosine insertion at position 3020 in exon 11, which gives rise to a stop codon and a truncated NOD2 protein (20). Several other mutations have been found as well. However, all mutations interfere with its ability to recognize MDP ligand. Therefore, mutant forms of NOD2 have a reduced capacity to induce NFkb activation upon stimulation with MDP (21, 22). Exactly how NOD2 mutations confer susceptibility to CD is not well understood, but a couple of hypotheses exist. First, NOD2 may function as a negative regulator of IL-12 production mediated by peptidoglycan (PGN) through TLR2. In the absence of this regulation, an excessive IL-12 response by antigen presenting cells (APCs) drives the inflammation in CD (14). The second hypothesis is intestinal epithelial cells expressing mutated NOD2 would have defective activation of NFkb in response to MDP, which would restrict the ability of the mucosal immune system to control the enteric bacteria in the gut (14). This is supported by evidence showing that Paneth cells in NOD2-deficient mice have impaired production of mRNA encoding  $\alpha$ -defensins, and these mice show increased infection on oral challenge with certain bacteria (23). Furthermore, almost all CD patients have reduced

expression of  $\alpha$ -defensin 5 and  $\alpha$ -defensin 6 (24). NOD2 mutations have also been shown to have an effect on the risk of developing systemic erythematosus lupus, another inflammatory disease.

### 1.1.1.3 Reactive oxygen metabolites in IBD

IBD is characterized by massive leukocyte recruitment into the lamina propria, which includes neutrophils and macrophages. Their major protective function is to destroy potentially harmful bacteria, which is mediated by a respiratory burst leading to the release of large amounts of reactive oxygen metabolites (ROM). The initial reaction releases excessive amount of superoxide anion, which itself is not damaging, however its neutralization reaction yields the more destructive metabolites hydrogen peroxide, hypochlorous acid and the hydroxyl radical. Hydrogen peroxide directly damages epithelial cells (25) and the hydroxyl radical depolymerizes GI mucins and inflicts DNA damage (26, 27). Hydrogen peroxide released by tumor-derived macrophages has been shown to substantially decrease T-cell proliferation (28, 29) and impair Tcell function (30). Hypochlorous acid can inactivate a host of essential enzymes (31), decrease the adhesive properties of extracellular matrix components (32) and increase endothelial permeability through the mobilization of cellular zinc (33). Peroxynitrite, formed by the reaction between superoxide and nitric oxide is particularly damaging and is capable of modifying and damaging virtually all cell and tissue components (34). During chronic inflammation, overproduction of ROM leads to excessive tissue damage and breaching the epithelial barrier, which allows the luminal contents further access to immune cells, thereby, exacerbating the disease.

#### 1.1.1.4 Lipid-derived eicosanoids in IBD

Eicosanoids are lipid-derived protein mediators that are synthesized during the early phases of an inflammatory response. They are critical in modulating the adaptive immune response through their effects on DCs and macrophages. Some of the earlier studies revealed a role for eicosanoids in the pathogenesis of IBD as rectal biopsy specimens from patients with IBD were found to produce high levels of prostaglandins (PGs) (35). Later this was found to be the result of increased cyclooxygenase-2 (COX-2) levels (36).

Phospholipase A2 catalyzes the release of arachidonic acid from membrane phospholipids and arachidonic acid is the precursor for the synthesis of two main families of eicosanoids, prostaglandins (PGs) and leukotrienes (LTs). PGs are formed by most cells in the body and are not stored, rather they are synthesized *de novo* by COX-1 and COX-2 forming PGG2 and PGH2. COX-1 is constitutively expressed and COX-1 derived PGs are involved in normal biological homeostasis of renal water and electrolyte balance, gastric cytoprotection and platelet aggregation (37). COX-2 synthesis is induced by inflammatory stimuli and increases the production of PGs. PGE2 is generated by either degradation of PGH2 or by a reaction catalyzed by PGE synthase (38). PGE2 induces DC maturation, chemotaxis and lymphocyte migration (39, 40). PGE2 regulates macrophage release of TNF- $\alpha$ , which is produced in excess in IBD. It was originally thought that inhibition of PGE2 could represent a therapeutic option for the treatment of IBD, however, inhibition was found to generate ulcers and cause reactivation of quiescent IBD (41, 42). It is now known that PGE2 has a dual function during inflammation with pro-inflammatory activity during the early phase and later during resolution it switches to anti-inflammatory activity and plays a role in promoting healing of mucosal injury (43). As PGE2 levels increase it feeds back to COX-2 and lipoxygenase to drive the synthesis of lipoxins and cyclopentenone PGs, which block neutrophil influx (44, 45), and can down-regulate IL-12

secretion by DC and up-regulate IL-10 independently (46, 47). Peroxisome proliferatorsactivating receptor  $\gamma$  (PPAR- $\gamma$ ) is upregulated in activated lymphocytes and DCs during inflammation and in vitro studies indicated that PPAR- $\gamma$  activation by cyclopentenone PGs, which are naturally occurring PPAR- $\gamma$  ligands, profoundly alters the immune properties of lymphocytes and DCs leading to the inhibition of immune responses (48).

LTs are short-lived lipid-mediators with potent pro-inflammatory biological activity. Three species of lipoxygenases; 5-lipoxygenase, 12-lipoxygenase and 15-lipoxygenase, convert arachidonic acid to leukotrienes. 5-lipoxygenase metabolizes arachidonic acid to 5hydroperoxyeicosatetraenoic acid (5-HPETE) which is further metabolized to 5hydroxyeicosatetraenoic acid (5-HETE) and LTA4 (49). LTA4 hydrolase converts LTA4 to LTB4, which is a potent chemoattractant that amplifies recruitment of neutrophils during inflammation (50). LTB4 activate endothelial cells by inducing P-selectin and E-selectin on the surface, which facilitates cellular adhesion and neutrophil binding. Neutrophil attachment through integrin binding plus stimulation with TNF and LTB4 triggers degranulation and a respiratory burst (50-52). 15-lipoxygenase metabolizes arachidonic acid to 15-HETE and lipoxins (LX). Cyclopentenone and lipoxins are endogenous anti-inflammatory mediators and execute the resolution phase of the inflammatory process. Lipoxins are generated through cellcell interactions by a process known as transcellular biosynthesis (53). Cyclopentenone is part of the J series of PGs and are formed by nonenzymatic dehydration of PGD2. The PGJs are unique from other PGs in that they have no known membrane receptors, rather they interact with a class of nuclear receptors called PPAR family (49). PPAR-y transrepress transcription factor activation, thereby, reducing pro-inflammatory gene expression. The importance of lipidderived mediators is highlighted by their role in the resolution phase of the inflammatory response.

The resolution phase is critical to end the inflammatory response and it is thought that this self-limiting response is triggered during the early phase of the inflammatory response (44, 45). The switch from tissue damage to tissue repair is thought to be initially mediated by neutrophils at the site of inflammation in pustules (54). Neutrophils are not synchronized and can produce many protein mediators at different time intervals depending on their cytokine environment as well as cell-to-cell contacts. Once stimulated to do so, they synchronize and begin the switch from production of pro-inflammatory LTs to anti-inflammatory lipoxins (55). In the presence of lipoxins, neutrophil recruitment ceases (56) and those present begin to undergo apoptosis, vascular permeability in reduced (57) and macrophages are induced to ingest apoptotic neutrophils (58), which stimulate macrophages to release anti-inflammatory mediators including tumor growth factor  $\beta$  (TGF- $\beta$ ) and IL-10 (59, 60).

# 1.1.1.5 Matrix metalloproteinases in IBD

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are regulated by inflammatory signals to mediate changes in extracellular matrix (61). Loss of regulation and excessive production of MMPs plays a major role in the pathogenesis of many inflammatory diseases. MMP-2, MMP-9 (gelatinases) and MMP12 (metalloelastase) are produced by macrophages and neutrophils. The collagenases (MMP-1 and MMP-13) and stromelysins (MMP-3 and MMP-10) are secreted by cytokine activated fibroblasts and MMP-10 is also made by epithelial cells. *In situ* hybridization and Western blotting has shown very high expression of these molecules in diseased tissues and around ulcers in IBD. Tissue macrophages are major producers of MMP-9, which is regulated by TNF- $\alpha$  and eicosanoids, and MMP-9 has been shown to increase proteolysis of the mucosa leading to ulceration and fistula formation in IBD (62). MMP-9 is also involved in the pathogenesis of multiple sclerosis and is responsible for disruption of the blood brain barrier and invasion of immune cells to the central nervous system as well as degrades myelin basic protein (MBP) enhancing the autoimmune response by release of degradation products (61). MMP-7 and MMP-3 have been shown to cleave E-cadherin and its fragments can disrupt other cells by acting as a competitive inhibitor of E-cadherin homotypic binding between cells. This causes a breach in the epithelial barrier and leads to exacerbation of the disease. The extracellular matrix plays a fundamental role in controlling cell survival, cell shape, growth, and differentiation. Therefore, MMPs impact on all of the above functions through their ability to alter the extracellular matrix. MMP inhibitors have been an active area of research for therapeutic strategies for IBD. However, their translation into clinical use has been problematic because of their side-effects.

#### 1.1.1.6 Regulatory T cell populations in IBD

Antigenic stimuli from the gut lumen are responsible for driving expansion of effector Tcells as well as regulatory T-cells, with the latter population responsible for keeping the gut in a state of what has been termed 'controlled inflammation'. Various populations of regulatory Tcells exist in the gut, including T-regulatory-1 (Tr1), T-helper-3 (Th3), CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD45RB<sup>low</sup>, CD8<sup>+</sup>CD28<sup>-</sup> and  $\gamma\delta$  T cells. However, the CD4<sup>+</sup> subsets are the most well characterized and they include Tr1, Th3 and CD4<sup>+</sup>CD25<sup>+</sup> cells. These populations of regulatory cells mediate suppressive function through cell-cell contact and/or production of IL-10 and TGF- $\beta$ . The importance of regulatory T-cell populations is highlighted by the profound autoimmune disorders in the absence of this population (63).

The Tr1 subset produces IL-10 and appears in vitro after repeated stimulation with antigen in the presence of IL-10. They have a cytokine profile and phenotype which is distinct from Th1 or Th2 cells. Tr1 cells produce high amount of IL-10 and low levels of IFN- $\gamma$ , little to no interleukin-4 (IL-4), and no interleukin-2 (IL-2) upon T-cell receptor (TCR)-mediated activators. They can inhibit both naïve and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells in an antigen specific manner, and can inhibit the production of immmunoglobulin by B cells (64), which is dependent on the production of IL-10 and TGF- $\beta$  as well as a contact-dependent mechanism that is not well defined. In vivo they are most likely controlled by IL-10 producing DCs and intestinal epithelial cells (65). They can be induced in response to specific infection, as intestinal infection with Helicobacter hepaticus has been shown to induce Tr1 differentiation and gut homeostasis occurred through the production of IL-10 (66). They can regulate Th2 pathology, and suppress serum IgE responses via the production of IL-10 (67). Tr1 cells have also been shown to be important in other inflammatory mediated diseases such as rheumatoid arthritis (68) and it is most likely the absence of this population that contributes to the establishment of colitis in IL-10 deficient mice. The importance of IL-10 as a regulator of mucosal immune responses is highlighted by its role in IL-10 deficient mice which spontaneously develop severe intestinal inflammation characterized by discontinuous transmural lesions (69). An adoptive transfer model of colitis also shows the importance for the suppressive function of IL-10 as well as the necessity of IL-10 to induce this population of regulatory T-cells. Transfer of CD4<sup>+</sup>CD45RB<sup>High</sup> T-cells induce a Th1-mediated colitis in severe combined immune deficiency (SCID) mice and this colitis can be prevented by cotransfer of the CD45RB<sup>low</sup> subset. However, when CD45RB<sup>low</sup> population was isolated from IL-10 deficient mice the protective effect was lost (70).

The Th3 regulatory T-cell population was originally identified in the peripheral blood of humans with multiple sclerosis and was later identified as mediating oral tolerance in mice that were fed autoantigens. The Th3 regulatory population predominantly mediates their suppressive function through the production of TGF- $\beta$ . They can suppress the activation of both Th1 and Th2 effector cells and they induce plasma cells to undergo class switching from an initial IgM to IgA isotype. These cells have been shown to regulate inflammation in the 2,4,6-trinitrobenzene sulphonic acid-induced colitis model (71) and they can prevent intestinal inflammation induced by oxazolone, which is a Th2 model of colitis (72). TGF- $\beta$  is expressed in large amounts in the intestinal mucosa and is a potent regulator of intestinal inflammation. TGF- $\beta$  signals through the family of Smad proteins and inflamed intestine from patients with IBD have marked overexpression of Smad7 and a reduction of Smad3. Blocking Smad7 with a specific antisense oligonucleotide restores TGF- $\beta$  signaling and its ability to inhibit proinflammatory cytokine production by the isolated mucosal lamina propria mononuclear cells (73).

The CD4<sup>+</sup>CD25<sup>+</sup> subset of T-cells consists of two subpopulations. They include a naturally occurring population which is generated in the thymus through negative selection with self peptides, and a second population that can be generated in the periphery. It is now known that the CD4<sup>+</sup>CD25<sup>+</sup> regulatory population can be distinguished by expression of the transcription factor FoxP3. Reduction of or functional alteration of this regulatory population leads to the development of various organ-specific autoimmune diseases, which include thyroiditis, gastritis and type-1 diabetes (74-77). These cells are also required to maintain balanced responses to environmental antigens in IBD (78). Their mechanism of action is still controversial; however, cell-cell contact with CTLA-4 and membrane-bound TGF- $\beta$  are important (78, 79) and production of IL-10 plays a role (80).

The actual identification of regulatory T-cells and their specific functions in humans is relatively limited and the bulk of our current understanding has been obtained from animal models of colitis. So far the data indicate that chronic intestinal inflammation is mediated by effector CD4<sup>+</sup> T cells, and in animal models, their function can be manipulated by the presence or absence of functional regulatory T-cell populations. In human IBD there appears to be an imbalance between effector T cells and regulatory T-cells, rather than a complete absence of regulatory populations, and the underlying cause of the imbalance is unknown. A complex three-way interaction exists in the intestine between microbiota, the epithelium and immune cells. A defect or abnormal response in any of the three components can result in this imbalance. This is seen in the  $mdr1a^{-/-}$  mouse, where deficient expression of the mdr transport protein in epithelial cells is sufficient to induce Th1-mediated colitis (81). It is also proposed that a microbial product may be suppressing the expansion or function of regulatory T-cells or the lack of a certain microbe may be responsible for the failure to expand the regulatory population. This hypothesis has been partially validated with the use of probiotic therapy as a treatment for IBD. Probiotics, including bacteria, such as Lactobacilli sp. and Bifidobacteria sp., some E. coli, Enterococci, and certain Saccharomyces spp. have been shown to provide beneficial effects in IBD (82). As probiotics colonize the intestinal tract for longer or shorter periods of time, they can act through a variety of mechanisms. They can directly act on the host through competition with pathogenic bacteria for binding sites on epithelial cells, thereby enhancing barrier function (83), or through effects on the epithelial cells and the mucosal immune system (84). They have also been shown to act through indirect mechanisms by producing antimicrobial compounds that antagonize pathogenic bacteria, via competition for ecological niches or substrates (85, 86). It

has not yet been determined if increases in regulatory T-cell populations occurs with probiotic therapy.

