INFLAMMATION ASSOCIATED CANCERS AND POTENTIAL THERAPEUTICS

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Abstract

Inflammation is a major contributing factor in approximately 20% of cancers, and two types of cancer in which inflammation plays a particularly crucial role are mesothelioma and colorectal carcinoma. Both mesothelioma and colon cancer are induced by the production of TNFα. Moreover, these two cancers are both largely driven by inflammation resulting from macrophage activation. Mesothelioma arises from the transformation of mesothelial cells induced by inflammatory mediators secreted during the aberrant activation of macrophages exposed to asbestos particles. Similarly, colorectal cancer involves the transformation of epithelial cells lining the intestine and tumor progression may be driven by inflammation resulting from macrophage activation by commensal bacteria of the gut. The work described in this thesis focuses on mouse models of mesothelioma and colorectal cancer in an attempt to dissect molecular mechanisms that regulate tumor progression and identify possible intervention strategies for each type of cancer.

In the case of mesothelioma we investigated intervention with increasing dietary concentrations of selenium in order to see if the increase in antioxidant enzyme activity could aid the anti-cancer immunity. Independent of any effects on anti-cancer immunity, we found that increasing dietary selenium altered the redox status of certain mesothelioma tumors in a manner that increased tumor progression. Select mesothelioma cell lines were identified that were capable of using higher levels of bioavailable selenium to grow more rapidly and this led to increased in vivo tumor progression.
In our second project involving colorectal cancer, we focused on calpain enzymes and their endogenous inhibitor, calpastatin. Our earlier work showed that calpastatin is dynamically regulated in the inflammatory activation of macrophages but not in other immune cell-types and that macrophages express calpain-2. This led us to our hypothesis that using a synthetic inhibitor to decrease calpain-2 activity beyond the normal regulation of calpain-2 activity via the endogenous inhibitor calpastatin will protect mice from colitis and inflammation driven colorectal cancer. Calpain-2 inhibitor treatment showed a significant protective effect against colitis and colorectal cancer in the mouse model of colitis and CAC.
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List of Abbreviations

MM- malignant mesothelioma
ROS- reactive oxygen species
ERK- extracellular regulated kinase
pERK- phospho extracellular regulated kinase
tERK- total extracellular regulated kinase
GSH- reduced glutathione
NAC- N-acetyl-cysteine
GPx- glutathione peroxidase
TAYE- threonine to alanine/tyrosine to glutamate mutant
DHCF- dihydrochlorofluorescein
MAPK- mitogen activated protein kinase
s.c.-subcutaneous
IBD – Inflammatory Bowel Disease
CAC – Colitis Associated Carcinoma
Ip – intra-peritoneal
IL- interleukin
TNF – tumor necrosis factor
MCP – monocyte chemotactant protein
AOM- azoxymethane
MFI- mean fluorescence activity
MUC2- mucin 2
SHP-1/2- Src homology region 2 domain-containing phosphatase-1
Ly5.1/5.2 - Protein tyrosine phosphatase receptor type 1 isoforms

DSS - Dextran sulfate sodium
Chapter 1: Introduction
1.1 Mesothelioma and selenium

Dietary selenium is an essential micronutrient that is important for many aspects of human health. The biological effects of selenium are exerted mainly through the actions of the selenoproteins. The human genome encodes for 25 selenoproteins and several of these are enzymes that play important roles in regulating cellular redox status. Within cells, dietary selenium may also be metabolized into intermediate compounds such as methylselenol that affect redox homeostasis and may exert toxic effects on cancer cells. An abundance of preclinical findings together with some clinical data have suggested that selenium supplementation may prove to be an affordable, effective means to prevent or treat a wide variety of cancers. However, the effectiveness of selenium supplementation for cancer prevention has been inconsistent and somewhat controversial. These inconsistencies may be due to differences in the forms of selenium supplementation used, baseline selenium status of the participants in different populations, or other study design factors. In addition, certain selenoproteins have been shown to actually promote tumor progression, which highlights the importance of identifying the molecular mechanisms by which dietary selenium influences the development of each type of cancer.

Malignant mesothelioma (MM) is a deadly cancer associated with asbestos or erionite exposure for which no successful therapies are currently available. MM is among the most aggressive tumors, arising from the mesothelial cells that line the pleura, peritoneum, and occasionally the
pericardium\textsuperscript{15}.

It is currently thought that asbestos exposure leads to accumulation of asbestos in the alveoli of the lungs or the gastric crypts of the intestines and causes physical damage to the cells. This physical damage leads to necrosis and the release of proteins not normally found in high concentrations outside of cells. One protein in particular, a nuclear protein HMGB1, has been identified as a protein that acts as an inflammatory cytokine and chemoattractant for macrophages\textsuperscript{14,15}(Figure 1). Macrophage driven inflammation and the production of cytokines such as TNF\(\alpha\) leads to NF-\(\kappa\)B activation in the mesothelial cells and promotes survival in DNA damaged cells and mesothelial transformation\textsuperscript{16}.
Figure 1 Mesothelioma is conventionally thought to arise from inflammation driven by macrophage response to asbestos induced necrosis.

This figure illustrates the current model suggested for the inflammation driven transformation of mesothelial cells to mesothelioma. In this model asbestos collects in the alveoli of the lungs and causes physical damage to the cells leading to necrosis. Necrosis leads to the release of nuclear proteins such as HMGB1. HMGB1 acts as an inflammatory signal that activates macrophages which then secrete TNFα. With excessive exposure to TNFα mesothelial cells that might otherwise undergo apoptosis are able to survive. If survival signals are activated in mesothelial cells with DNA damage to a tumor suppressor or oncogene, there may be a transformation event leading to mesothelioma. 

Dietary selenium has been proposed to play a chemopreventive role in the treatment of MM, although the data are limited. In one study involving a MM cell line, selenium was shown to inhibit MM cell growth and induce apoptosis in a dose-dependent manner. However, the levels of selenium required for these effects were extremely high, and other in vivo studies involving selenium, as well as vitamins A and E did not show beneficial effects from any of these dietary antioxidants on tumor development. The key difference between these studies helps to shine some light on the different mechanisms at work. In vitro, selenium levels exert their antioxidant protective effects between 10-100 nM concentrations while its cytotoxic effects are likely to occur in the µM range of concentrations. The physiological nM concentrations of selenium are determined by the ranges in which supplementation increases the production of selenoproteins. This is defined as such because increases in dietary selenium in vivo lead to increases in selenoprotein production.

This is important because the daily recommended maximum intake of selenium is 400 µg/day oral intake while the current treatments with selenium for cancer patients involves the use of 350 µg/day intravenous injection. This high dosage circumvents the normal physiological metabolism of selenium and allows a higher level of bioavailable selenium for cancer tissue. In this manner, the selenium is not only metabolized into antioxidant selenoproteins but is also shunted into selenium metabolites that are pro-oxidant and toxic to cancer cells. As a result of these high concentrations, cytotoxic effect of selenium will also be toxic to the normal cells of a patient. In fact, the trials showing some successful
anti-cancer effects are doing so by delivering bolus doses of selenium directly to the bloodstream at toxic levels which suggests these approaches more resemble chemotherapy than a dietary treatment.

These cytotoxic effects of selenium are due to the dual redox nature of selenium in living organisms. As stated above, selenium can be incorporated in the selenocysteine residue through a recoding of the conventional UGA stop codon. This selenocysteine residue is incorporated into selenoproteins at the site of UGA due to cis-elements in the 3’ noncoding region of the mRNA and protein factors that redirect translational machinery to insert selenocysteine. Many selenoproteins utilize the selenocysteine residue to direct oxidoreductase reactions that result in the reduction of reactive oxygen species or oxidized macromolecules. Examples include the glutathione peroxidase family, thioredoxin reductase family, and methionine sulfoxide reductase.

The other side of the selenium reactive oxygen species story is the fact that selenium in excess of that which can be shuttled to and used by the selenoprotein synthesis machinery may be metabolized into inorganic and organic selenium species that actually have pro-oxidant activity (Figure 2). The counter-intuitive aspect of this process is that both the anti- and pro-oxidant processes involving selenium largely involve reduced glutathione (GSH).
Figure 2 Selenium exerts both anti and pro oxidant action depending on concentration

This figure shows the pathways of resulting disease for selenium deficient, and selenium excessive diets. Under selenium deficient conditions, selenoprotein concentrations fall and oxidative stress rises resulting in diseases such as White muscle disease and Keshan’s disease. Selenium excess results in thiol and glutathione oxidation decreasing the cells ability to regulate reactive oxygen species leading to DNA damage and apoptosis.
Reduced GSH is a tripeptide of cysteine, glycine, and glutamate with the sulfur of the cysteine being the target residue for its reducing activity. Under physiological selenium conditions glutathione peroxidase catalyses the reduction of hydrogen peroxide into water using reduced GSH as a cofactor and thereby generating oxidized glutathione (GSSG). Under excessive selenium conditions selenium reacts directly with GSH to form GSSG and superoxide. The formation of superoxide and reduction of GSH and reduced thiols is one of the mechanisms by which selenium exerts its cytotoxic effect.

Given that the physiological effects of selenium vary under different circumstances depending on the complex redox role this micronutrient may play and that the role of redox in MM is only beginning to be understood, it is essential that insight be gained into how selenium status affects MM tumor development and progression. MM incidence is on the rise in the U.S. and other parts of the world. Selenium intake varies in different geographic regions and is affected by behavioral and environmental factors. With selenium supplementation being considered as a potential treatment approach for MM and other cancers the question of efficacy and risks is of immediate importance and significance.

An emerging perspective of the role that selenium may play in chemoprevention has focused on the different stages of tumor development: the initial carcinogenesis events involving DNA repair in which increased selenium may be beneficial, and the subsequent progression of established tumors for which the effects of increasing selenium intake may vary. This differentiation is
important to consider because the effects of an antioxidant on carcinogenesis would presumably differ from tumor progression. If an antioxidant can protect from DNA damage, then increasing dietary intake should reduce the formation of potential cancers. However, the effects of selenium in dietary concentrations on the progression of an established tumor depends on the type of cancer cells arising as well as their redox state. To more specifically address the issue of whether different levels of selenium intake influence the progression of MM tumors after they have been established, we utilized several different MM cell lines generated by administration of asbestos particles into mice. Surprisingly, we found that increasing selenium intake did not limit the growth of any of the MM cell lines and higher selenium levels actually promoted proliferation, mobility, and in vivo tumor progression for some MM cell lines. We subsequently identified molecular mechanisms affected by higher selenium intake involving redox sensitive signaling pathways converging at the point of ERK phosphorylation.