#### 1.1.1.7 Current therapeutic options for IBD

Effective therapy of IBD often requires the use of multiple pharmacologic agents, and this is due to the diversity of the etiologies of disease. Topical inhibitors of inflammation include aminosalicylates, which have been shown to inhibit intestinal epithelial cell injury and Systemic inhibitors of inflammation include apoptosis due to oxidant stress (87, 88). glucocorticoids, which have been shown to have a variety of effects. They can inhibit the production of inflammatory cytokines TNF- $\alpha$  and IL-1, chemokines such as IL-8, repression of transcription of the genes for inducible nitric oxide synthase (iNOS), phospholipase A2 and COX-2. This results in the blockade of leukocyte migration and inhibits the lipid-derived mediators of inflammation. Prolonged usage of these drugs is associated with serious sideeffects, which include hyperglycemia, abnormal liver chemistry and osteoporosis. Immunosuppressants such as azathioprine, methotrexate and cyclosporine are used frequently for the treatment of IBD. However, prolonged use leads to an impaired systemic immunity and susceptibility to various infections. Antibiotics are used as a therapeutic modality mostly to reduce the enteric bacterial load in the host. Lastly, cytokine modulators, including Infliximab, an antibody that blocks TNF- $\alpha$ , have had favorable clinical efficacy, though not in all patients.

#### 1.1.2 Summary

IBD is a chronic inflammatory disease affecting the GI tract. Although the etiology of IBD is unknown, the currently accepted view is that intestinal inflammation that characterizes this disease is the result of a dysregulated mucosal autoimmune response directed toward intestinal antigens in genetically predisposed persons. It is now appreciated that multiple factors underlie IBD pathogenesis, including dysfunction of the epithelial cell barrier, excessive production of ROM, NOD2 mutations, alterations in TLR signaling, inappropriate T-cell responses to the intestinal microflora and alterations in apoptosis. Current therapies do not work on all patients due to the heterogeneity of the disease hence the design of new treatment modalities is an active area of IBD research.

### **1.2 CHRONIC INFLAMMATION AND CANCER**

Chronic inflammatory diseases have been associated with an increased incidence of cancer development in the affected tissue or organ. This includes association of chronic bronchitis and emphysema with lung cancer, chronic esophagitis with carcinoma of the esophago-gastric junction (89), chronic inflammation resulting from exposure to asbestos fibers with mesothelioma (90) and IBD with colon cancer (91, 92).

A link between inflammation and cancer was made over a century ago when Rudolf Virchow noted that cancers tended to occur at sites of inflammation (93). It was initially believed that the immune infiltrate represented an attempt by the host immune system to eliminate the aberrant cells. This idea was further promoted by William Coley and his use of "Coley's Toxins". Coley found a correlation between remission in sarcoma patients and the development of erysipele, a severe skin infection that is caused by *Streptococcus pyogenes* (94, 95). Coley would vaccinate sarcoma patients with a mixture of heat-killed *Streptococci* and *Serratia marrescens*. Indeed, there is extensive evidence that infiltration of NK and T cells, effectors of innate and adaptive immunity, is associated with better clinical outcome in cancer patients (96, 97). However, infiltration of macrophages and mast cells in human breast carcinoma, lung adenocarcinoma and melanoma have all been associated with less favorable clinical outcome (98, 99).

Cancer is a multistep process during which cells acquire a malignant phenotype through the acquisition of genetic alterations. Hanahan *et al* proposed six essential alterations that characterize the cellular malignant phenotype: self-sufficiency of growth signals, insensitivity to growth inhibitory signals, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (100). This process takes years to unfold, which suggests that host mechanisms prevent aberrant cells from accumulating and causing harm to the host, the immunosurveillance theory initially proposed by Burnet more than thirty years ago (101).

This dual role of the immune system, sometimes preventing and other times facilitating cancer growth, has been an area of intense research raising fundamental questions. This includes how does a transformed cell harness the inflammatory process to avoid death and facilitate its malignant phenotype; and at what point in the inflammatory process, presumably started as a tumor rejection process, does this change occur? The answer to these questions should suggest therapeutic strategies directed towards preventing this change from a protective to tumor promoting immune response. In this section, we will examine how chronic inflammation can facilitate the progression of cancer by looking at colitis-associated colon cancer, which is thought to result from chronic inflammation of the GI tract.

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#### **1.2.1** Colitis-associated colorectal cancer (CACC)

IBD, which includes UC and CD is clinically characterized as a chronic relapsing and remitting inflammatory condition affecting the GI tract. Patients with IBD have an increased incidence of developing colitis-associated colorectal cancer (CACC) and the risk is thought to increase with duration and extent of colonic disease. The microenvironment in IBD is thought to promote the development of cancer by having a direct effect on cellular pathways and tissue homeostasis.

The leading theory on how chronic intestinal inflammation leads to the progression of colon cancer it is through oxidative stress. Inflamed tissues from patients with IBD have increased expression of ROM, which are the result of a respiratory burst associated with chronically activated macrophages and neutrophils. ROM can target DNA, RNA, proteins and lipids, and as such, have the ability to affect many cellular metabolic pathways. ROM can cause alterations in DNA such as base hydroxylation, deoxyribose damage and strand cleavage, which can result in gene mutations and subsequent malignant transformation. ROM can cause epigenetic changes such as DNA methylation (102), and can induce protein oxidation which introduces new functional groups, such as hydroxyls and carbonyls which contribute to altered protein function and degradation (103). Hydrogen peroxide has been shown to impair T cell functions in cancer patients by reduction in expression of TCR- $\zeta$  chain (30). Therefore, immune surveillance mechanisms in chronic inflammation are unable to eliminate the formation of aberrant cells.

Intestinal epithelial cell turnover is important in maintaining the integrity of the epithelial barrier and this occurs at a higher frequency in patients with IBD. Colonic mucosal biopsies taken from patients with IBD have higher rates of mitosis as well as apoptosis (104). Although

epithelial cell turnover does not cause cancer, when it occurs in an environment that facilitates the development of cellular mutations and inhibits elimination of aberrant cells, then it can drive cancer progression.

The major tissue destruction in IBD is from the production of MMPs, which degrade the extracellular matrix. The matrix plays a fundamental role in controlling cell survival, growth and differentiation, and by altering the matrix components MMPs can regulate tissue homeostasis. MMP-7 has been shown to protect tumor cells from apoptosis by shedding membrane-bound FasL, which increases the production of soluble FasL(105). MMP-7 and MMP-3 have been shown to cleave E-cadherin and the fragments can disrupt other cells by acting as a competitor inhibitor of E-cadherin homotypic binding between cells. This may have important implications is the formation of cancer because E-cadherin is not only important in maintenance of epithelial integrity but also functions as a tumor suppressor (106). Up-regulation of COX-2 occurs in IBD as well as colon cancer and is associated with activation of MMP-2, which can facilitate tumor invasion and angiogenesis (107).

Increased posttranslational modifications of p53 are associated with increased iNOS activity in inflamed tissues from IBD patients (108). p53 mutations occur fairly early in CACC and allelic deletion occurs in 50-80% of CACC (109). Interestingly, a high frequency of p53 mutations were also found in inflamed mucosa of IBD patients who did not have cancer, indicating that chronic inflammation may drive neoplastic progression in cells that have acquired a mutated phenotype.

Altered glycosylation of glycoproteins occurs in IBD, adenomatous polyps, metaplastic polyps and colon cancer (110). This shortening of oligosaccharide side chains leads to the expression of oncofetal T- and Tn antigens in the colon. This change can be induced in cultured

goblet cell-differentiated colon cancer lines by TNF- $\alpha$  (110). TNF- $\alpha$  has been shown to upregulate expression of MUC1 mRNA (111). MUC1 has been shown to interact with  $\beta$ -catenin in carcinoma cells via a peptide motif in the cytosolic domain (112), and  $\beta$ -catenin has been shown to play a role in the progression of colorectal adenocarcinoma (113). The role of aberrant glycosylation in IBD is not well known, but it could potentially play a role in the pathogenesis of IBD and subsequent CACC by providing enhanced binding for bacterial lectins, providing new glycoepitopes for immune cell recognition and aberrant signaling via  $\beta$ -catenin pathway.

### 1.2.2 Immunosurveillance

Tumor-specific T-cells and antibodies can be found in patients with cancer, suggesting that a mechanism exists that allows the initial recognition of tumor cells. The concept of cancer immunosurveillance was initially proposed by Burnet more than thirty years ago (101) who suggested for the first time that the immune system has the ability to recognize and eliminate developing tumor cells. In an attempt to give credence to an immune surveillance mechanism and to explain the formation and progression of spontaneous tumor cells, several groups tested the hypothesis that animals with compromised immune systems would develop tumors more frequently than wild type animals. Experiments that compared chemically induced tumor formation in nude mice and wild type mice showed no statistically significant difference between the two groups (114). Subsequent experiments have shown that the basic concept of immunosurveilance is indeed valid and that the many constituents of an intact immune system participate in the recognition and control of primary tumor formation. Tumor development is a multi-step process requiring years for a single transformed cell to become a malignant cell mass with distant metastases. During this time, there are many interactions between the tumor and the

immune system and the tumor variants that persist acquire numerous mutations to facilitate their continued growth and survival. A process termed 'cancer immunoediting' has been proposed that hypothesizes that the immune surveillance system can function to select tumor variants that have found ways to subvert the host immune system (115). This process is analogous to the evasion mechanisms employed by many persistent viral and bacterial infections, where the selective pressure of drug intervention propagates a more virulent outgrowth.

#### **1.2.3** Tumor escape

Through both active and passive processes, tumor cells have developed mechanisms to escape the immune system (116). Some tumors can drastically reduce or lose expression of classical MHC molecules (117, 118) while expressing non-classical MHC molecules, thereby inhibiting both T-cell and NK cell activity (119). Tumor cells have also been shown to lose expression of tumor antigens (120). Loss of TAP transporter proteins occurs frequently in tumors (121), which interferes with peptide delivery to MHC class I molecules resulting in decreased surface expression of MHC molecules including those bearing tumor-specific peptides. Apoptosis of activated tumor infiltrating lymphocytes has been shown to occur through the up-regulation of Fas-L on the surface of tumor cells that binds to Fas molecules on the surface of activated T-cells (122, 123). Tumor cells also secrete soluble cytokines that can directly interfere with T-cell function. TGF- $\beta$  has been shown to suppress T-cell activation and proliferation, as well as increase angiogenesis facilitating tumor growth (124, 125).

#### **1.2.4** Failure to induce an anti-tumor response

An anti-tumor immune response can fail during the earliest stage of cancer development, where premalignant cells are only beginning to acquire a transformed or malignant phenotype. These cells can evade the immune system simply by negatively affecting the initial priming phase of the immune response, thus suppressing the generation of effector T-cells.

In the context of cancer, the majority of peptides presented to the adaptive immune system by APCs are self-antigens. The innate immune system, important in facilitating the priming process, is activated only in the presence of danger signals primarily derived from pathogens (126). These are not always present during processing and presentation of selfpeptides or tumor peptides. Signal one (tumor antigen) in the absence of signal two (costimulation) renders T cells anergic, which results in tolerance to tumor antigens. Another factor that influences the initial priming step in tumor-specific immunity is the quality of the available T-cell repertoire. During T-cell maturation in the thymus, T-cells that react to selfpeptide/MHC molecules with high affinity are deleted from the repertoire. Thus, T-cells in the periphery have low affinity TCRs for self-antigens. These T-cells are unable to generate or sustain signal 1, which results in their inability to mature into effector cells. Although this prevents unwanted immune responses, it leaves a T-cell repertoire that is ill equipped to eliminate some tumor cells.

Tumor cells are also able to suppress the initial priming process by acting as APCs. Tumor cells express MHC class I molecules on their surface and are able to present their tumor antigens to T-cells. However, since they do not express co-stimulatory molecules, their encounter with tumor antigen-specific naïve T-cells results in T-cell elimination or anergy rather than activation. Cancer patients respond to the presence of the tumor with an initial activation of the innate immune system, with neutrophils and macrophages as the first responders. The main effector mechanism used by these cells is the respiratory burst which releases several ROM. However, chronic release of ROM can impair T cell function (30). Hydrogen peroxide released by tumor-derived macrophages has been shown to substantially decrease T-cell proliferation (28, 29). Interestingly, neutrophils isolated from patients with rheumatoid arthritis, also a chronic inflammatory disease, have been shown to decrease T-cell proliferation (29). The failure of T-cells to respond is due to inhibition of TCR- $\zeta$  chain expression (127), and this has actually been found to occur in many diseases with excessive hydrogen peroxide production (128-131). In essence, the innate immune system is responding to the aberrant cell with continuously increasing levels of ROM, which impairs specific adaptive immune responses.

# 1.2.5 Summary

In the previous section we have described how chronic intestinal inflammation can facilitate progressive tumor growth through the production of ROM, MMPs, increase in cellular proliferation and alteration of protein glycosylation. All this leads to, in some way, the suppression of adaptive tumor-specific immunity. There are several mechanisms that impede the ability of the immune system to eliminate premalignant cells. Early tumor cells can escape destruction by the adaptive immune system, and the chronic intestinal inflammation that ensues can continue to promote tumor growth by the cytokines produced as well as through the continuous impairment of T-cell function.

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### **1.3 MUC1 IMMUNOBIOLOGY**

MUC1 is overexpressed on the majority of adenocarcinomas (132), which represents over 80% of all human cancers, and cancer patients have been shown to have anti-MUC1 antibodies as well as T-cell responses directed against hypoglycosylated (tumor) MUC1. Importantly, MUC1 on normal epithelial cells is quite distinct from MUC1 that is expressed on tumor cells. These characteristics make MUC1 an attractive target for the prevention of cancer.

MUC1 is a large transmembrane O-linked glycoprotein that is present in low levels on normal ductal epithelial cells restricted to the apical surface facing the lumen. The peptide backbone of MUC1 consists of a variable number of tandem repeat (VNTR) region, which consists of 20 amino acids (GVTSAPDTRPAPGSTAPPAH). Due to its polymorphic nature the VNTR region can vary between 20-120 repeats in individuals (133, 134). Within the VNTR region there are two serines and three threonines representing five potential O-glycosylation sites. The VNTR region is flanked by short regions containing several degenerate repeat peptides. The N-terminal domain contains a signal peptide and a splice site yielding two alternative splice products. The C-terminal domain outside the VNTR region has a transmembrane domain (28 aa) and an intracellular domain (72 aa) (Fig. 1). The physiological role of MUC1 is undetermined, but as a member of the mucin family it is thought to function in lubrication and protection of epithelial surfaces, due in part to its excessive carbohydrate content. The carbohydrate content on the mature molecule can account for 50-90% of its weight and the presence of long, branched sugar chains results in an extended rigid molecule that is hydrated and protease resistant.


#### Figure 1: The structure of normal MUC1.

The cytoplasmic tail is colored purple, the transmembrane domain is green and the extracellular non-VNTR region is pink. The VNTR region is blue and has a high concentration of branched O-linked carbohydrates represented by the circles and lines. The N-terminal is colored gold and contains a hydrophobic signal sequence.

## 1.3.1 MUC1 glycosylation

*O*-glycosylation is a post-translational modification which proceeds via distinct steps and is initiated in the *cis* Golgi and reaches a final state after passage through the *trans* Golgi. Premature MUC1 recycles several times from the cell surface to the *trans* Golgi, which occurs by clathrin-mediated endocytosis. The final addition of sialic acid occurs in the *trans* Golgi and the final glycoform of MUC1 completes approximately 10 cycles before it is completely sialylated (135). The availability of peptide substrate and glycosyltransferases, which are site specific and differentially expressed in human tissues, determine glycosylation of the MUC1 peptide.

There are five potential *O*-glycosylation sites within the VNTR region and the glycosylation of MUC1 is built-up by chain elongation. The most common glycosylation addition to these amino acids is a core 2 structure, which is an N-acetylgalactose with a galactose branching from its third carbon, and N-acetylglucose branching from its sixth carbon.

On tumor cells, MUC1 is quite distinct compared to normal MUC1. MUC1 loses its apical polarization, becomes overexpressed and *O*-glycosylation becomes prematurely terminated leading to the accumulation of truncated sugars attached to the protein backbone (Fig. 2).



#### Figure 2: The structure of tumor MUC1.

The cytoplasmic tail is colored purple, the transmembrane domain is green and the extracellular non-VNTR region is pink. In contrast to normal MUC1, O-glycosylation is prematurely terminated resulting in truncated sugars attached to the protein backbone in the VNTR region (blue). O-linked carbohydrates are represented by the circles and lines.