This research is timely and important when we consider that dietary interventions are being considered for many cancers. Current research has suggested that supernutritional levels of selenium may protect against some cancers including mesothelioma. Due to the fact that conventional cancer treatments such as surgery and chemotherapy have been largely unsuccessful in the case of mesothelioma new treatments are constantly being considered in advanced patients. Overall, the data presented herein provide crucial insight into the mechanisms by which dietary selenium affects MM tumor progression and suggests that the use of selenium supplementation to treat MM may in some
cases be more harmful to the patients.

1.2 Inflammatory bowel disease and calpastatin

We have previously discussed the inflammatory environment mediated by macrophages that leads to the development of MM. MM lung cancer is caused by the combination of tissue damage induced by asbestos and the inflammation caused by responding inflammatory macrophages. In contrast CAC is caused by the inflammation (IBD) resulting from macrophage activation by gut microbiota. This is thought to occur due to loss of normal mucin lining in the gut, aberrant activation of immune cells, and/or alterations in the composition of gut microbiota.

Inflammatory bowel diseases (IBD) are comprised of the chronic inflammatory disorders Crohn’s disease and ulcerative colitis. The specific tissues of the gut affected by these two disorders are different, but both types of IBD share symptoms indicative of chronic intestinal inflammation. Recent progress has been made in understanding the onset and progression of IBD pathology, particularly how interactions between the mucosal immune system and the microbiota drive the initiation and progression of the diseases. IBD arises due to the interaction of microbiota and the immune system through the mucosal barrier and therefore when considering these diseases, one should consider these three factors as the major points of interest.

Emerging evidence suggests that one way IBD may arise is from a disruption of the normal Mucin layer produced by the cells of the gut.
Genome wide association studies of IBD have found a link to MUC2 mutations and suggest that low MUC2 production by the goblet cells of the gut and improper MUC2 structure are major contributors to the disease \(^{33}\) \(^{34}\). There is also evidence that disturbances in the composition of the different gut flora can act to alter the Mucin expression and stability \(^{32}\).

Mucin production has been demonstrated to be upregulated in the presence of certain gram positive bacteria and is therefore an important contributor to homeostasis that is based on feedback from the gut environment \(^{35}\) \(^{36}\). This balance is sensitive to homeostatic disturbance as illustrated by studies showing that dietary and environmental factors can alter balances of normal gut flora and result in IBD \(^{37}\) \(^{38}\). Some clinicians have turned to dietary modulation in an attempt to influence the populations of gut microbiota with limited success \(^{39}\) \(^{40}\) \(^{41}\). There is also evidence that the involvement of both normal and abnormal gut microbiota can serve as initiators of pathology. IBD arising in the presence of normal gut microbiota, in the absence of a sufficient Mucin layer, or with the absence of regulatory cytokine signaling demonstrates the importance of all three factors \(^{42}\) \(^{43}\). One example that highlights the delicacy of this balance is that the removal of the anti-inflammatory cytokine IL-10 alone has been demonstrated to be enough of a release of normal restraints on the immune system to cause spontaneous colitis in mice \(^{44}\). However, colitis does not arise in IL-10 deficient mice maintained in pathogen-free conditions, which emphasizes the etiological role of commensal bacteria in the disease process.
Many of the same factors that are involved in initiation of IBD also serve to exacerbate the disease. Absence of immunomodulators or excess of inflammatory stimulators can obviously have significantly negative effects on IBD progression. Many cytokines exhibit pleiotropic activity. This means that they can function in different ways in different contexts. Among these pleiotropic cytokines are IL-10, TNFα, and IL-6. IL-10 can act as a modulator of inflammation and low serum levels have been shown to be involved in some cases of IBD but treatment with IL-10 does not significantly alleviate the symptoms of IBD in clinical trials. This result was very surprising given that IL-10 knock out in mice resulted in spontaneous colitis and treatment with IL-10 was enough to alleviate the disease. These odd results are suspected to be due to the inflammatory effects of IL-10 signaling and demonstrate the complexity of cytokine signaling in disease. Because introduction of “anti-inflammatory” cytokines like IL-10 have not produced promising results, the focus for treatment options has fallen primarily on intervention strategies that interrupt inflammatory disease progression.

In order to mimic the normal progression of the human disease a mouse model was developed that involves the use of Dextran Sodium Sulfate to disrupt the Mucin lining of the gut. The loss of Mucin allows the invasion of the epithelial tissue by gut commensal bacteria leading to infection and an inflammatory response. This inflammatory response is triggered by the activation of dendritic cells and macrophages through TLR2 receptor recognition of pathogen associated molecular patterns (PAMPs) and resulting activation of
inflammatory response through NF-κB signaling\textsuperscript{48 49}. The PAMPs are derived from the gut microbiota and primarily include bacterial peptidoglycan, lipoteicoic acid, and lipoproteins\textsuperscript{50 51}. Then, through antigen processing/presentation and INFγ, IL-6, IL-12, IL-18, and IL-24 cytokine signaling the inflammatory macrophage and T cell response follows\textsuperscript{49}. It has been suggested that the lingering chronic inflammation produced by macrophage production of TNFα and IL-6 may exacerbate the disease\textsuperscript{52}. In order to effectively treat this disease more information is needed regarding cellular factors in immune cells that regulate disease susceptibility or pathology in IBD patients\textsuperscript{53 54}. Identification of specific factors that function to limit hyperactivation of intestinal immune cells is a crucial step toward understanding and treating these and other chronic inflammatory disorders.

Recent publications suggest that some inflammatory conditions may be driven by the actions of calpains, which are cytosolic Ca\textsuperscript{2+}-activated cysteine proteases that cleave specific targets to modulate cellular functions\textsuperscript{55 56 57 58}. There are two major isoforms of this enzyme, μ-calpain (or calpain-1) and m-calpain (or calpain-2), which require µM and mM Ca\textsuperscript{2+} concentrations for activity, respectively\textsuperscript{59}. Calpain enzymes are comprised of an 80 kDa catalytic subunit and a 30 kDa regulatory subunit. Activation occurs after Ca\textsuperscript{2+}-binding induces conformational changes that lead to auto cleavage of the N-terminal inhibitory domain of the 80 kDa subunit\textsuperscript{60 61}. Following activation by Ca\textsuperscript{2+}, calpain cleaves a specific subset of cellular proteins, including cytoskeletal proteins, membrane receptors, calmodulin binding proteins, G proteins, protein kinase C and other
signal transduction, and certain transcription factors\textsuperscript{62}. Because the Ca\textsuperscript{2+}-induced activation of calpain is an irreversible reaction, an additional mechanism has evolved to modulate its activity. This mechanism involves the protein calpastatin (CAST), which is an endogenous inhibitor that binds strongly to calpains. CAST contains four tandem repeats of a calpain inhibitory domain and each CAST molecule is capable of inhibiting more than one calpain molecule\textsuperscript{63}. Given that calpains are involved in inflammatory activation of immune cells and CAST regulation of calpain activity may inhibit this inflammatory function of calpains, it is critical that we develop a greater understanding of the system and its underlying mechanisms. This knowledge may lead us to a better understanding of inflammatory diseases such as IBD and a framework to develop therapeutics targeting this mechanism.

1.3 IBD, CAC, and calpain

IBD is on the rise in developed nations and across the globe and as chronic inflammatory disease increases so does the resulting inflammation associated cancers. This disease has an estimated health care cost of $1.7 billion dollars in the United States and the prevalence rate is 400/100,000 worldwide\textsuperscript{64}. The patients show disease onset from ages 15-30. It is a painful disease and often results in bloody diarrhea. These patients also have a 2-3 fold increased likelihood of developing CAC as a result of the chronic inflammation\textsuperscript{65}. The cancers are thought to be driven by imbalances in cytokines, growth factors, reactive oxygen species, and reactive nitrogen species\textsuperscript{66}\textsuperscript{67}. There have been
many intervention strategies and one among them has been an attempt to regulate inflammation. Regulation of inflammation has gained much biomedical research attention as the integral involvement of inflammation in diverse disease such as obesity/diabetes, cancer, asthma, and arthritis has become more clearly defined. Our lab has recently demonstrated that the calpain proteases are involved in macrophage driven inflammation and due to the dynamic endogenous regulation of this protease it may serve as a potential target for intervention.

The calpain family of calcium dependent cysteine proteases consists of 15 isoforms with calpain-1 and calpain-2 being the most highly and ubiquitously expressed. These proteases differ from other proteases in that they act as modulators of protein function through their proteolytic activity rather than as degraders of protein. Calpains proteolytically modulate target proteins by cleaving between domains, and substrates identified thus far include more than one hundred proteins such as talin, vinculin, p53, c-fos, PKC, rac, IκB, and more. Calpains 1 and 2 are the most abundantly expressed enzymes in most tissues and these isoforms are the focus of our studies. However, it should be noted that other calpain isoforms that have been defined in certain tissues such as the eye and brain and may need to be considered in future studies for their potential effect on inflammatory disease.

Overall, calpain enzymes are found at high levels throughout various tissues. Calpains 3 and 5 can be found in skeletal muscle and the lens and retina. There has been some data showing that the cleavage products of calpains 2 and 3 in the eye can lead to cataract development while calpain 5 has been
associated with vitreous inflammation. Calpains 8 and 9 are especially relevant enzymes to consider for the purposes of this study due to their expression in the gut. However, calpains 1 and 2 are more highly expressed in the gastrointestinal tract compared to these other isoforms, particularly in the immune cells of the gut. It has been previously demonstrated in mouse KO models that calpains 8 and 9 form a complex and that knockout of either enzyme can lead to greater alcohol induced gastric lesions. The mechanism of this gastric protection has not been elucidated but the authors demonstrated that neither granule production nor Mucin production by the pit cells were affected.

Our laboratory has previously demonstrated that calpain-2 is the predominant calpain in macrophages and that its activity is dynamically regulated by CAST during macrophage activation. We have shown that CAST protein increases within 30 min of activation and peaks by 2 h, leading to rapid inhibition of calpain-2 activity. Our previous work suggested that this increased CAST was required for the 'proper' level of activation to occur in macrophages, and the focus of our current studies are to determine how impairment in this CAST upregulation process may affect macrophage activation and diseases driven by macrophage activation like IBD.

Macrophages are one of the largest cellular contributors to the inflammatory cytokines that lead to pathology in IBD and other inflammatory diseases. IL-6 and TNFα have been shown to be integral cytokines in IBD and colitis associated carcinoma along with NF-κB and STAT3 signaling. It has been demonstrated, in the mouse model of dextran sodium sulfate induced colitis
and inflammation induced CAC, that with inactivation of IKKB/NF-κB \(^{82}\), TNFR \(^{83}\), or IL-6 \(^{84}\) there is less colitis and less CAC. In these models, the inactivation of cytokines were immune system specific, implying that cells such as macrophages, when inhibited in NF-κB signaling, TNFα production, or IL-6 production, produce less IBD which results in less CAC.