The aberrant glycosylation is thought to be the result of changes in the levels of glycosyltransferases in tumor cells (136). This hypoglycosylated form of MUC1 exposes the shorter monosaccharide Tn antigen (GalNAc $\alpha$ Thr/Ser) or dissacharide T antigen (Gal $\beta$ 3GalNAc) as well as their sialylated forms sTN and sT attached to the peptide backbone. Overexpression and changes in glycosylation result in an immunologically distinct molecule as the peptide backbone, as well as new glycoepitopes, can serve as targets for the immune system.

#### **1.3.2** MUC1 is a tumor antigen

The level of MUC1 expression is increased on the surface of cells in virtually all adenocarcinomas (137-147). The first evidence that MUC1 can serve as a tumor antigen came from studies on breast and pancreatic cancer patients. Cytotoxic T lymphocytes isolated from draining lymph nodes of breast and pancreatic cancer patients were found to have an  $\alpha/\beta$  TCR and recognized MUC1 on the surface of tumor cells in an MHC-unrestricted manner. It was found that this specific recognition was directed against the APDTRP epitope within the tandem repeat region (148, 149). MHC-unrestricted recognition was the result of MUC1 multiple repeat epitopes that can cross-link the TCR on T-cells and induce intracellular signaling events similar to MHC-restricted recognition (150). MHC-restricted T-cells have also been isolated from They have been shown to recognize a nine amino acid peptide, breast cancer patients. STAPPAHGV, from the tandem repeat region bound to class I HLA-A11 (151). MUC1-specific antibodies have been found in sera of breast, colon, and pancreatic cancer patients (152), however antibody responses are of IgM isotype and low titer, indicating the lack of a CD4<sup>+</sup> T helper response. MUC1 is also associated with premalignant disease. MUC1 expression is correlated with high grade dysplasia in early colorectal cancer (153-155). MUC1 is upregulated in Barrett's esophagus, a premalignant condition of the esophagus (142).

## 1.3.3 MUC1 signaling

The cytoplasmic domain of MUC1 is highly conserved among species (156) and the cytoplasmic tail contains multiple tyrosine, serine and threonine residues as potential phosphorylation sites. Meerzaman *et al.* constructed a chimeric receptor by replacing the

extracellular and transmembrane domains of human MUC1 with those of human CD8 (157). Treatment with anti-CD8 antibody showed phosphorylation of four tyrosine residues resulting in the activation of extracellular signal-regulated kinases (ERK1/2) (157, 158). At least six residues in the cytoplasmic tail have now been shown to be phosphorylated in vitro. Phosphorylated tyrosine residues in the cytoplasmic tail of MUC1 provide binding sites for the SRC homology 2 (SH2) domains of many kinases and adaptor proteins. Pandey et al. identified phosphorylation at Y(60) leading to MUC1 association with adaptor proteins Grb2 and Sos (159). Li et al. identified Y(46) was phosphorylated by the epidermal growth factor (EGF) receptor (ErbB1), c-Src and Lyn kinases (160, 161). Glycogen synthase kinase 3β (GSK3β) has been shown to phosphorylate MUC1 on serine in a SPY site (162). MUC1 binds directly to  $\beta$ catenin and MUC1 blocks GSK3β-mediated phosphorylation of β-catenin, resulting in increased  $\beta$ -catenin levels in the cytoplasm and nucleus of carcinoma cells (163). MUC1 has been shown to directly bind to the estrogen receptor  $\alpha$  (ER $\alpha$ ) DNA binding domain and this interection stabilizes  $ER\alpha$  by blocking its ubiquination and degradation (164) and this interaction is increased by 17β-estradiol (E2) stimulation. MUC1 can bind intrercellular adhesion molecule-1 (ICAM-1) and this interaction is immediately followed by a calcium influx in MUC1<sup>+</sup> tumor cells and increased migration (165). This suggests that the binding of ICAM-1 to MUC1<sup>+</sup> on tumor cells triggers a pro-migratory signal, thus facilitating tumor cell metastasis.

Overexpression of MUC1 in a fibroblast cell line activates the antiapoptotic phosphoinositide-3-kinase/Akt (PI3K/Akt) and Bcl-xL pathways (166). Oxidative stress activates MUC1 gene transcription resulting in increased levels of MUC1 protein. This leads to reduced  $H_2O_2$  intracellular level that is mediated in part by upregulation of superoxide dismutase, catalase and glutathione peroxidase expression (167). Therefore, the apoptotic response to

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cellular oxidative stress is attenuated by overexpression of MUC1. It is thought that carcinoma cells may exploit this mechanism by overexpressing MUC1 to obtain a survival advantage under conditions of oxidative stress (167).

#### **1.3.4 MUC1 and inflammation**

MUC1 has been identified as a marker of several chronic inflammatory diseases. Increases in serum levels of MUC1 and specific antibodies directed against the peptide backbone of MUC1 have been reported in interstitial pneumonitis, active pulmonary fibrosis and ulcerative colitis (168-170). MUC1 is expressed around areas of ulceration in the gut suggesting that it may play a role in ulcer healing throughout the gut (171). MUC1 is rarely present in normal gallbladder epithelium. However, in gallbladder specimens with more severe inflammation, immunoreactivity for MUC1 could be found on cells in the deep mucosal folds (172). It has been proposed that MUC1 may play a role in inflammation and tumorigenesis through altering intracellular signaling, facilitating adhesion and migration of tumor cells and increasing resistance to apoptosis.

Although the physiological role of MUC1 is undetermined, as a member of the mucin family, it is thought to function in protection of epithelial surfaces. Studies in animal models have shown that Muc1 (MUC1 in humans and Muc1 in nonhumans) expressed by hamster and mouse epithelial cells is a receptor for *Pseudomonas aeruginosa* (PA) (173, 174). PA is an opportunistic pathogen responsible for morbidity and mortality associated with cystic fibrosis and pneumonia in immunocompromised patients. The binding of PA, mediated through flagellin, leads to phosphorylation of the Muc1 cytoplasmic domain and activation of ERK1/2 (175). In response to intranasal instillation of PA or flagellin, Muc1<sup>-/-</sup> mice showed greater

airway recruitment of neutrophils and higher levels of TNF- $\alpha$  in bronchoalveolar lavage fluid (176). This suggests that MUC1/Muc1 may play an early anti-inflammatory role in lung infection or exposue to bacterial products. In contrast, female Muc1<sup>-/-</sup> mice housed under normal conditions display chronic infection and inflammation of the uterus as a result of the normal bacterial flora of the GI tract. Furthermore, Muc1-/- mice have increased eye inflammation as a result of bacterial infection with *Staphylococcus, Streptococcus* and *Corynebacterium spp.*, compared with wild type littermates (177). MUC1 is also expressed on activated human leukocytes. Studies showed that in response to chemokines, activated human T-cells upregulated and concentrated MUC1 at the leading edge of the T-cell (178). This suggests that MUC1 may be involved in early interactions between T-cells and endothelial cells at inflammatory sites.

### 1.3.5 Summary

MUC1 is overexpressed by virtually all adenocarcinomas. The tumor form of MUC1 is quite distinct from normal MUC1 and the immune system is capable of discriminating between the two forms. Immune responses directed against MUC1 have been found in cancer patients; however, antibody titers are low and CTL responses are weak. The tumor form of MUC1 has also been detected in premalignant disease. Therefore, the immune response to MUC1 is most likely shaped early in the disease process. MUC1 overexpression and glycosylation changes are associated with inflammatory diseases and it has been proposed that MUC1 may play a role in inflammation and tumorigenesis through altering intracellular signaling, facilitating adhesion and migration of tumor cells and increasing resistance to apoptosis.

#### 1.4 COMMON MUCOSAL IMMUNE SYSTEM

The following section discusses the two components of the common mucosal immune system Nasopharynx-associated lymphoid tissue (NALT) and Gut-associated lymphoid tissue (GALT), as both have prominent roles on the prevention and pathogenesis of IBD and CACC. This is followed by a section which highlights several animal models of intestinal inflammation.

The common mucosal immune system (CMIS) maintains immunological defense along the enormous epithelial surface which includes oral and nasal cavities, respiratory, intestinal and genitourinary tracts. The CMIS has the formidable task of discriminating between potentially harmful pathogens and innocuous antigens that we encounter on a daily basis. CMIS can be functionally divided into inductive sites, where antigen is sampled from the mucosal surfaces and presented to B and T cells; and effector sites, where primed cells return to exert their effector functions. Inductive sites for the CMIS consist of mucosal-associated lymphoid tissue (MALT), which can be further subdivided according to anatomical regions into the NALT, which is the major inductive site for the generation of mucosal immunity through inhalation of antigens, and GALT, which is the major inductive site for the generation of mucosal immunity through the gastrointestinal tract. The CMIS is an integrated network of immune cells that communicate with each other and function to protect the mucosal surfaces of the body.

### 1.4.1 Nasopharnynx-associated lymphoid tissue (NALT)

Little is known about the generation of tolerance to nasal exposed antigen. However, it has been shown that protective immunity can be generated through administration of nasal

vaccines. As such, NALT may be a viable route of administration for prophylactic vaccination for the prevention of IBD and colon cancer.

In rodents, NALT is found dorsal to the cartilaginous soft palate and is considered analogous to Waldeyer's ring in humans. Recently, a structure of lymphocyte aggregates that form follicles was identified in nasal mucosa in the middle concha, which share many similarities to rodent NALT structure (179). NALT contains all the components for the induction and regulation of mucosal immune responses that are delivered via the nasal cavity. Characterization of mRNA that encodes Th1 and Th2 cytokines in CD4<sup>+</sup> T cells isolated from mouse NALT revealed a predominant Th0 cytokine profile (180). Nasal administration of protein antigens with cholera toxin (CT), used as an adjuvant, induced antigen-specific Th2 responses. This promoted the generation of antigen-specific IgA-producing B cells in nasal passages as well as distal mucosal effector sites such as genitourinary, respiratory and intestinal tract (181). By contrast, nasal administration with antigen-expressing recombinant Bacillus Calmette-Guerin (rBCG) resulted in Th1-mediated immunity (182). Nasal vaccination with reovirus has been shown to generate antigen-specific IgA in the respiratory and intestinal tracts as well as high frequency antigen-specific cytotoxic T lymphocytes (183).

## 1.4.2 Gut-associated lymphoid tissue (GALT)

The GALT comprises Peyer's patches (PPs), the appendix, mesenteric lymph nodes, isolated lymphoid follicles throughout the GI tract, as well as the epithelial cell barrier lining the GI tract. The lower gastrointestinal tract, which includes the small intestine and colon, can harbor greater than 500 different species of bacteria. It falls on the various components of the

GALT to discriminate between potentially harmful pathogens or innocuous microbes and to mount a protective immune response or maintain tolerance, respectively.

A single layer of intestinal epithelial cells (IEC) separates the luminal contents of the gut from the largest lymphoid organ in the body. The epithelial cells not only provide a barrier, with epithelial tight junctions composed of claudins and occludins, but the epithelial layer also functions as a sensor that can detect microbial pathogens and respond by sending cytokine and chemokine signals to the underlying DCs. The epithelial lining is protected by a layer of glycocalyx, which is formed from mucins that bind the apical membrane of IEC, and an additional semi-permeable thick protective layer of mucus, comprised of various mucin glycoproteins and trefoil factor peptides. Goblet cells, which are mucus producing cells, are present in both the crypts and villus epithelium throughout the small intestine, colon and rectum (184). Bacteria are unable to penetrate these mechanisms unless they express mucinase and The IEC regulate the apical density of adherence, colonization and invasion factors. microorganisms through the release of antimicrobial peptides. The two main families are  $\alpha$ defensins and  $\beta$ -defensins. The  $\alpha$ -defensins are produced by specialized cells located at the bottom of the crypts in the small intestine called Paneth cells. The  $\beta$ -defensions are ubiquitously expressed throughout the GI tract, including the colon. Another class of antimicrobial peptides is the cathelicidins of which there is one member, LL37 (also known as CAMP), which is constitutively expressed in the intestinal epithelium (184).

Lymphoid follicles and their associated follicle-associated epithelium (FAE) are distributed throughout the GI tract as large visible aggregates, such as PPs located mostly in the distal ileum, or as single follicles dispersed in the colon and rectum. The epithelium communicates with the underlying immune system by way of the FAE, which contains M

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(microfold) cells. The M cells, which translocate antigens and microorganisms from the lumen into the basolateral side of the epithelium, are thought to be the predominant way that luminal antigens are brought into direct contact with immune cells (184). However, recent evidence shows that luminal contents are constantly being sampled by DCs that extend pseudopods across the IECs of the lining into the lumen (185). The lack of intestinal inflammation despite the massive population of bacterial microflora that exists in the GI tract does not appear to reflect immunological ignorance, rather, represents finely tuned immune responses that balance tolerance mechanisms with effector mechanisms. Even the colon which encounters the highest proportion of commensal microorganisms shows a very minor infiltrate of neutrophils, which is considered the hallmark of an inflammatory response.

The signaling mechanisms that determine tolerance versus immune response in the GI tract are mediated by a large family of receptors called pattern recognition recptors (PRRs). PRRs sense conserved structural motifs on microbes called pathogen-associated molecular patterns (PAMPs), which include lipopolysaccharide (LPS), lipoprotein, peptidoglycan (PGN), lipoteichoic acid, flagellin and CpG-containing (unmethylated) DNA. The two most well studied families of PRRs that are associated with intestinal disease are the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) family.

TLRs comprise a family of 11 individual transmembrane PRR, which are differentially expressed in a variety of cell types. TLRs represent the first point in an inflammatory response where the immune system tailors its response to specific pathogens and this functional specialization occurs by differential distribution of TLRs and further specialization exits at the level of the receptor itself. TLR1, TLR2 and TLR6 recognize and bind lipoproteins, TLR3 recognizes and binds dsRNA, TLR4 recognizes and binds LPS, TLR5 recognizes and binds flagellin, TLR7 and TLR8 recognize and bind ssRNA, TLR9 recognizes and binds CpG DNA, and TLR11 is activated by uropathogenic bacteria but a specific ligand has not yet been identified (186). The "classical" activation pathway is mediated by recruitment of the adaptor molecule MyD88 to the TIR domain, activation of serine/threonine kinases of the interleukin 1 receptor associated kinase (IRAK) family, degradation of inhibitor kB (IkB) and subsequent translocation of nuclear factor  $\kappa B$  (NF $\kappa B$ ) to the nucleus. Downstream signaling molecules result in the activation of several transcription factors, including NFKB, AP-1, ELK-1, CREB, STATs, followed by the transcriptional activation of genes encoding pro- and anti-inflammatory cytokines, chemokines and costimulatory molecules (186). Several mechanisms exist in IECs which allow the gut to maintain lack of excessive immune cell activation: relatively low levels of TLR2 and TLR4 on IECs during normal tissue homeostasis (9), high expression of Tollip, which inhibits IRAK activation (187), and ligand-induced activation of peroxisome proliferators activator receptor y (PPARy) (188, 189). IL-1-receptor-associated kinase M (IRAK-M) also has been shown to mediate tolerance properties. It is a negative regulator of TLR signaling (190) and can inhibit TLR association with TRAF6, thereby inhibiting TLR signals.

The NOD family comprises more than 20 different mammalian proteins that are intracellular pattern recognition receptors, and two members, NOD1 and NOD2, have been identified as playing a role in inflammatory bowel disease. NOD1 is constitutively expressed in may tissues and cells, and NOD2 has been shown to be constitutively or inducibly expressed in monocytes, macrophages, T- and B-cells, DCs, IECs, and Paneth cells (186). On ligand stimulation, both NOD1 and NOD2 enter into CARD-CARD interactions with the serine/threonine kinase Rip2/RICK/CARDIAK (191), which leads to NF $\kappa$ B activation and enhanced caspase induced apoptosis (192). Several lines of evidence have suggested that NOD

mutations may span a spectrum of diverse phenotypes from complete "loss of function" on one end of the spectrum to "gain of function" on the opposite end. Importantly, NOD knockout mice or NOD frameshift mutants do not develop spontaneous colitis; and in the DSS model of colitis, NOD knockout mice have enhanced disease and NOD mutants do not (23, 193, 194). This suggests that the NOD mutation alone is most likely not responsible for the etiology of CD.