Activation of the NF-κB/STAT3 signaling axis in macrophages and dendritic cells leads to the production of the inflammatory cytokines TNFα, IL-6, and IL-23. This creates the initial inflammatory environment leading to the activation of macrophages and T-cells which produce TNFα, IL-6, IL-12, IL-23, and IL-32β \(^{85}^{86}\). IL-6 is important for T-cell survival \(^{87}\). IL-12 is an inflammatory cytokine and it was previously suggested that IL-12 and TNFα were the most likely cytokines to drive inflammation in IBD \(^{88}\). Interestingly, monoclonal antibody therapies targeting these cytokines have shown mixed results \(^{88}\). IL-23 is involved in Th17 cell differentiation and recruitment \(^{89}\). TNFα and IL-6 may both act to promote carcinogenesis and tumor growth. TNFα stimulates NF-κB signaling and allow escape from some normal proliferation checks in the cell cycle. This increases the chance that cells with DNA damage might escape normal tumor suppression mechanisms. IL-6 production and autocrine signaling and chemotherapy resistance is well documented \(^{90}^{91}\). All of this taken together draws a picture in which inflammatory macrophages accumulate in the colon, call other immune cells to the site of inflammation, and ultimately produce unchecked inflammation and IBD/colitis associated carcinoma \(^{92}^{93}^{94}\).
As a result of this cytokine signaling in IBD, one of the most effective treatment regimens has been the use of anti-TNFα and other inhibiting antibodies \(^{95-96}\) (Figure 3). These drugs have taken the form of monoclonal antibodies such as Remicade and Humira or the soluble receptor Enbrel \(^{97-98}\). They have been effective in treating IBD in some studies but it is recognized that they may increase risk for infection \(^ {99-100}\). TNFα inhibition potentially leads to a weakening of the immune system but paradoxically some patients receiving TNFα inhibition therapy have been shown to develop auto-immune disease with the treatments \(^{101-103}\). These include psoriasis and arthritis in IBD patients undergoing anti-TNF treatments \(^{104}\).

This suggests that new treatments and more options for patients experiencing these types of side effects are needed. The calpain/CAST system may represent a unique opportunity for intervention. Calpain proteases have many targets in different tissues and during development. We have previously demonstrated that CAST acts to regulate inflammatory response in macrophages. The primary pathways for the inflammatory activation of macrophages through TLRs involves NFkB or AP-1 signaling. We previously interrogated the mediators of AP-1 activation; ERK, p38, and JNK. We found that the activation of these was unaffected in CAST KO mice suggesting that the calpain/CAST system worked primarily through NFkB. Calpain-2 proteolytically activates the inflammatory response through cleavage of the NFkB regulatory subunit IkB and CAST regulates this pathway through inhibition of calpain-2. This suggests that the use of a calpain-2 specific inhibitor to treat colitis may act to
alleviate the macrophage driven symptoms of IBD and help to prevent the inflammation driven colon cancers derived from chronic inflammation.
Figure 3 Inflammatory cytokines are increasingly targeted with inhibiting antibodies for IBD treatment

The current treatments for IBD are focused on inhibitory antibodies which have shown significant promise. These antibodies often target such proteins as TNFα, IL-6, and IL-12. These are all produced by inflammatory macrophages and could possibly be regulated more effectively with a calpain-2 inhibitor at the source of the inflammation.
Chapter 2: The effect of dietary selenium on mesothelioma tumor development
2.1 Abstract

To determine how increasing dietary selenium influences the progression of malignant mesothelioma (MM), four different MM cell lines were cultured in media containing increasing amounts of sodium selenite (30, 50, and 80 nM). Increasing selenium increased density dependent proliferation and mobility for MM cell lines CRH5 and EKKH5 but not AB12 and AK7. Comparison of these cell lines revealed that ERK phosphorylation was sensitive to increases in selenium in CRH5 and EKKH5 but not AB12 and AK7 cells. Stable expression of a dominant negative mutant ERK eliminated the effects of increasing selenium. Because ERK is redox sensitive, we compared the MM cell lines in terms of glutathione levels and the capacity to reduce exogenous hydrogen peroxide. Increasing selenium led to higher glutathione and reducing capacity in CRH5 and EKKH5 but not AB12 and AK7.

The reducing agent N-acetyl-cysteine eliminated the effects of selenium on ERK activation, proliferation, and mobility. Mice fed diets containing increasing levels of selenium (0.08, 0.25, 1.0 ppm) exhibited increased tumor progression for CRH5 but not AB12 MM cells, and in vivo N-acetyl-cysteine treatment eliminated these effects. These findings suggest that the effects of dietary selenium on MM tumor progression depend on the arising cancer cells' redox metabolism, and those tumors able to convert increased selenium into a stronger reducing capacity actually benefit from increased selenium intake.
2.2 Introduction

Dietary selenium is an essential micronutrient. It has been shown to act primarily through the action of Selenium containing proteins collectively referred to as Selenoproteins. These proteins are often reducing enzymes due to the selenium residues’ ability to transfer hydroxide groups in an intermediate to sulfur containing reducing agents such as thioredoxin or glutathione.

We hypothesized that dietary selenium through its antioxidant activity, in combination with its known general cancer and mesothelioma specific cytotoxicity might be protective against mesothelioma tumor growth.

One possible mode of the protective effects of selenium could be immune response modulation. We and others have previously demonstrated that increasing dietary selenium can significantly augment immune cell function. One of the hallmarks of mesothelioma and cancers in general is the escape from normal immune surveillance and the alteration of immune cell function to suit the needs of a growing tumor. We hoped that selenium augmentation of inflammatory response might allow the immune system to overcome the suppressive effects of a growing mesothelioma tumor.

Another possible role for dietary selenium might be in causing tumor specific reactive oxygen stress. This might occur through the selective localization of excess selenium in the cancer cells leading to the generation of oxidative stress through the direct action of selenium on GSH to produce superoxide as noted in chapter 1.
2.3 Materials and methods

2.3.1 Mice, cell lines, and reagents

C57BL/6 and Balb/c mice originally obtained from Jackson Laboratories were used to generate colonies. Male weanling mice were maintained on diets containing different levels of selenium (0.08, 0.25, 1.0 ppm selenium) for a minimum of 4 wks to effectively establish selenium status as previously described. At 8-10 wks of age, mice were used for experiments and all animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee. The MM cell lines AB12, AK7, CRH5, and EKKH5 were generated as previously described by the peritoneal injection of asbestos, and cultured in F-12 media 10% FBS (GIBCO/Invitrogen, Waltham, MA). The selenium content of the FBS was determined by IC-MS (West Coast Analytical Service, Santa Fe Springs, CA), and increasing levels of selenium established in the culture media by addition of sodium selenite (Sigma, St. Louis, MO) to the complete F-12 media at a final concentration of 30 nM, 50 nM, and 80 nM. Cells were maintained under these conditions for 1 wk prior to experimentation to establish selenium status. The reducing agent N-acetylcysteine (NAC) was purchased from Sigma.
2.3.2 GPx Activity and GSH assays

After cells were cultured for at least 1 wk in low (30 nM), medium (50 nM), or high (80 nM) levels of selenium, cell pellets were collected and cells lysed and GPx activity analyzed using a Bioxytech® GPx-340 Colorimetric Assay Kit (Oxis Research, Foster City, CA). Total protein content was measured using a Bradford Assay (Bio-Rad, Berkeley, CA). GPx activity was normalized to protein concentration for each sample. For GSH measurement, cell pellets were harvested in a similar manner and GSH levels measured using a Bioxytech® GSH-400 (Oxis Research) and normalized to protein concentration.

2.3.3 Cell proliferation and mobility assays

After one week of maintenance in media with low (30 nM), medium (50 nM), or high (80 nM) selenium, cell lines were plated at a density of $10^3$ cells/well in a 96-well plate in 200 μL of fresh media. Media was replaced every 24 hr to prevent selenium depletion. Every 24 hr cells were lysed and quantified using Cyquant Fluorescent Dye (Invitrogen) and fluorescence detection on a Spectramax M3 (Molecular Devices, Sunnyvale, CA). After cells were cultured for 1 wk in low (30 nM), medium (50 nM), or high (80 nM) levels of selenium, cells ($10^6$ cells/well) were plated in 6-well plates 24 hr before performing the scratch assay. For this assay, a 200 μL pipette tip was used to create 4 vertical scratches in the adherent monolayer. The cells were washed with PBS and then covered with 2 mL of low, medium, or high selenium media. A Ziess Axioscope with mounted camera was used to capture images for 10 fields of each cell line in
each condition at 0 and 6 hr post-scratch. The images were then analyzed using ImageJ (NIH) to compare the change in area of the scratch at each point \(^1\).  

**2.3.4 Western blots and real-time PCR**

Cell pellets were harvested and lysed in CellLytic MT buffer (Millipore, Billerica, MA) using a probe sonicator. In the case of measuring pAKT and pERK, cells were cultured in 0.2% FBS media for 24 hr prior to protein isolation. Protein concentration in the lysates was measured by Bradford assay reagent (Bio-Rad) and 15 \(\mu\)g total protein was combined with reduced Laemeli buffer, boiled at 95°C for 10 min, cooled on ice, and loaded into wells of 10 - 14.5% polyacrylamide gels (Bio-Rad). After gel electrophoresis, protein was transferred to PVDF membranes, which were blocked for 1 hr with low fluorescence blocking agent (Li-Cor, Lincoln, NE) and incubated with primary antibodies including anti-pAKT, anti-total AKT, anti-pERK, anti-total ERK (Cell Signaling, Inc., Beverly, MA), anti-GPx1 (Laboratory Frontier, Inc., Antioch, CA), or anti-\(\beta\)-actin (Li-Cor, Inc.). After washing with PBS, membranes were incubated with secondary antibodies from Li-Cor for 1 h, membranes washed with PBS, and signals detected with densitometry conducted using the Li-Cor Odyssey imaging system. Real-time PCR was performed as previously described \(^2\) using primers: c-fos fwd CTCCCGTGTCACCTGTACT; c-fos rev TTGCCTTCTCTGACTGCTCA; actin fwd TGACAGGATGCAGAAGGAGA; actin rev CGCTCAGGAGGAGCAATG.
2.3.5 Subcutaneous Tumor Growth

Weanling mice were fed low (0.08 ppm), medium (0.25 ppm), and high (1.0 ppm) selenium diets for 4 wks. These mice were then subcutaneous injected with $10^6$ AB12 or CRH5 MM cells. The mice were monitored for 6-7 weeks and tumor volume measured using digital calipers as previously described \(^3\). The mice were sacrificed when tumors reached volumes that threatened the health of the mice (volumes >1,500 mm\(^3\)).

2.3.6 Stable transfection

The CRH5 cell line was transfected with a plasmid encoding the dominant negative form of ERK (TAYE-ERK; a kind gift of Dr. James Turkson, University of Hawaii Cancer Center, Honolulu, HI) and Lipofectamine reagent (Invitrogen). Stably transfected cells were selected by culturing cells in media containing G418 (Sigma). A killing curve of 100 \(\mu\)g/mL - 1000 \(\mu\)g/mL revealed that 300 \(\mu\)g/mL was optimal dosage for selection and G418 was removed after the TAYE-ERK CRH5 cell line was established.