One of the most important features of immune regulation in GALT is the diversity of specialized subsets of DCs that are located in MLN, PPs, as well as in the lamina propria (LP). Intestinal DCs are a heterogenous population of cells that migrate from all regions of the intestine via the lymph to draining mesenteric lymph node (MLN) (195). The MLN DCs appear to have a more mature phenotype which most likely reflects their migration from LP or PPs. MLN DCs have the propensity to produce IL-10 and TGF- $\beta$  and preferentially stimulate CD4<sup>+</sup> T-cells to produce IL-4, IL-10 and TGF-β (196). Antigen recognition in the MLN has been shown to occur within a few hours after feeding protein antigen (197). MLN DCs were shown to contain apoptotic bodies from intestinal epithelial cells in the steady state, suggesting that DCs in the gut continuously sample apoptotic epithelial cells for cross-presentation to T-cells in the MLN (198). The PPs contain subsets of DCs that are CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> and CD8 $\alpha$ <sup>+</sup>CD11b<sup>-</sup>, which are the conventional myeloid and lymphoid subsets, respectively, as well as a large subset of CD8 $\alpha$  CD11b. PP DCs, when stimulated with CD40L or receptor activator of NF $\kappa$ B (RANK) produce IL-10, in contrast to spleen DCs which produce IL-12 (199). PP DCs induce T-cells that predominantly produce high levels of IL-4 and IL-10 and lower levels of IFN- $\gamma$  (199, 200). LP DCs are located just below the basement membrane. Very few studies have been done on LP DCs, however, they appear to be the same subsets as in PPs. Importantly, these DCs have been shown to extend their processes into the intestinal lumen and sample antigen (185), and this is

thought to be a mechanism which helps maintain tolerance in the gut. When compared to splenic DCs, all populations differ in their cytokine profile. PPs and MLN DCs have been shown to specifically induce the mucosal homing receptor  $\alpha 4\beta 7$  as well as the chemokine receptor-9 (CCR9) on T-cells (201), which have important implications for lymphocyte homing to the gut.

Naïve lymphocytes can be primed in PPs or MLN, and those primed in PPs drain to MLN and undergo further differentiation. After priming, lymphocytes exit into the bloodstream and enter the thoracic duct. Lymphocytes primed in the GALT lose expression of L-selectin and selectively upregulate  $\alpha 4\beta 7$  integrin and CCR9. These lymphocytes will emigrate from the blood to the LP effector site by way of the ligands for  $\alpha 4\beta 7$  and CCR9, mucosal addressin celladhesion molecule-1 (MAdCAM-1) and thymus expressed chemokine (TECK/CCL25), respectively. MAdCAM-1 is constitutively expressed in the intestinal mucosa and TECK/CCL25 is expressed in the small intestine (202, 203). This chemokine pattern is distinct from T-cell primed in peripheral lymph node organs, which selectively upregulate  $\alpha 4\beta 1$  integrin (VLA4) and the chemokine receptor-4 (CCR4), so these T-cells are unable to migrate to mucosal surfaces (204, 205). Upon entry in the mucosa, the lymphocytes redistribute. The B-cell blasts migrate into the LP where they mature into IgA-producing plasma cells. The CD4<sup>+</sup> T-cells migrate into the LP, but distribute more evenly between the crypt and villus. The CD8<sup>+</sup> T-cells distribute into both the LP and epithelium.

At least 80% of the body's Ig-producing cells are located in the intestinal mucosa, which constitutes the largest effector organ providing adaptive and humoral immunity (206). Mucosal plasma cells produce dimers and large polymers of IgA collectively called pIgA. Through a process of transcytosis, the complex is carried through the cell cytoplasm to the luminal surface. The pIgA is cleaved, thus releasing pIg , which is nonfunctional and is degraded, and secretory

component bound to IgA (sIgA) is released into the lumen (207). sIgA promotes entrapment of microorganisms in the mucus, which prevents direct contact of pathogens with the mucosal surface, know as 'immune exclusion' (208). Dimer IgA that remains can bind pathogens that have breached the epithelial barrier and can mediate antibody-dependent cell-mediated cytotoxicity (ADCC), which is the destruction of antibody coated cells by natural killer (NK) cells (209). Immune complexes composed of antigen and IgA antibodies do not exhibit an efficient complement-activating capacity compared with other isotypes. The abundance of IgA isotype in the GI tract contributes to the control of complement-dependent inflammation.

## 1.5 ANIMAL MODELS OF INTESTINAL INFLAMMATION

Animal models of intestinal inflammation can be divided into four categories: spontaneous models, inducible models, adoptive transfer models and genetically engineered models. Each has contributed partially to the global understanding of intestinal inflammatory diseases but each also has a different missing component of the immune response.

### 1.5.1 Spontaneous models

#### 1.5.1.1 C3H/HeJBir

The C3H/HeJBir is a novel mouse strain that is bred at the Jackson Laboratory (210). These mice develop a spontaneous, pathogen-independent, colitis that is mediated by CD4<sup>+</sup> T-cells that are reactive against enteric bacterial flora, but not food antigens (211). This T-cell response is directed at protein antigens and is MHC class II restricted. The spontaneous colitis affects the cecum and colon with onset in the third to fourth week of life and resolving by 10-12

weeks of age. There was acute and chronic inflammation, ulceration, crypt abscesses, and epithelial regeneration, but no thickening of the mucosal layer or granulomas (210). They have been a valuable tool for studying and identifying genetic susceptibility factors in the pathogenesis of IBD.

#### 1.5.1.2 SAMP1/Yit

SAMP1/Yit mice develop inflammation in the small intestine with no colonic involvement. They develop discontinuous lesions as early as 10 weeks of age and show 100% penetrance by 30 weeks of age (212). Phenotypic analyses of inflammatory infiltrates have demonstrated increases in activated T-cells. The endogenous bacterial flora plays a role in the development of disease as mice raised in germfree environment fail to develop intestinal inflammation. The cytokine profile is characterized by increased production of IFN- $\gamma$  and TNF- $\alpha$ , and treatment with neutralizing antibodies to either TNF- $\alpha$  or IL-12 suppressed disease incidence and severity. Administration of antibodies to adhesion molecules, such as E-selectin, P-selectin and  $\alpha$ 4 integrin have also been shown to attenuate disease development in these mice.

#### **1.5.2** Inducible colitis models

#### 1.5.2.1 Indomethacin

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAIDs). Subcutaneous injections or oral administration of indomethacin in rats causes chronic ulceration and transmural inflammation in the small bowel (213, 214). This represents a model of epithelial barrier destruction and can be used to evaluate consequences of immune activation against enteric bacterial flora.

#### 1.5.2.2 Dextran sulfate sodium colitis (DSS)

Administration of DSS in the drinking water of rats and mice induces an acute left-sided colitis characterized by bloody diarrhea, ulcerations, histological damage and infiltration of neutrophils (215). This is predominantly a model of acute inflammation; however, in susceptible strains, administration of DSS in several cycles can induce lesions with CD4<sup>+</sup> lymphocytes and fissuring ulcers. This model has also been used to study the effects of inflammation on cancer development as pretreatment of mice with azoxymethane leads to development of multiple colorectal tumors in inflamed areas of the colon (216). The main limitation of this model is that it represents a nonspecific injury model and does not require T- and B-cells. Thus, it can not be used to address immunologic or therapeutic issues involving the adaptive immune system.

#### 1.5.2.3 Trinitrbenzene sulfonic acid (TNBS)/dinitrobenzene sultonic acid (DNBS) colitis

In susceptible strains of mice, luminal administration of TNBS or DNBS in 30-50% ethanol can induce colitis. The ethanol breaks the mucosal barrier and is a crucial component, as no colitis develops if TNBS is given alone. The type of inflammation that ensues is highly strain dependent as C57Bl/6 mice are resistant and this treatment requires strain-specific optimization. For the most part, this is also an acute model of inflammation associated with mucosal permeability as a consequence of necrosis and myeloperoxidase activity mediated by macrophages and granulocytes (217). A more chronic type of inflammation can be induced in the SJL/J strain of mice, which is characterized by a transmural granulomatous inflammation with severe diarrhea, weight loss and thickening of the bowel wall and increased lymphocytes in the lamina propria at the end stage (218). This has been a useful model for study of cytokine secretion patterns, cell adhesion and mechanisms of oral tolerance.

#### 1.5.2.4 Oxazolone colitis

Enema administration of the contact sensitizing agent oxazolone in 50% ethanol induces distal colitis in mice. The oxazolone-induced lesions are characterized by superficial inflammation of the mucosa associated with severe epithelial loss, focal ulceration and severe edema in the submucosal layers. Colitis in this model is mediated by CD4<sup>+</sup> T-cells that produce excessive amounts of the Th2 cytokines, IL-4 and IL-5. There is also increased myeloperoxidase activity leading to epithelial damage and ulceration. The disease in mice shares aspects of pathology similar to human ulcerative colitis (72, 219).

#### **1.5.3** Adoptive transfer model

CD4<sup>+</sup>CD45RB<sup>high</sup> T-cells from wild-type donor mice transferred to severe combined immunodeficient (SCID) mice or recombination activating gene (RAG<sup>-/-</sup>) deficient mice cause a wasting syndrome with transmural intestinal inflammation (220). Recipient mice that are repopulated with CD4<sup>+</sup> lymphocytes or CD4<sup>+</sup>CD45RB<sup>low</sup> lymphocytes do not develop colitis. When CD4<sup>+</sup>CD45RB<sup>high</sup> T-cells are transferred to SCID recipients with a reduced flora or to recipients treated with antibiotics, the colitis is ameliorated (221, 222). This model has been instrumental in the understanding of regulatory T-cell populations and cytokine production in the pathogenesis of IBD.

#### 1.5.4 Genetically engineered models

### 1.5.4.1 N-cadherin model

Dominant negative N-cadherin mice emphasize the importance of an intact epithelial barrier for the maintenance of gut mucosal homeostasis (223). This model provides support to

the idea that primary abnormalities of epithelial barrier function may cause chronic intestinal inflammation. A dominant negative N-cadherin mutant lacking an extracellular domain was transfected into 129/Sv embryonic stem cells which were then introduced into normal C57Bl/6 blastocytes. The chimeric mice have patches of mutant 129/Sv epithelium dispersed in normal C57Bl/6 epithelium. Two types of chimeras were generated using different promoters. One causes expression of the dominant negative N-cadherin in both the crypt and villus cells, and the other causes expression only in villus cells. Interestingly, focal inflammation and adenomas only occurred in the chimeras which expressed the epithelial defect in both crypt and villus cells. It is thought that antigen coming through the crypts induces immune responses different than antigen coming through villus epithelium. This model supports the idea that primary abnormalities of the epithelial barrier could result in secondary inflammation because there is no direct effect of dominant negative N-cadherin on the immune system.

## 1.5.4.2 Gai2-deficient mice

Hetreotrimeric G proteins mediate many signal transduction processes via adenylate cyclase. Gi2 is widely distributed in most cell types, including gut epithelial cells and lymphocytes. Mice with a targeted disruption of the  $\alpha$ -subunit of Gi2 develop severe chronic colitis and high incidence of adenocarcinomas with some clinical features that resemble human ulcerative colitis patients (224). In the inflamed colon there are increased numbers of memory CD4<sup>+</sup> T-cells and IgG-producing plasma cells in the lamina propria and elevated levels of cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and IL-12p40 mRNA. The development of the disease depends on the genetic background of the mouse; however, this model appears to exclude environmental factors as breeding under pathogen-free conditions does not ameliorate inflammation.

#### 1.5.4.3 IL-10 knockout mice

IL-10 is a well known immunoregulatory cytokine that is produced by T-cells, certain B cells, macrophages, thymocytes and keratinocytes. IL-10 is a direct inhibitor of macrophage function and an indirect inhibitor of Th1 and natural killer (NK) cells. Mice with a targeted disruption in the IL-10 gene spontaneously develop chronic colitis with massive infiltration of lymphocytes, activated macrophages and neutrophils into the lamina propria and submucosa (225). The disease is progressive and does not remit. Approximately 30-60% of mice surviving 6 months or more develop colon adenocarcinomas. The effector cell mediating colitis in the IL-10 deficient mouse is the CD4<sup>+</sup> T-cell. Transfer of CD4<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup> T-cells isolated from the lamina propria of IL-10<sup>-/-</sup> mice into RAG-2<sup>-/-</sup> recipients results in colitis. However, transfer of CD8+ lamina propria T-cells does not induce colitis. The colitis is mediated by an overproduction of inflammatory cytokines IL-1, IL-6, TNF- $\alpha$ . Administration of anti-IFN- $\gamma$ , anti-IL-12 or IL-10 has been shown to attenuate colitis in IL-10 deficient mice (226-228). Although IL-10 deficiency in these mice is global, the lesions are mostly confined to areas of the colon, which is thought to occur because of the large quantities of bacteria in the colon. Mice raised in a germ-free environment do not develop colitis suggesting that the enteric microflora is important for initiation and perpetuation of intestinal pathology. The bacterial species that are involved remain unclear

#### STATEMENT OF THE PROBLEM

Various chronic inflammatory diseases have an increased incidence of cancer development in the affected organ or tissue. Examples include pancreatitis and pancreatic cancer, schistosomiasis and bladder cancer, pelvic inflammatory disease and ovarian cancer, and inflammatory bowel disease and colon cancer. This has led to the widely accepted hypothesis that chronic inflammation leads to the development of cancer.

Extensive research in the field of tumor immunology has reported that the lack of a sufficient inflammatory response, due to various mechanisms imposed by the tumor and its environment, result in the failure of the immune system to effectively eliminate aberrant cancer cells. This leaves us with a conundrum of how does an overzealous immune response lead to cancer formation if in fact the lack of an immune response also leads to cancer formation.

An alternative hypothesis could be proposed where cancer comes first and is recognized by the immune system, but the immune system is unable to eliminate the aberrant cells. This in turn leads to the presence of a driving stimulus which perpetuates chronic inflammation, which now becomes detrimental to the host and facilitates the progression to cancer.

The following chapters examine the pathogenesis of inflammatory bowel disease, colitisassociated colon cancer and the role that MUC1 plays in the pathogenesis of both diseases, thereby contributing to a better understanding of the link between chronic inflammation and cancer.

## 1.6 MATERIALS AND METHODS

1.6.1 Mice.

IL10<sup>-/-</sup> mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and MUC1-Tg mice on a C57BL/6 background were purchased from The Mayo Clinic (Scottsdale, AZ). All animals were housed at the University of Pittsburgh (Pittsburgh, PA) animal facility. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh. Human MUC1 transgenic mice express full length human MUC1 in the same spatial and tissue distribution as the endogenous protein in humans. IL10<sup>-/-</sup> mice develop spontaneous enterocolitis with pathological changes similar to human inflammatory bowel disease. The double transgenic mouse MUC1<sup>+</sup>/IL10<sup>-/-</sup> develops MUC1 expressing IBD and develops CACC.

### 1.6.2 Polymerase Chain Reaction (PCR) Screening

PCR was used to identify MUC1 transgene as well as IL-10 transgene. The primer pairs for 5'-CTTGCCAGCCATAGCACCAAG-3' 5'-MUC1 Tg are and CTCCACGTCGTGGACATTGATG-3'. The 5'-IL-10 primers are GTGGGTGCAGTTATTGTCTTCCCG and 5'-GCCTTCAGTATAAAAGGGGGGACC and 5'-CCTGCGTGCAATCCATCTTG-3'. The amplification program for MUC1 Tg consisted of 10 min at 95°C and 40 cycles of 1 min each at 94°C, 59°C, and 72°C followed by one cycle of 10 min at 72°C. The amplification program for IL-10 consisted of 10 min at 94°C and 35 cycles of 30 s each at 94°C, 66°C, and 72°C followed by one cycle of 3 min at 72°C. The PCR product of each reaction was analyzed through a 1% agarose gel. MUC1 resulted in a 500-bp fragment and IL-10 resulted in 200-bp (IL- $10^{+/+}$ ), 200-bp and 450-bp (IL $10^{+/-}$ ) and 450-bp (IL- $10^{-/-}$ ).