2.3.7 Statistical analyses

GraphPad Prism version 4.0 (GraphPad, La Jolla, CA) was used to compare means of two groups using an unpaired Student’s \(t\) test. For three groups, a one-way Anova was used followed by a Tukey post-test to compare means. Standard curves and regression analyses were also conducted using
GraphPad Prism version 4.0. All comparisons were considered significant at P < 0.05.

2.4 Results

2.4.1 Certain MM cell lines derive a proliferative and migratory advantage from increasing selenium concentration

MM cell lines AB12, AK7, CRH5, and EKKH5 were cultured in media containing increasing levels of selenium. Activity of the selenoenzyme, glutathione peroxidase (GPx), was measured to confirm biological effects of increasing selenium in the media and the effects of increasing selenium on proliferation was then evaluated (Figure 4a). While all cell lines equivalently utilized the bioavailable selenium for increasing GPx activity, the effect of selenium levels on proliferation differed between cell lines (Figure 4b). The MM Cell lines CRH5 and EKKH5 exhibited two stages of growth characterized by an early selenium-independent phase and as the cells approach confluency showing higher proliferation with increasing selenium concentration (Figure 4). In contrast, proliferation of AK7 and AB12 cell lines were not influenced by increasing selenium levels. We next evaluated the MM cell lines for the effects of selenium.
Figure 4 Increasing selenium levels increases the proliferation of select MM cell lines

Cellular GPx activity increases with increasing selenium levels [low (30 nmol/L), medium (50 nmol/L), and high (80 nmol/L)] in all MM cell lines (AK7, AB12, EKKH5, and CRH5). (a) A total of 10^3 cells/well were plated and allowed to grow to confluence with media changes daily. (b) Proliferation of AK7 and AB12 cells was not affected by selenium levels, whereas CRH5 and EKKH5 cells showed an increase in proliferation with increasing levels of selenium during the late stages of growth. Data represent means± SEM. Means of each selenium group were compared at each time point. Each experiment was repeated at least twice. *P < 0.05. MFI= mean fluorescence intensity.
Figure 5 Increasing selenium levels affect the mobility of CRH5 and EKKH5 MM cell lines similar to proliferation

Four different MM cell lines were cultured in media containing 30, 50, and 80 nmol/L selenium for 1 week, cells were replated, and allowed to grow to confluency. The plates were scratched with a 20 µl pipette tip. Each experiment was repeated twice. Images from the scratch assays captured at 0 and 6 hours show mobility. (a) Representative images at 6 hours are shown. (b) Quantification of the scratch volume was calculated by ImageJ software. Data represent means ± SEM. *P < 0.05.
2.4.2 ERK phosphorylation is increased with increasing selenium in the CRH5 and EKKH5 MM cell lines

To investigate signaling pathways affected in the selenium sensitive versus insensitive MM cell lines, we evaluated two important pro-growth signaling molecules associated with proliferation and mobility in mesothelioma, ERK and AKT. CRH5 and EKKH5 cells that responded to higher levels of selenium with increased proliferation and mobility above also showed by Western blot analyses to have elevated phosphorylated ERK (pERK) with increasing selenium (Figure 6a). The cell lines that did not functionally benefit from increased selenium intake above, AB12 and AK7, did not exhibit changes in pERK with increasing selenium. Only CRH5 cells showed slightly increased pAKT with increasing selenium levels, while selenium levels did not affect pAKT in the other cell lines.

Since the effects of increasing selenium on proliferation and mobility shown above correlated most consistently and clearly with ERK activation, we next generated a CRH5 cell line stably expressing the ERK dominant negative TAYE (CRH5-TAYE). The effects of expressing the dominant negative ERK in the CRH5-TAYE cell line was confirmed as these cells exhibited lower levels of mRNA for the pERK target gene, cFos (Figure 6b). Unlike the CRH5 cell line, the CRH5-TAYE cell line showed no differences in either proliferation or mobility in response to increased selenium (Figure 6 c and d.).
The effects of selenium on proliferation and mobility of MM cell lines is mediated by ERK activation

(a) Four different MM cell lines were cultured in media containing low (30 nM), medium (50 nM), and high (80 nM) selenium for 1 wk and cell lysates (15 μg total protein per well) analyzed for different proteins by Western blot. CRH5 and EKKH5 showed increases in pERK levels with increasing selenium while AK7 and AB12 did not. CRH5 also showed slight increases in pAKT with increasing selenium. Total ERK and AKT levels were equivalent as was β-actin (loading control). Levels of GPx1 confirmed equivalent utilization of bioavailable selenium for each MM cell line. Data are representative of two independent experiments.

(b) CRH5 cells stably transfected with a plasmid encoding a dominant negative form of ERK (TAYE-ERK) were lower compared to CRH5 cells for cFos mRNA expression using real-time PCR to confirm functional effects on ERK activation. cFos mRNA was compared to housekeeping β-actin mRNA for each sample. (c-d). The TAYE-ERK CRH5 cells were then cultured in media containing 30, 50, and 80 nM sodium selenite for 1 wk and evaluated for mobility using a scratch assay and for proliferation. Data represent mean ± S.E. and means were compared using a student’s t test, *P < 0.05. Each experiment was repeated in two independent experiments.
Overall, these data suggest that ERK activation represents an important signaling event in mediating the effects of increasing selenium in those MM cells that are responsive to changes in levels of this micronutrient.