#### 1.6.3 Histology

Mice were sacrificed and colon removed and rinsed with cold PBS to remove fecal material, dried and weighed for colon mass. The colon was divided into the ascending colon, descending colon and cecum, and fixed in 10% buffered formalin and embedded in paraffin. 5um-thick sections were stained with hematoxylin-eosin. Colitis scores (0-4) were determined by a staff pathologist who was blinded to the experimental protocol using the criteria reported by Berg et al. Briefly, 40 separate microscopic fields are evaluated for each mouse by a pathologist (A.R. Sepulveda) using the following criteria: (grade 0) no change from normal tissue; (grade 1) one or a few multifocal mononuclear cells in the lamina propria with minimal epithelial hyperplasia and slight to no mucus depletion; (grade 2) more frequent lesions or lesions involving more intestine than in grade 1. Mild hyperplasia and mucin depletion are seen; (grade 3) moderate inflammation involving more area than grade 2 and involving submucosa but not transmural. Moderate epithelial hyperplasia, mucin depletion, few ulcers and crypt abscesses are seen; (grade 4) severe inflammation sometimes transmural with crypt abscesses and ulcers. Epithelial hyperplasia is severe with crowding of epithelial cells in elongated glands. Few mucin containing cells are seen. A total inflammation score for each sample was determined by taking the sum of the fields divided by the number of fields.

#### 1.6.4 Immunohistochemistry

5-μm-thick tissue paraffin sections were deparaffinized by baking overnight at 59°C. Endogenous peroxidase activity was eliminated by treatment with 30% H<sub>2</sub>O<sub>2</sub> for 15 min at room temperature. Antigen retrieval was performed by microwave heating in 0.1% citrate buffer. Nonspecific binding sites were blocked with Protein Blocking Agent (Thermo-Shandon Corp). The anti-MUC1 antibody HMPV (IgG1) recognizes the epitope APDTR was purchased from BD Pharmingen. The anti-MUC1 antibody VU4H5 (IgG1), which preferentially recognizes hypoglycosylated MUC1, was purchased from Santa Cruz Biotechnology. Staining was performed by the avidin-biotin-peroxidase complex (ABC) method with a commercial kit (Vectastain ABC kit, Vector Laboratories). Color development was performed by 3,3'Diaminobenzidine (DAB) kit (BD Pharmingen).

## 1.6.5 MUC1-specific ELISA

Blood samples were collected after sacrifice and the serum was tested for the presence of MUC1-specific antibodies using a MUC1-specific ELISA. Briefly, 96-well plates were coated overnight at 4°C with 13µg/ml of TnMUC100mer in PBS. Plates were washed three times with PBS and incubated with serial dilutions of the mouse serum for 1 h at room temperature. After three washes with 0.5% Tween 20, plates were incubated with goat anti-mouse peroxidase-conjugated secondary antibody for 1 h at room temperature. The goat anti-mouse IgG1 and IgG3 were from Southern Biotech (Birmingham, AL) and IgM was from Sigma. The plates were washed three times with 0.5% tween 20 and then incubated with substrate reagent A & B (BD

OptEIA) from BD Bioscience for 15 min. The reaction was stopped with 2.5M sulfuric acid, and the absorbance was read at 450nm.

#### 1.6.6 Generation of bone marrow derived dendritic cells

Femurs and tibias were removed from C57BL/6 mice. The epiphyseal plates were cut off and bone marrow flushed out using 23 gauge needle and complete DMEM. Cells were centrifuged at 1200 rpm and cell pellet resuspended in RBC lysis buffer (Sigma, MO) for 2 min. Cells were washed three times in complete DMEM. Cells were counted and resuspended 1.5-2 million cells/ml in AIM V with 10ng/ml each of GM-CSF and IL-4 (a generous gift from Immunex, WA) and transferred into T75 flasks at 30 million cells in 15 ml. Cells were fed every two days by adding 5 ml of AIM V supplemented with 10ng/ml GM-CSF and IL-4. On day 5 of culture, cells were harvested using 2mM EDTA and separated using Nycoprep (Accurate Chemical, NY). Purified DCs were loaded for 4-6 hours in polypropylene tubes with 30 ug/ml of synthetic TnMUC1-100mer in AIM V at 1 million cells per ml. Loaded DCs were washed once with AIM V and added to lymphocyte cultures for *in vitro* stimulation.

## 1.6.7 Isolation and *in vitro* stimulation of lymph node and spleen cells

Cells were isolated from inguinal lymph nodes, mesenteric lymph nodes and spleen by mechanical disruption and passed through a 70 µm filter. Splenocytes were centrifuged and cell pellets resuspended in 1 ml RBS lysis buffer (Sigma, MO) for 2 min. Cells were washed three times with complete DMEM. Cells were plated at 3 million cells per 2 ml in 24-well Linbro plates in complete DMEM containing 20U/ml mIL-2 (Immunex, WA). TnMUC1-100mer

loaded DCs were added to lymphocytes at a ratio of 1:10. Cultures were fed every two days by replacing half of the media with fresh complete DMEM containing 20U/mL mIL-2.

#### **1.6.8** Intestinal tissue explant cultures

Sections (1 cm each) of the transverse colon from individual mice were washed with PBS to remove fecal contents, shaken at 280 rpm at room temperature for 30 minutes in RPMI 1640 plus 50  $\mu$ g/ml gentamicin and cut into small fragments. Tissue fragments were dried by covering them with a paper towel and a heavy object on top for ten minutes. Tissue fragments, 0.5 g dry weight were incubated in 1.0 ml of complete DMEM supplemented with 50  $\mu$ g/ml gentamicin (Gibco Life Technologies, Grand Island, NY), and supernatants were collected after 24 and 48 hours for cytokine production.

## 1.6.9 Chromium-release assay

 $10^{6}$  RMA and RMA-MUC1 target cells were labeled for 1 hour with radioactive sodium chromate (Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>), then washed three times with DMEM, resuspended at  $10^{6}$  cells/mL and plated in 96-well V-bottom microtiter plate at 2,000 per well in 100µl. Effector LN cells were added in triplicate in DMEM at  $10^{5}$  for 4 hours. Supernatant was harvested and analyzed on a Cobra II auto-gamma counter (Perkin-Elmer, MA). Specific lysis was calculated as (lysis – spontaneous lysis)/(total lysis – spontaneous lysis ). Average of triplicates was plotted.

#### 1.6.10 Interferon-gamma (INF-γ) ELISPOT

Lymph node cells were mixed with peptide-pulsed DC (at a ratio of 10:1) in Multiscreen 96-well filtration plates (Millipore, Bedford, MA) precoated with anti-IFN- $\gamma$  capture antibody (BD PharMingen, CA). Plates were incubated for 40 h at 37°C. After three washes with PBS/0.1% Tween 20, plates were incubated with 2 µg/well of biotin-labeled anti-IFN- $\gamma$  antibody (BD PharMingen, CA) at 37°C. Plates were washed and spots developed with Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Spots were counted and analyzed on ImmunoSpot counter (Cellular Technology Ltd., Cleveland, OH).

## **1.6.11 Vaccination protocol**

MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice received 20  $\mu$ l (10  $\mu$ l/ nare), which consisted of 30  $\mu$ g of TnMUC1-100mer plus 3  $\mu$ g of adjuvant E6020 (gift from EISAI, Boston, MA) in PBS, or adjuvant alone. Mice were vaccinated between 4-5 weeks of age and boosted twice at two week intervals.

### 1.6.12 Peptide synthesis

The TnMUC1-100mer peptide used for immunization corresponds to five tandem repeats of a 20-aa sequence from the extracellular variable number of tandem repeat (VNTR) region of MUC1. The amino acid repeat sequence is GVTSAPDTRPAPGSTAPPAH. The peptide was synthesized in the University of Pittsburgh Peptide Synthesis facility. Briefly, enzymatic addition of GalNAc to the peptide was prepared using recombinant UDP-GalNAc:polypeptide N-acetyl-galactosaminyltransferases rGalNAc-T1. Detailed description of the biochemistry can be found in Brokx *et al* (229).

The SAPDTRPA peptide corresponds to an eight amino acid sequence from the extracellular variable number of tandem repeat (VNTR) region of MUC1. The peptide was synthesized and purified on a Phenomenex C-18 Analytical column at the University of Pittsburgh Peptide Facility.

## 1.6.13 Flow Cytometry

Isolated cells from lymph nodes and spleens were washed and resuspended in FACS buffer (2% FBS in PBS) and plated at 0.5 x 106 cells per well. Surface Fc receptors were blocked with the addition of anti-CD16 antibody diluted 1:50 in FACS buffer for 30 mins. Without washing, cells were stained for 30 mins using antibodies specific for surface antigens, CD3 and CD4 from (BD Biosciences, CA). Antibodies were diluted 1:50 in FACS buffer. After 30 min incubation, cells were washed three times in FACS buffer. Cells were stained intracellularly for FoxP3 using a mouse FoxP3 staining kit (eBioscience, CA) as per instructions. Cells were resuspended in 400 µl FACS buffer and analyzed on a LSR II Flow cytometry (BD Bioscience), running FACSDiva software.

# 2.0 CHARACTERIZATION OF MUC1 EXPRESSION IN IBD AND COLITIS-ASSOCIATED COLON CANCER

(Adapted from manuscript Pamela Beatty, Antonia Sepulveda, Scott Plevy and Olivera Finn, Department of Immunology, University of Pittsburgh School of Medicine submitted for publication).

## 2.1 INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract and manifests as two clinically distinct diseases, ulcerative colitis (UC) or Crohn's disease (CD) (2). Although the etiology of IBD is unknown, the currently accepted view is that intestinal inflammation that characterizes this disease is the result of a dysregulated mucosal autoimmune response directed toward intestinal antigens in genetically predisposed persons. It is now appreciated that multiple factors underlie IBD pathogenesis, including dysfunction of the epithelial cell barrier, inappropriate T cell responses to the intestinal microflora and Nod2 mutations implicating both environmental and genetic factors (1). IBD is clinically characterized by relapsing and remitting chronic inflammation, and patients with IBD have an increased incidence of colitis-associated colorectal cancer (CACC) (230-232). CACC and sporadic colon cancer follow the same dysplasia-carcinoma sequence with similar frequencies in chromosomal abnormalities, microsatellite instability and glycosylation changes.

of approximately 50% (233). However, the macroscopic appearance of dysplasia between the two forms of colon cancer differ, with the majority of sporadic colon cancer arising from polyps and CACC more often developing from flat dysplastic mucosa.

Animal models have contributed in part to understanding some of the immunobiology and pathology of chronic intestinal inflammation. However, none of the current models accurately represents human IBD or CACC. The chemically induced mouse models of acute colitis due to disruption of the epithelial barrier (234) can occur in the absence of T- and B-cells, and therefore, while it allows examination of innate immune mechanisms, it excludes the contribution of adaptive immunity in the pathogenesis of IBD. The adoptive T-cell transfer has been used extensively to study the role of pathogenic and regulatory T-cells in intestinal inflammation (234), however, in this model it is difficult to examine the participation of innate immunity in the disease pathogenesis. The IL-10<sup>-/-</sup> mouse that spontaneously develops IBD is another well established model (228). Since it has been shown that the complete loss of the regulatory cytokine IL-10 is not the major underlying cause of IBD, this model allows the examination of adaptive as well as innate factors in the pathogenesis of IBD. Very importantly, however, none of these models are useful to address the role of the cell surface glycoprotein MUC1 mucin that may be an important participant in IBD.

MUC1 is not expressed in normal colonic epithelium, but it has been reported to be *de novo* expressed in IBD as well as on all colorectal adenocarcinomas. In addition to being described as a tumor antigen (149, 150, 235-237), MUC1 has many important functions in normal and abnormal epithelial cell biology. On normal epithelial cells, MUC1 is expressed at low levels as a highly glycosylated molecule with complex branched *O*-linked oligosaccharides. In contrast, on tumor cells MUC1 is overexpressed and hypoglycosylated (238) with short monosaccharides, Tn antigen (GalNAc $\alpha$ Thr/Ser), or dissacharides, T antigen (Gal $\beta$ 3GalNAc), and their sialylated forms sTN and sT (155). This "tumor" form of MUC1 can be recognized by both the innate (237) and the adaptive immune system (155, 239).

Altered glycosylation of proteins leading to expression of T and Tn antigens on MUC1 is also seen in IBD (110, 240). Most importantly, antibodies specific for MUC1 have been eluted from inflamed colonocytes of patients with IBD (241) suggesting that this molecule as well as an immune response against it may play a role in IBD and/or IBD associated colon cancer.

We report here on a new mouse model, a cross between IL-10<sup>-/-</sup> mouse and human MUC1 transgenic mouse, that shows *de novo* expression of MUC1 in IBD and thus better mimics human disease. MUC1 expression increases with the severity of inflammation, which recapitulates what is seen in human IBD. Importantly, MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice develop both chronic colonic inflammation and colon cancer at an earlier age than the IL10<sup>-/-</sup> mice, with increased tumor burden suggesting that MUC1 plays an important role in IBD pathogenesis and progression to CACC. The role of MUC1 in human IBD has not been investigated to date. Because of the important role that this molecule plays in other diseases, we believe that understanding its function in IBD may lead to a better understanding of mechanisms that promote chronic inflammation and cancer and may suggest novel approaches to therapy.

## 2.2 RESULTS

# 2.2.1 Characterization of MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice

The IL10<sup>-/-</sup> mouse is a well established model to study IBD and it is thought that development of intestinal disease in this mouse is the result of an exaggerated immune response to intestinal microflora due to the lack of IL-10 mediated immunoregulation. At 3-6 months of age, 30-60% of the IL10<sup>-/-</sup> mice on the C57BL/6 background develop spontaneous enterocolitis characterized by inflammatory cell infiltration, goblet cell depletion, crypt abscess formation and epithelial hyperplasia (228). All of these characteristics are similar to the pathology of human IBD, with the exception of MUC1 expression. The role of MUC1 in human IBD has only been noted but not studied. In order to be able to study the participation of this molecule in the disease process, we took advantage of the existence of transgenic mice on the same C57Bl/6 background that carry human MUC1 transgene. MUC1-Tg mice express full length human MUC1 in the same spatial and tissue distribution as the endogenous protein in humans (242), with low expression on normal epithelial cells and overexpression of the hypoglycosylated "tumor" form on tumor cells (243, 244). We bred MUC1-Tg mice with IL10<sup>-/-</sup> mice, extracted DNA obtained from tail snips and used PCR to identify animals that carried the MUC1 transgene but lacked IL-10 genes. MUC1-Tg mice are heterozygous for MUC1 expression and thus only 25% of the cross had the required genotype. Other mice resulting from the cross were used as controls (Fig. 3).



Figure 3: Development of MUC1<sup>+</sup>/IL10<sup>-/-</sup> mouse.

 $IL10^{-/-}$  mouse was crossed with MUC1 Tg mouse resulting in progeny that are herterozygous for IL-10 (MUC1<sup>+</sup>/IL10<sup>+/-</sup>) and homozygous (MUC1<sup>+</sup>/IL10<sup>-/-</sup>).

We were particularly interested in the comparison between the IL10<sup>-/-</sup> mice and the mice with the MUC1<sup>+</sup>/IL10<sup>+/-</sup> and MUC1<sup>+</sup>/IL10<sup>-/-</sup> genotype. MUC1<sup>+</sup>/IL10<sup>+/-</sup> mice were sacrificed at various time points and colonic tissue was stained with H&E to assess morphologically the presence of colonic inflammation, and with MUC1-specific antibody (HMPV) for MUC1 expression. We confirmed that the addition of human MUC1 to the genetic background of the IL10<sup>+/-</sup> mice did not change their resistance to IBD. MUC1<sup>+</sup>/IL10<sup>+/-</sup> mice do not develop colonic inflammation and we see little to no MUC1 expression in the colon (Fig. 4).



#### Figure 4: Colon section and MUC1 expression in the absence of inflammation.

(A) Colon section from IL10<sup>-/-</sup> mouse prior to the development of inflammation and stained with anti-MUC1 antibody (HMPV). (B) Colon section from MUC1<sup>+</sup>/IL10<sup>+/-</sup> mouse stained with the anti-MUC1 antibody (HMPV). Arrows indicate drak brown MUC1 stain. MUC1<sup>+</sup>/IL10<sup>+/-</sup> mice do not develop inflammation and expresses low levels of MUC1 (dark brown). Arrows indicate MUC1 expression. Isotype control sections were negative for MUC1 (not shown). Magnification is 200x.

MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice, expected to develop IBD, were monitored for symptoms of disease, such as weight loss, diarrhea and rectal prolapse. In our experience with this animal model, rectal prolapse is the first external sign of disease in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice, and we used this throughout our study as a marker to monitor disease onset and duration. MUC1<sup>+</sup>/L10<sup>-/-</sup> mice were sacrificed upon observation of rectal prolapse and colons were removed for histological

assessment of colonic inflammation and MUC1 expression. We found that all MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice with rectal prolapse had areas of segmental, patchy colonic inflammation (Fig. 5), consistent with histological inflammation observed in IL10<sup>-/-</sup> mice (not shown). Colon sections were stained with anti-MUC1 antibody to assess MUC1 expression in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. The affected colons from MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice were scored by our pathologist for severity of inflammation according to the established scoring system for human IBD. We found that MUC1 expression at sites of more severe inflammation in contrast to low levels of MUC1 expression in areas with no inflammation (Fig. 6).



Figure 5: MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice develop segmental patchy colonic inflammation.

MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice were sacrificed after the development of rectal prolapse and colon stained with H&E. Colon sections exhibited segmental, patchy colitis characterized by areas with (A) no inflammation in contrast to areas with (B) severe inflammation. Magnification is 200x and inserts show crypt area at 1000x.



#### Figure 6: Higher grades of inflammation are characterized by higher levels of MUC1 expression.

Colon sections from MUC1+/IL10-/- mice were scored by a pathologist for severity of inflammation according to the established scoring system for human IBD (see material and methods). Colon section with (A) 0-1 inflammation score, (B) 2-3 inflammation score and (C) 3-4 inflammation score were stained with anti-MUC1 antibody (HMPV). Isotype control sections were negative for MUC1 (not shown). Magnification is 200x and inserts show crypt area at 1000x.
We compared the MUC1 staining pattern in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice with human colon samples and found a similar staining pattern with high level of MUC1 expression in inflamed colon sections from IBD patients in contrast to low level of MUC1 expression in normal human colon (Fig. 7).



#### Figure 7: MUC1 expression in human colon.

Human colon sections were stained (drak brown) with anti-MUC1 antibody (HMPV). (A) Low level of MUC1 expression in normal colon in contrast to (B) high level of MUC1 expression in inflamed colon section from IBD patient. Isotype control sections were negative for MUC1 (not shown). Magnification is 1000x.

Given previous reports of the important role of MUC1 in other diseases, *de novo* expression of MUC1 concurrent with inflammation and its highest expression at sites with the highest inflammations score, begged the question of whether MUC1 may be contributing to the disease initiation and progression. To test this we compared the course of the disease in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice with the disease in IL10<sup>-/-</sup> mice. We found that MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice develop disease, as measured by first appearance of rectal prolapse, earlier than IL10<sup>-/-</sup> mice (Fig. 8).

Median time to rectal prolapse was 11 weeks for  $MUC1^+/IL-10^{-/-}$  mice compared with 16 weeks for  $IL10^{-/-}$  mice.



**Figure 8:**  $MUC1^+/IL10^{-/-}$  mice develope rectal prolapse at an earlier age than  $IL10^{-/-}$  mice.  $MUC1^+/IL10^{-/-}$  and  $IL10^{-/-}$  mice were monitored for the first sign of rectal prolapse.  $MUC1^+/IL10^{-/-}$  mice (n=13) develop earlier onset of IBD as measured by rectal prolapse compared to  $IL10^{-/-}$  mice (n=11). \*\* P< 0.001.

To evaluate and compare early inflammatory changes in the colon between MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice, prior to rectal prolapse, we sacrificed mice at 5-6 weeks of age and examined the colons. Due to the segmental and patchy pattern of colitis histological results were evaluated and reported in two different ways. First, 20-40 random fields distributed over the entire length of colon for each mouse were graded for inflammation by a pathologist blinded to the

experimental group, using a standard scoring system (228). Briefly, each field is given a score from 0 to 4, representing no inflammation (colitis score of 0), mild to moderate inflammation (colitis score of 1 to 2) or severe inflammation (colitis score of 3 to 4). Results are presented as the sum total for three separate areas of the colon: cecum, ascending colon and descending colon (Fig. 9).



#### Figure 9: Diagram of mouse colon.

Mouse colon is cut into three separate sections: (1) cecum, (2) ascending colon and (3) descending colon.

We found that MUC1<sup>+</sup>IL10<sup>-/-</sup> mice have a higher colonic inflammation score in all three areas of the colon compared to age-matched IL10<sup>-/-</sup> mice (Fig. 10). In the cecum, the median inflammation score in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice was 1.2 compared with 0.47 from IL10<sup>-/-</sup> mice (Fig. 10A). In the ascending colon, the median inflammation score was 1.9 in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice compared with 0.55 in IL10<sup>-/-</sup> mice (Fig. 10B). In the descending colon, the median inflammation score was 1.3 in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice compared with 0.32 in IL10<sup>-/-</sup> mice (Fig. 10C).



Figure 10: MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice have higher colonic inflammation score compared with IL10<sup>-/-</sup> mice.

MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> colon sections were scored for inflammation by a pathologist blinded to the experimental protocol. Briefly, 20-40 fields were given a score from 0 to 4 representing no inflammation to severe inflammation, respectively. The summation of scores for each colon section (cecum, ascending colon and descending colon) was divided by the total number of fields for that section. The individual scores for each mouse were plotted. All mice were sacrifed between 5 and 6 weeks of age.

To represent the spectrum of histological changes in individual mice, results are also presented as the percentage of total fields per mouse with no histological inflammation (colitis score of 0), mild to moderate inflammation (colitis score of 1 and 2), and severe inflammation (colitis score of 3 and 4). According to this scoring system as well, we see that MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice have fewer fields with no evidence of inflammation (0) and significantly more fields with moderate inflammation compared to IL10<sup>-/-</sup> mice (Fig. 11).



Figure 11: MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice have more areas of colonic inflammation compared with IL10<sup>-/-</sup>.

 $IL10^{-/-}$  mice have more colonic fields with no inflammation (0) and  $MUC1^+/IL10^{-/-}$  mice have more colonic fields with moderate inflammation (1-2).  $MUC1^+/IL10^{-/-}$  (n=4) and  $IL10^{-/-}$  (n=5). All mice were sacrificed between 5 and 6 weeks of age. \* P<0.05.

Consistent with the more severe disease,  $MUC1^+/IL10^{-/-}$  mice have lower median body mass compared with age-matched  $IL10^{-/-}$  mice (Fig.12).  $MUC1^+/IL10^{+/-}$  mice represent body mass in MUC1 Tg mice in the absence of inflammation.



Figure 12: MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice have lower body mass consistent with more severe disease.

 $MUC1^+/IL10^{+/-}$ ,  $MUC1^+/IL10^{-/-}$  and  $IL10^{-/-}$  mice were weighed between 5-6 weeks of age. Plots represent individual mice.

We also compared total cell numbers in the lymph node and spleens between MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice at the time when they were visibly sick with severe rectal prolapse. Lymph nodes and spleens were enlarged in both MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL0<sup>-/-</sup> mice compared to MUC1<sup>+</sup>/IL10<sup>+/-</sup> mice, however, cell counts showed that draining lymph nodes from MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice were still 1.5 fold higher than in IL10<sup>-/-</sup> mice. In contrast, MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice had fewer total cells in the spleen compared to IL10<sup>-/-</sup> mice.

# 2.2.2 Colitis-associated colon cancer (CACC) in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice.

Considering that our results suggested that the presence of MUC1 was associated with the earlier onset and more severe disease, we wondered what effect, if any, this might have on the colon cancer incidence. MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice were sacrificed at various time points after the first sign of rectal prolapse. Colons were cut into three separate sections: cecum, ascending colon and descending colon and each section placed onto a glass slide and examined under a dissecting microscope for the presence of tumors (Fig.13).



# Figure 13: MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice develop CACC.

Colons were removed from mice after the development of rectal prolapse. Colons were cut into sections, mounted on glass slides and examined under a dissecting microscope for the macroscopic appearance of tumors. (A) One tumor in the ascending colon, (B) four tumors in the ascending colon and (C) one tumor in the cecum. A, B and C represent colon sections from three different MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice.

We found that 89% of  $MUC1^+/IL10^{-/-}$  mice had developed colon tumors compared with 20% of  $IL10^{-/-}$  mice. In addition,  $MUC1^+/IL10^{-/-}$  mice had a much higher tumor burden (Fig.14), with an average of two tumors per mouse.



**Figure 14: MUC1<sup>+</sup>/IL10<sup>-/-</sup> have increased tumor burden compared with IL10<sup>-/-</sup> mice.** Whole mount colon sections were examined for the number of tumors per mouse. Each bar represents one mouse.

Whole mounts of the colons were fixed, embedded in paraffin, sectioned and stained with H&E and two MUC1-specific antibodies. Histological assessment by our pathologist revealed that the tumors in MUC1+/IL10-/- mice are adenocarcinomas (Fig.15), and immunohistochemistry showed increased MUC1 staining in the adenocarcinomas as well as increased staining for the hypoglycosylated form of MUC1 (Fig.16).



# Figure 15: Histology of CACC in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice.

Tumor sections were stained with H&E and examined by a pathologist blinded to the experimental protocol. Tumor sections were determined to be adenocarcinomas. A, B and C represent three tumors from three different animals.



#### Figure 16: MUC1 expression in adenocarcinomas.

Adenocarcinomas were stained with two different anti-MUC1 antibodies (dark brown). (A-B) Adenocarcinoma stained with the glycosylation-independent anti-MUC1 (HMPV). (C-D) Adenocarcinoma stained with glycosylation-dependent anti-MUC1 (vu4H5), which preferentially recognizes the hypoglycosylated "tumor" form of MUC1. Isotype controls were negative for MUC1 (not shown). (A&C) Magnification is 200x and (B&D) 1000x.

## 2.3 DISCUSSION

IBD and CACC have been studied extensively, both in patients and in mouse models. Because the disease at the time of diagnosis is characterized by massive infiltration of leukocytes, massive overproduction of cytokines and the presence of many IFN- $\gamma$  producing T cells, it has been characterized as the Th1 type autoimmune disease.

MUC1 is a tumor-associated antigen that is overexpressed on the majority of adenocarcinomas of the breast, lung, colon, pancreas, stomach, prostate and ovary (132). On normal epithelial cells MUC1 is a heavily glycosylated glycoprotein and provides protection against physiological, chemical and biological stress and its expression pattern varies depending on the histological site. However, during tumor progression MUC1 becomes overexpressed and there are changes in MUC1 glycosylation that results in novel B and T cell epitopes (239). MUC1 is found in the majority of adenomatous dysplasia and almost all colorectal adenocarcinomas (132, 247) and is an independent prognostic factor with immunoreactivity correlated with tumor progression and poor survivor probability (153). Interestingly, mucin glycosylation changes have been reported in patients with IBD (110, 233, 240) and MUC1-specific antibodies have been detected in patients with IBD (170, 241).

This led to our hypothesis that MUC1 may play a role in the pathogenesis of IBD and CACC. More specifically, we believe that *de novo* expression of MUC1 on early premalignant lesions forming in the colon may activate the innate immune system characterized by acute inflammation. This response fails to trigger effective adaptive immunity capable of clearing the lesion, and the persistence of the MUC1 expressing lesion leads to chronic inflammation which in turn could facilitate cancer progression. If this hypothesis is correct, then MUC1 could be

used as a vaccine target to boost MUC1-specific adaptive immunity. Thus, eliminating MUC1<sup>+</sup> premalignant lesions and preventing chronic intestinal inflammation and colon cancer. In this study, our goal was to develop a mouse model that expresses the human MUC1 molecule, develops intestinal inflammation and progresses to colon cancer, then use this model to test our hypothesis.

We bred MUC1-Tg mice with IL10<sup>-/-</sup> mice generating MUC1<sup>+</sup>/IL10<sup>+/-</sup> and MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. Using immunohistochemistry, we examined colonic MUC1 expression in control MUC1/IL10+/- mice to determine baseline levels in the absence of inflammation. We detected low colonic MUC1 expression using a MUC1-specific antibody (HMPV). Previous studies have reported varied MUC1 staining patterns in normal colonic mucosa (154, 248). It is thought that this slightly contradictory staining pattern is due to variations in the glycosylation of MUC1 in the colon. MUC1 in colon is thought to be expressed at low levels, but is more extensively glycosylated compared with epithelia in other organs, preventing staining by certain anti-MUC1 specific antibodies (249). In our study, to eliminate inconsistencies when comparing normal, inflamed and neoplastic colonic tissue we used the glycosylation-independent anti-MUC1 antibody (HMPV) to detect colonic MUC1 expression. We also used a glycosylationdependent anti-MUC1 antibody (vu4H5) to detect the hypoglycosylated (tumor) form of MUC1. Next, we monitored MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice for external symptoms of colonic inflammation, as well as histological assessment for inflammation. We found that MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice develop colonic inflammation and histological examination of colonic tissue revealed markedly increased MUC1 staining in the presence of colonic inflammation compared with control MUC1/IL10+/mice. Further characterization showed that MUC1 colonic staining increased with severity of inflammation in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. Using MUC1-specific ELISA and serum from

MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice with colonic inflammation we were able to detect MUC1-specific antibodies that were IgM isotype indicating an early immune response directed against changes in MUC1 glycosylation and expression (data not shown). Our initial characterization of MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice shows that this model shares similarities to human IBD and suggests MUC1 plays a role in the pathogenesis of IBD.

To confirm the role of MUC1 in the pathogenesis of IBD we compared disease progression between MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice. We hypothesized that if MUC1 plays a role in IBD pathogenesis then we would observe differences in disease progression between MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice. Using several parameters to assess colonic inflammation we found accelerated disease in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice compared with IL10<sup>-/-</sup> mice in support of our hypothesis. First, we monitored MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice for initial development of rectal prolapse, which is an external marker of chronic intestinal inflammation. We found that MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice develop accelerated disease as measured by rectal prolapse. Next, we looked at very early time points (5-6 weeks of age) to determine the earliest time points that we could detect accelerated disease in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. We found that as early as 5-6 weeks of age MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice have lower body mass compared to IL10<sup>-/-</sup> mice and accelerated disease could be detected in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice as measured by total colonic inflammation scores as well segmental inflammation scores within the colon. Lymph node and spleen were extremely enlarged in both MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice, however, live cell counts showed that draining lymph nodes from MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice had 1.5 fold increase compared with IL10<sup>-/-</sup> mice suggesting greater cellular proliferation. MUC1 epitopes that are exposed on the tumor-derived molecule have been shown to be chemotactic for immature dendritic cells (DC) (237). Changes in MUC1 glycosylation and expression levels in the colon could attract immature DC, which

would traffic to the draining lymph nodes and result in proliferation of T-cells that recognize hypoglycosylated MUC1 as a foreign molecule. Using flow cytometry we compared CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and Foxp3<sup>+</sup> cell in the draining lymph node of MUC1<sup>+</sup>/IL10<sup>-/-</sup> versus IL10<sup>-/-</sup> mice, however, we found no consistent differences between the two groups (data not shown). This came as a surprise as we expected the increased cell count was due to an increase in effector CD8<sup>+</sup> cells in the lymph nodes of MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. After eliminating CD3<sup>+</sup> cells we now believe the increased cell count is due to an increase in DC and myeloid suppresser cells (MSC) in the lymph nodes.

Lastly, we compared MUC1<sup>+</sup>/IL10<sup>-/-</sup> and IL10<sup>-/-</sup> mice for the development of colitisassociated colorectal cancer and found that 89% of MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice with chronic intestinal inflammation developed CACC. In contrast, 20% of IL10<sup>-/-</sup> mice with chronic intestinal inflammation develop CACC. In addition, we found that the MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice developed more tumors per mouse compared with IL0-/- mice and immunohistochemistry revealed that the tumors from MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice react with both glycosylation dependent and glycosylation independent antibodies.

Together, these findings clearly show accelerated IBD and higher frequency of CACC as well as increased tumor burden in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice compared with IL10<sup>-/-</sup> mice. Our findings raise the fundamental questions as to why MUC1 becomes overexpressed during IBD and importantly is overexpression an initiating event or a result of chronic inflammation and how does this affect colon cancer development?

A single layer of epithelial cells line the digestive tract and are responsible for tight junctions, separating luminal contents from the largest lymphoid organ in the body. Mucins in the GI tract provide the single layer of epithelial cells protection against physiological, chemical

and biological stress. We speculate that increased MUC1 expression in IBD is a compensatory mechanism to provide increased protection to the injured epithelial layer during chronic inflammation and this is supported by our findings that MUC1 expression increases with severity of inflammation. Studies have shown a STAT-binding element in the promoter region of MUC1; and IL-6 and IFN- $\gamma$ , important cytokines that are increased in IBD, both induce MUC1 expression (247). TNF- $\alpha$ , another important cytokine in IBD pathology, has been shown to stimulate expression of MUC1 mRNA in human nasal epithelial cells, which is thought to contribute to the pathogenesis of human inflammatory diseases of the upper respiratory system (111). IFN- $\gamma$  and TNF- $\alpha$  have also been shown to synergistically induce MUC1 expression in normal and malignant human breast epithelial cells. The STAT-binding site is conserved in the promoter of murine muc1. However, the immunogenic variable number tandem repeat (VNTR) is located in the human MUC1 extracellular domain, which contains very little sequence homology with murine Muc1. Therefore, we would not expect murine muc1 to contribute to the observed acceleration of inflammation in the MUC1<sup>+</sup>/IL10<sup>-/-</sup> model or the development of CACC.

Overexpression of MUC1 on cancer cells results from the same mechanisms reported for inflammation, induction of promoter by IL-6, IFN- $\gamma$  and TNF- $\alpha$ . Cancer cells have also been shown to have deficiencies in one or more glycosyltransferases, enzymes responsible for adding the extended carbohydrates to the MUC1 peptide backbone. This leads to the accumulation and expression of hypoglycosylated MUC1 on the the surface of cancer cells (136). Overexpression of MUC1 on cancer cells is thought to confer a selective advantage to these cells by destabilization of cell-cell adhesion from the basement membrane, migration and subsequent adhesion to endothelial cells, which would facilitate their metastatic capacity. This is supported by studies showing that MUC1 functions as a ligand for intercellular adhesion molecule-1 (ICAM-1) enabling tumor cell binding to endothelium (165). In fact, transendothelial migration was shown to be even higher when MUC1-expressing tumor cells were in the presence of proinflammatory cytokines. MUC1 has been shown to interact with various signaling molecules, including  $\beta$ -catenin and c-Src, which have been shown to be involved in neoplastic transformation in colon cancer (250, 251). With this mind, it has been suggested that MUC1 be considered tumorigenic, rather than a passive facilitator of metastasis (165).

Together, these findings suggest that in IBD and CACC, MUC1 can contribute to the pathogenesis by way of two distinct mechanisms. In the first mechanism, MUC1 is initially overexpressed as a compensatory mechanism in IBD in response to inflammation, characterized by IL-6, IFN- $\gamma$  and TNF- $\alpha$  cytokine production and disruptions in the epithelial barrier. Massively increased MUC1 mRNA will overwhelm the glycosyltransferases leading to greater expression of hypoglycosylated MUC1 versus normal MUC1 on the epithelial cell surface. Although initially started as a protective mechanism, an increase in hypoglycosylated MUC1 on the surface will eventually amplify and drive the inflmammtory response by providing chemotactic epitopes for immature DCs and subsequent B and T-cell epitopes (237, 239). In the second mechanism, the premalignant cell is already present and acquiring mutations that will confer a selective growth and survival advantage. Deficiencies in one or more glycosyltransferase will lead to surface expression of hypoglycosylated MUC1, which will amplify and drive inflammation. In both mechanisms, the inflammation is amplified and chronic with MUC1 providing a driving stimulus for innate and adaptive immunity, characterized by overproduction of cytokines in the GI tract.

In summary, these findings highlight the importance of MUC1 as a unique molecule involved in the pathogenesis of IBD and CACC with important clinical implications for both IBD and colon cancer. First, we have established a model that more accurately represents human IBD and CACC. Second, we show that MUC1 is involved in the pathogenesis of IBD and CACC, and we propose this is mediated by two distinct mechanisms. Importantly, these findings show MUC1 can be pursued as a vaccine target to boost MUC1-specific adaptive immunity in the colon for the prevention and/or therapy of IBD and CACC. Lastly, the MUC1<sup>+</sup>/IL10<sup>-/-</sup> model can be used to explore the link between inflammation and cancer development from the perspective of a well characterized molecule, MUC1.

# 3.0 IMMUNIZATION AGAINST MUC1 EARLY IN LIFE SLOWS IBD PROGRESSION AND HAS AN ANTI-TUMOR EFFECT

#### **3.1 INTRODUCTION**

The new animal model of MUC1<sup>+</sup> IBD that we developed and characterized allowed us to begin to test the alternative hypothesis about the relationship between inflammation and cancer. We were able to make this hypothesis more specific by proposing that de novo expression of MUC1, known to be a colon tumor antigen, is related to the appearance of early premalignant lesions. MUC1 expression on these lesions may initiate acute inflammation consistent with its documented ability to attract and activate cells of the innate immune system (237). This initial response in the case of an infection with a pathogen would lead to the activation of adaptive immunity that would eliminate the pathogen and resolve the inflammation. In the case of a premalignant lesion, if the adaptive immune response triggered by the initial inflammation, such as MUC1-specific T- and B-cells, fails to eliminate the lesion, the persistence of the MUC1 expressing cells will lead to chronic inflammation which in turn could facilitate progression of that lesion to colon cancer. If this hypothesis were correct, we could predict that a stronger adaptive immunity would clear the lesion and reduce inflammation as well as its effects on cancer progression. Having the new mouse model of MUC1<sup>+</sup> IBD and IBD-related MUC1<sup>+</sup> colon cancer, we had an opportunity to enhance MUC1-specific adaptive immunity through vaccination and observe the effects of immunization on inflammation and cancer.

We vaccinated MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice early in life, and therefore either prior or early in disease, with nasal administration of synthetic TnMUC1-100mer peptide and adjuvant. Nasal administration is known to prime lymphocytes in the nasal mucosal immune system (NALT), which we expected would facilitate antigen-specific lymphocyte migration to the colon. We show below that, indeed, boosting the efficacy of tumor antigen-specific immunity led to profound changes in the outcome of IBD and CACC.

## 3.2 RESULTS

# 3.2.1 MUC1 vaccination slows disease progression to IBD

MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice were vaccinated between 4-5 weeks of age with nasal administration of TnMUC1-100mer plus adjuvant or adjuvant alone, and boosted twice at two week intervals. The synthetic TnMUC1-100mer peptide (Fig. 17) is comprised of five 20 amino acid-long repeats from the MUC1 tandem repeat region with the monosaccharide GalNAc linked to three out of five (two serines and three threonines) *O*-glycosylation sites within each tandem repeat. This synthetic peptide represents the hypoglycosylated form of MUC1 that is found in IBD as well as in colon cancer. The adjuvant is E6020 (a kind gift from EISAI, Boston, MA), which is a synthetic lipopolysaccharide (LPS) analog that is a weak TLR4 receptor agonist and has been shown to induce mucosal IgA in nasal and vaginal washes when administered with ovalbumin or tetanus toxoid antigens (252).

# (HGVTSAPDTRPAPGSTAPPA) × 5

#### Figure 17: Diagram of MUC1 and synthetic TnMUC1-100mer peptide.

Synthetic Tn-MUC1-100mer is comprised of five 20 a.a. repeats from the MUC1 tandem repeat region, with the monosaccharide GalNAc linked to three sites within the each repeat.

Vaccinated animals were followed for 16 weeks and monitored for the initial development of rectal prolapse. We found that MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice that received TnMUC1-100mer plus adjuvant developed rectal prolapse at a slower rate compared to untreated mice (Fig.18A) or mice treated with adjuvant alone (Fig.18B). In fact, the administration of adjuvant alone accelerated the development of rectal prolapse. Although TnMUC1-100mer vaccination slowed the development of IBD, it did not completely prevent it, as mice eventually developed rectal prolapse at later time points.



Figure 18: Vaccination with TnMUC1-100mer plus adjuvant slows IBD progression.

Kaplan-Meier curves reflect the onset of rectal prolapse in  $MUC1^+/IL10^{-/-}$  mice. Each mouse in the vaccination (n=19) and adjuvant only (n=13) groups received nasal administration of 20 µl of TnMUC1-100mer (30 µg) plus adjuvant (3 µg) in PBS or adjuvant only (3 µg) in PBS. Untreated group (n=30). Mice were monitored for the development of rectal prolapse. \* P=0.042.

Our previous work showed that untreated MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice with rectal prolapse also developed CACC. We examined TnMUC1-100mer vaccinated MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice after their development of rectal prolapse to see how the vaccine affected the development of CACC. Colons were removed and cut into three separate sections; cecum, ascending colon and descending colon. Colon sections were place onto glass slides and examined under a dissecting microscope for the macroscopic appearance of tumors. Tumors were sectioned and stained with H&E and evaluated by a pathologist blinded to the experimental protocol. We found that TnMUC1-100mer vaccination provided an anti-tumor effect (Fig. 19). Six out of eight vaccinated MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice remained tumor free after the development of rectal prolapse. In contrast, only one out of eight untreated mice was tumor free after the development of rectal prolapse. Although TnMUC1-100mer vaccination was unable to completely prevent colonic inflammation, it did prevent the development of CACC.



Figure 19: Vaccination with TnMUC1-100mer plus adjuvant prevents CACC development.

Colons were removed from mice after the development of rectal prolapse. Colons were cut into sections, mounted on glass slides and examined under a dissecting microscope for the macroscopic appearance of tumors. In the vaccine group, 6 out of 8 mice were tumor free. In the untreated group, 1 out of 8 mice was tumor free. In the adjuvant alone group, 3 out of 3 mice developed colon tumor. \* P=0.0406, Fishers exact two-tailed.

# 3.2.2 Antibody and T cell responses in vaccinated mice

Although antibody production is not thought to play a major effector role in anti-tumor immunity, we were interested in the humoral responses elicited by TnMUC1-100 vaccine. Antibody production is not only a direct reflection of B-cell responses, but is also an indirect reflection of CD4<sup>+</sup> T-cell responses. Serum was collected from vaccinated, adjuvant alone and untreated MUC1<sup>+</sup>IL10<sup>-/-</sup> mice and analyzed using a MUC1-specific ELISA assay. We looked for

IgM, IgG1 and IgG3 MUC1-specific antibody isotypes. MUC1-specific IgM isotype was detected in all groups, independent of treatment, suggesting that *de novo* MUC1 expression in the disease can elicit a spontaneous, T-cell independent B-cell response. The vaccine did not appear to induce detectable serum levels of IgG1 or IgG3 T-cell-dependent isotypes (Fig. 20). MUC1<sup>+</sup>IL10<sup>-/-</sup> mice that received adjuvant alone had consistently higher levels of MUC1-specific IgM compared to untreated mice (Fig.17), while the Tn-100mer vaccinate mice had the lowest levels of IgM of all three groups. This suggests vaccine induced changes in the immunized MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. We are currently looking for MUC1-specific IgA antibody in both serum and fecal samples.



# Figure 20: Spontaneous development of MUC1-specific IgM in response to disease and no detectable vaccineinduced isotype switching.

(A) TnMUC1-100mer vaccine does not elicit T-cell dependent antibody isotype switching and has similar T-cellindependent IgM antibody levels compared with (B) untreated mice. Mice that received (C) adjuvant alone had higher T-cell-independent IgM compared with vaccinated (A) and untreated (B). Serum used in A, B and C is from one mouse per group. Data shown are representative of three separate experiments. The main effector cells that mediate anti-tumor immunity are cytotoxic T-cells (CTLs), with CD4<sup>+</sup> T cells providing the requisite help to elicit CTLs. Although we were unable to detect CD4<sup>+</sup> T-cell-dependent antibody isotype switching, we looked in MLN of TnMUC1-100mer vaccinated mice for IFN- $\gamma$  producing MUC1-specific T-cells. Animals were sacrificed at various time points after vaccination, MLN were removed and cells isolated by mechanical disruption. Cells isolated from MLN were *in vitro* stimulated once with DCs loaded with TnMUC1-100mer peptide plus the addition of exogenous IL-2. After six days, isolated MLN cells were harvested and plated at 10<sup>5</sup> cells per well in an IFN- $\gamma$  ELISPOT assay. Isolated MLN of xecinated mice. These cells were MUC1-specific IFN- $\gamma$  producing T-cells in the MLN of vaccinated mice. These cells were MUC1-specific, as stimulation with DCs in the absence of TnMUC1-100mer peptide resulted in a significantly lower number of background spots in the ELISPOT assay (Fig. 21). In contrast, untreated mice had no difference in the number of spots between stimulation with DCs in the presence or absence of TnMUC1-100mer peptide.



Figure 21: IFN-y producing MUC1-specific T cells are present in MLN of vaccinated mice.

Cells were harvested from MLN from (A) vaccinated MUC1<sup>+/</sup>IL10<sup>-/-</sup> mouse and (B) untreated MUC1<sup>+/</sup>IL10<sup>-/-</sup> mouse. Cells were incubated with DCs alone or DCs loaded for four hours with 20µg of TnMUC1-100mer peptide. Data shown are representative of three separate experiments.

Using a standard <sup>51</sup>Cr-release assay, we looked for the ability of the TnMUC1-100mer vaccine to elicit MUC1-specific CTLs in the LN of vaccinated mice. Mice were sacrificed at various time points after vaccination, inguinal and MLN removed and cells isolated by mechanical disruption and pooled. Isolated LN cells were *in vitro* stimulated once with DCs loaded with TnMUC1-100mer peptide plus the addition of exogenous IL-2. After five days, cells were harvested and tested for their ability to lyse MUC1<sup>+</sup> tumor cells. The mouse tumor cell line RMA transfected with full length human MUC1, served as target cells. Transfected

RMA-MUC1 cells express epitopes from both normal and hypoglycosylated forms of MUC1. We found that vaccination with TnMUC1-100mer induced CTLs in the MLN of immunized MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. These MUC1-specific CTLs were capable of lysing MUC1<sup>+</sup> tumors at an effector to target ratio of 50 to 1 (Fig. 22).



# Figure 22: Vaccination with TnMUC1-100mer induces CTLs capable of lysing MUC1<sup>+</sup> tumors.

MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice received TnMUC1-100mer plus adjuvant, adjuvant only or untreated. Cells were isolated from LN and plated at various effector to target ratios. The LN cells were pooled from two mice per group. RMA and RMA-MUC1 served as targets.

Isolated LN cells from vaccinated MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice were also tested for their ability to recognize and lyse MUC1 peptide (SAPDTRPA) loaded target cells in a standard <sup>51</sup>Cr-release assay (Fig.23). Isolated LN cells from vaccinated mice were *in vitro* stimulated with TnMUC1-100mer loaded bone-marrow-derived DCs. After 5 days, LN cells from vaccinated mice were incubated for 40 hours with either RMA or RMA/S cells loaded with the 8 amino acid peptide (SAPDTRPA). The Tap 2-deficient T-cell lymphoma RMA/S cells were pulsed for three hours with the eight amino acid peptide (SAPDTRPA) from the tandem repeat region of MUC1 prior to incubation with LN effector cells. We found that vaccination with TnMUC1-100mer induced MUC1-specific CTLs in the LN of immunized MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. The CTLs were specific for an 8 amino acid peptide (SAPDTRPA) as CTLs incubated with unloaded RMA/S cells did not result in specific target cell lysis.



Figure 23: Vaccination induces CTLs capable of lysing MUC1 peptide loaded targets.

Cells were isolated from LNs of MUC1<sup>+/</sup>IL10<sup>-/-</sup> mice vaccinated with TnMUC1-100mer plus adjuvant. Cells were plated at various effector to target ratios. RMA/S and RMA/S loaded for three hours with MUC1 peptide (SAPDTRPA) served as targets. LN cells were pooled from two mice.

### 3.2.3 Cytokine production in the colon and MLN

We anticipate that the *de novo* expression of hypoglycosylated MUC1 in the colonic epithelium might provide a source of continuous stimulation for cells of the innate immune system that could be analyzed by the cytokines they produce. Similarly, we expect that TnMUC1-100mer vaccination may provide increased numbers or cells of the adaptive immune system in the affected colon that produce another set of cytokines. The effect of the immunization on the disease progression may be due in part to stimulating a different cytokine environment in the colon. Colon samples were collected from TnMUC1-100mer vaccinated and untreated MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice as well as control MUC1<sup>+</sup>/IL10<sup>+/-</sup> mice and incubated in tissue culture medium to release the cytokines. We collected supernatant at 24 and 48 hour time points from the colonic tissue explant cultures and used the Luminex® multianalyte Profiling (MAP) technology to analyze cytokine production in the colon. Briefly, Luminex® MAP technology color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead can be coated with a reagent specific to a particle bioassay, allowing the capture and detection of specific analytes from a sample. We tested for four analytes: IFN- $\gamma$ , IL-12p70, IL-13 and TNF- $\alpha$ . For IFN- $\gamma$  and IL-13, we found that vaccinated mice had higher levels of these cytokines in the colon compared to untreated and control mice (Fig. 24). We were surprised to find no consistent differences in levels of IL-12p70 between vaccinated, untreated and normal mice. Luminex® analysis was unable to detect TNF- $\alpha$  in any colonic explant samples and we suspect that this might have been a problem with the Luminex  $\mathbb{R}$  assay, as excess TNF- $\alpha$  is responsible for mediating intestinal inflammation in the IL10<sup>-/-</sup> mouse. We also looked at the MLN for differences in cytokine production between vaccinated, untreated and normal mice. Isolated cells from the MLN were plated and incubated with phorbol myristate acetate (PMA) and ionomycin. PMA is a protein

kinase C activator and ionomycin is a  $Ca^{2+}$  ionophore. Both reagents are nonspecific stimulators for T-cell cytokine production. We found no consistent differences between groups (Fig.25).



#### Figure 24: Analysis of colonic cytokines.

Colonic tissue explants were cultured in tissue culture medium to release cytokines. Supernatant was collected at 24 h and 48 h time points and cytokines were analyzed by Luminex®. Each bar represents one mouse.



#### Figure 25: Analysis of MLN cytokines.

Cells isolated from MLN were plated and stimulated with PMA/ionomycin. Supernatant was collected at 24 h and 48 h time points. Cytokines were analyzed by Luminex®. Each bar represents one mouse.

# 3.2.4 Regulatory T-cells are not changed by vaccination

The population of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells plays an important role in maintaining immune homeostasis in the colon. We hypothesized that our vaccine may delay the onset of IBD in part by increasing the number of antigen-specific CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells. We

used flow cytometry to look at CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells in the LN and spleen of vaccinated mice, mice treated with adjuvant alone and untreated mice. Isolated cells were stained with anti-CD3 and anti-CD4 antibodies as well as intracellularly stained with anti-FoxP3. We determined the percentage of Foxp3<sup>+</sup> cells within the double positive CD3<sup>+</sup>CD4<sup>+</sup> population. We found no significant difference in the percentages of FoxP3<sup>+</sup> cells between vaccinated and untreated mice (Table 1). In the single "adjuvant only" treated mouse we found a much higher percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells in both the MLN and the spleen (Table 1). However, this experiment represents a small number of animals (2 per group) and does not allow a firm conclusion at this point. Future experiments will look at 4 mice per group.

# Table 1 : FoxP3<sup>+</sup> regulatory cells in MLN and spleen.

Cells were stained with anti-CD3, anti-CD4 and anti-Foxp3. Cell counts are reported as the percentage of FoxP3+ cells in the  $CD3^+CD4^+$  population.

Treatment	Age (wk)	MLN (%)	Spleen (%)
Vaccine	14	5.8	3.6
Vaccine	13	3.5	6.2
No treatment	12	1.6	4.8
No treatment	41	7.9	8.3
Adjuvant	8	15.6	17.4

# 3.3 DISCUSSION

We previously showed that intestinal inflammation in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice mimics human IBD and CACC, characterized by colonic inflammation as well as increased colonic tumor burden compared to IL10<sup>-/-</sup> mice, and the addition of MUC1 plays a role in both diseases. With this in mind, we used hypoglycosylated MUC1 as a target for nasal vaccination to impact on the inflammatory and neoplastic changes that occur in IBD and CACC. Our hypothesis was that during early neoplastic transformation, aberrant cells begin to express hypoglycosylated MUC1, thereby activating the innate immune system. This facilitates the development of chronic inflammation as the innate immune system is unable to eliminate the aberrant cells and the specific adaptive immune system is either slow to develop or impaired. We hypothesized that MUC1 could be used as a vaccine target to effectively prime the specific-adaptive immune system to eliminate the MUC1-expressing aberrant cells and restore the cytokine balance in the colon, thereby preventing or ameliorating chronic inflammation as well as preventing the progression to colon cancer.

Results from our vaccine study show that nasal administration of TnMUC1-100mer peptide plus a mucosal adjuvant, administered early in life (4 weeks of age) and in the disease process, is capable of delaying the onset of IBD and preventing CACC. These results have significant implications in our current understanding of the pathogenesis of IBD and CACC as well as current therapeutic treatments for IBD. Importantly, this work shows that IBD can be delayed and CACC prevented by engaging specific adaptive immunity early in an immune response and directing it against an antigen such as MUC1, that is known to be present at the site of the disease and on the resulting malignancy. We show in the first set of experiments that this might occur through the induction of MUC1-specific CTLs. We found MUC1-specific CTLs in LN of MUC1<sup>+</sup>/IL10<sup>-/-</sup> vaccinated mice, which were able to recognize and lyse MUC<sup>+</sup> tumor cells as well as target cells expressing an 8 amino acid MUC1 peptide (SAPDTRPA). We also detected MUC1-specific IFN- $\gamma$  producing T-cells in the MLN of vaccinated mice, suggesting the induction of the CD4<sup>+</sup> helper T-cell compartment to provide the requisite help for the induction of CTLs. Although we were unable to detect significant differences between vaccinated and untreated mice in the timing and level of colonic or MLN cytokines IL-12p40, TNF- $\alpha$ , and IL-13, we expect that TnMUC1-100mer vaccination has an impact on cytokine production. Our lack of data to support this change is most likely due to the extent of disease in mice at the time of sacrifice as some clearly had more extensive inflammation than others. In future experiments, we will look at colonic cytokine production at three separate time points. Mice will be sacrificed at one week after rectal prolapse, three weeks after rectal prolpase and eight weeks after rectal prolpase. This will allow us to examine the cytokine production during early and late colonic inflammation. We also compared the percentage of Foxp3<sup>+</sup> regulatory T-cells in LN and spleen between vaccinated and untreated mice. However, we did not detect an increase in Foxp3<sup>+</sup> cells.

This work has also shown that NALT is a viable priming site for nasal administration of TnMUC1-100mer peptide vaccination and is capable of imprinting T-cells for homing to the colon. This is supported by our detection of MUC1-specific CTLs and IFN- $\gamma$  producing T-cells in the MLN of vaccinated mice. This is most likely enhanced by the use of the mucosal adjuvant E6020, which has been shown to induce mucosal antibodies in nasal and vaginal washes when administered with ovalbumin or tetanus toxoid antigens (252).

In summary, we found that MUC1-specific vaccination slows the progression to IBD and has an anti-tumor effect in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice, and this is mediated through the induction of MUC1-specific CTLs. We were able to generate mucosal CTLs at the MLN through nasal
administration of TnMUC1-100mer vaccine. Collectively, this work shows that engaging adaptive immunity early in an immune response, chronic intestinal inflammation can be delayed and CACC prevented. Further work in this model will be directed towards identifying and further confirming specific immune mechanisms and their relative importance in this process.

## 4.0 SUMMARY

This work was undertaken in order to provide further insight into the relationship between chronic inflammation and cancer. The current hypothesis is that chronic inflammation causes malignant transformation and promotes tumor progression. However, a cause and effect relationship between chronic inflammation and cancer has not been established. We chose to examine the suspected relationship between inflammatory bowel disease (IBD) and colitisassociated colon cancer (CACC). We believed that we could contribute to the understanding of these two particular diseases because of our knowledge of how the innate and the adaptive immune systems perceive an important molecule found in IBD and in colon cancer, i.e. the tumor antigen MUC1.

MUC1 has been extensively studied by our group as well as others and MUC1 overexpression and glycosylation changes have been proposed to play a role in inflammation and tumorigenesis through altering intracellular signaling, facilitating adhesion and migration of tumor cells and increasing resistance to apoptosis. MUC1 is found in the majority of adenomatous dysplasia, almost all colorectal adenocarcinomas (246, 251), is expressed around areas of ulceration in the gut (171) and MUC1-specific antibodies have been detected in patients with IBD (170, 241). Furthermore, mucin glycosylation changes have also been reported in patients with IBD (110, 240). This led to our hypothesis that *de novo* expression of MUC1 on premalignant lesions forming in the colon are recognized and activate the innate immune system

characterized by acute inflammation (Fig. 26). This response fails to trigger effective adaptive immunity capable of clearing the lesion, and the persistence of the MUC1 expressing lesion leads to chronic inflammation, which in turn could facilitate cancer progression. We proposed that if this hypothesis was correct, then MUC1 could be used as a vaccine target to boost MUC1specific adaptive immunity. Thus, eliminating MUC1<sup>+</sup> premalignant lesions and preventing chronic intestinal inflammation and colon cancer. Given the known involvement of MUC1 in colon cancer and inflammatory diseases, we were surprised when our review of the work published on IBD did not show any reference to the importance of this molecule. Furthermore, among the number of animal models of IBD, or those connecting IBD and colon cancer, none included MUC1. Therefore, the first aim of this thesis was to develop a mouse model that expresses MUC1, develops IBD and CACC. Here, we report the derivation of a new mouse model of IBD and CACC, a MUC1<sup>+</sup>/IL10<sup>-/-</sup> mouse, which more accurately mimics both human IBD and CACC. Our second aim in this study was to use this model to characterize MUC1 expression in the presence and absence of intestinal inflammation. Using this model, we have shown that MUC1 plays a role in the pathogenesis of both IBD and CACC, as the addition of MUC1 accelerates both IBD and CACC in MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice. In addition, we detected MUC1-specific antibodies in the serum from MUC1<sup>+</sup>/IL10<sup>-/-</sup> mice that have IBD. In the absence of inflammation, we do not detect MUC1-specific antibodies. This shows that the immune system is recognizing *de novo* expression of MUC1 in the colon. Our third and final aim in this thesis was to characterize the impact of MUC1-specific vaccination on both IBD and CACC. Importantly, we have shown that we can intervene in the disease process by priming the adaptive immune system, through vaccination against hypoglycosylated MUC1. Thus, slowing the development of IBD and preventing CACC. We show that the MUC1 vaccine mediates its

effects in part through the induction of MUC1-specific cytotoxic T-cells (CTLs) and IFN- $\gamma$  producing T-cells. These findings support our initial hypothesis that aberrant MUC1 expression on the surface of premalignant intestinal epithelial cells can drive the development of chronic inflammation and CACC in the absence of specific adaptive immunity. Furthermore, by preparing the adaptive immune system to recognize aberrant MUC1<sup>+</sup> early in the course of disease, we can ameliorate chronic inflammation and eliminate CACC.

Although not shown, we propose that MUC1 most likely plays a role in the pathogenesis of IBD and CACC via mechanisisms other than in its ability to serve as a direct inflammatory stimulus. First, MUC1 may have a role in the epithelial barrier function. A single layer of epithelial cells line the GI tract and is responsible for tight junctions separating luminal contents from the underlying immune system. This is an important mechanism to maintain unwanted immune responses directed against the commensal gut bacterial flora. The epithelial barrier is a dynamic structure that is actively regulated and maintained and alterations in barrier function have been shown to preced the appearance of IBD (253). We expect that overexpression of MUC1 on the epithelial cells would eventually lead to disruption of the epithelial barrier, thus allowing luminal bacteria to come in contact with the underlying mucosal immune system. Furthermore, alterations in MUC1 glycosylation have been shown to enhance the ability of some pathogenic bacteria to bind to epithelial cells. Together, these changes in the epithelia barrier would lead to exaggerated mucosal immune responses and chronic inflammation. We also expect that MUC1 plays a role in the pathogenesis of CACC through its ability to function as an oncoprotein. MUC1 has been shown to play a role in tumorigenesis through alterations in intracellular signaling, facilitating adhesion and migration of tumor cells and increasing resistance to apoptosis. Specifically, MUC1 has been shown to promote epithelial cell

transformation through its ability to bind to and block the degradation of  $\beta$ -catenin (163, 254). Importantly, accumulation of cytoplasmic  $\beta$ -catenin and subsequent translocation to the nucleus leads to constitutive target gene activation, which has been linked to the majority of colon cancers.



## Figure 26: MUC1 plays a role in the pathogenesis of IBD and CACC.

Alterations in the glycosylation of MUC1 and overexpression of MUC1 on premalignant intestinal epithelial cells lead to its recognition by the immune system. This response fails to trigger effective adaptive immunity capable of clearing the lesion, and the persistence of the MUC1 expressing lesion leads to chronic inflammation which in turn facilitates cancer progression.

This work has many important implications for the prevention/treatment of IBD as well as CACC. It is now appreciated that multiple factors underlie disease pathogenesis in IBD, which contribute to the immunologic diversity seen in IBD. This includes patient's genetic background, disease stage and environmental factors, which impact on patient responsiveness to treatment. In the setting of chronic inflammation, an early cancer diagnosis can be difficult to determine leading to late stage cancer diagnosis for those patients that progress to CACC. This has led to the search for the identification of highly predictive markers of IBD and dysplasia as well as broadly defined targets for therapy or disease prevention. Our results suggest that MUC1 can function as both a marker of disease as well as a target for disease treatment and cancer prevention.

Collectively, the work presented in this thesis strongly supports a new paradigm on the relationship between inflammation and cancer as illustrated in figure 27, where premalignant lesions come first and cause chronic inflammation. Cellular changes that are associated with the formation of premalignant cells can be recognized by and activate the immune system. This in turn leads to the presence of a driving stimulus that perpetuates chronic inflammation, which now becomes detrimental to the host and facilitates the progression to cancer. Importantly, this work has shown the utility of early vaccination for the prevention of cancer.



Figure 27: A new paradigm on the relationship between inflammation and cancer.

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