2.4.3 Selenium affects certain MM cells by increasing reducing capacity

Because the phosphorylation of ERK has in some cases been shown to be a redox sensitive event, we next investigated the role of redox status in the effects of increasing selenium on MM cell growth and proliferation. CRH5 and EKKH5 cells showed increasing levels of reduced glutathione (GSH) with increasing selenium, while AB12 and AK7 did not (Figure 7a.). We did note that the baseline levels of GSH in the low selenium media for both of the selenium insensitive cell lines was as high as or higher than the high selenium media. This implies that the insensitive cell lines may have alter redox metabolisms that allows them to increase redox capacity independent of selenium. To determine if the reducing equivalents of GSH were utilized differently by the selenium sensitive and insensitive cells, 250 mM \( \text{H}_2\text{O}_2 \) was added and ROS levels evaluated using dihydrochlorofluorescein (DHCF). Interestingly, here we noted that the higher levels baseline levels of GSH did not correlate with greater ability to eliminate ROS.

Results showed that increasing selenium lowered ROS levels in CRH5 and EKKH5, but not in AB12 and AK7 (Figure 7b). No differences in basal ROS levels (i.e. no \( \text{H}_2\text{O}_2 \) added) were found with increasing selenium for the MM cell lines (data not shown). To determine if this difference in reducing capacity contributed to effects of selenium on ERK activation and proliferation/mobility, we
used N-acetyl cysteine (NAC) to eliminate the differences in redox status, and then compared all four MM cell lines. NAC was chosen because it is a precursor to GSH and can elevate the levels of GSH of the cell lines to eliminate the ROS stress that may be effecting the ERK phosphorylation and proliferation and mobility of the cells. It is also cell permeable while GSH itself is not. Western blot analyses demonstrated that treatment with NAC led to equivalent pERK and pAKT in all four MM cell lines, regardless of selenium levels (Figure 7c). NAC treatment also resulted in equivalent proliferation and mobility in the MM cell lines regardless of selenium levels (Figure 7 d & e).
Figure 7 Reducing capacity with increasing selenium is a crucial mechanism by which proliferation and mobility are affected in selenium sensitive cells.

Four different MM cell lines were cultured in media containing low (30 nM), medium (50 nM), and high (80 nM) selenium for 1 wk and (a) cellular GSH was measured (b) cells were challenged with 250 mM H$_2$O$_2$ and evaluated for ROS levels using DHCF. (c) Four different MM cell lines were cultured in media containing low (30 nM), medium (50 nM), and high (80 nM) selenium for 1
wk and 0.4 mM NAC was added as a reducing agent for 14 hr prior to protein extraction and Western blot analysis. No differences were found in pERK or pAKT for any of the cell lines, and levels of GPx1 confirmed equivalent utilization of bioavailable selenium for each MM cell line. Data are representative of two independent experiments. All four MM cell lines cultured in increasing selenium with NAC added as described above exhibited no differences in proliferation (d) or mobility (e). Data represent mean ± S.E. and means were compared using a student's t test, *P < 0.05. Each experiment was repeated in two independent experiments.
2.4.4 *In vivo* CRH5 MM tumor progression is increased with increasing dietary selenium in a manner that depends on reducing capacity

Balb/c mice were fed defined diets containing 0.08, 0.25, and 1.0 ppm selenium as sodium selenite for 4 wks to establish low, medium, and high selenium status as previously described. These mice were then subcutaneously injected with the selenium sensitive (CRH5) and selenium insensitive (AB12) MM cell lines. The mice were maintained on the selenium diets and tumor volume monitored over time. Similar to the *in vitro* data described above, CRH5 MM tumor progression was increased with increasing dietary selenium while AB12 MM tumors showed no significant effect from increasing dietary selenium (Figure 8). NAC treatment throughout the tumor measurements eliminated the effects of dietary selenium on CRH5 tumor progression. Thus, together with the *in vitro* data described above, these results suggest that certain MM tumors benefit from increasing selenium due to increased reducing capacity exerted by this micronutrient.
Figure 8 Increasing dietary selenium in vivo promotes redox dependent tumor progression of CRH5, but not AB12 MM cells.

Male Balb/c mice were fed defined diets containing low (0.08 ppm), medium (0.25 ppm), and high (1.0 ppm) selenium as sodium selenite for 4 wk, and then $10^6$ AB12 or CRH5 MM cells were injected s.c. and tumor volume measurements taken weekly. CRH5 were also injected in mice maintained on drinking water containing 0.4 mM NAC for the remainder of the experiment. Mice were sacrificed when tumor volumes threatened the health of the mice. Data represent mean ± S.E. and means were compared using a student's t test, *P < 0.05. Each experiment was repeated in two independent experiments with N = 5 per group.

Treatment of mice with NAC eliminates differences in progression of CRH5 tumors between dietary selenium groups. Male Balb/c mice were fed defined diets containing low (0.08 ppm),
medium (0.25 ppm), and high (1.0 ppm) selenium as sodium selenite for 4 wk, and then $10^6$
CRH5 MM cells were injected s.c. and mice maintained on drinking water containing 0.4 mM NAC
for the remainder of the experiment. Tumor volume measurements were taken weekly and mice
were sacrificed when tumor volumes threatened the health of the mice. Data represent mean ±
S.E. and means were compared using a student's $t$ test, *$P < 0.05$. 


Chapter 3: Calpastatin in macrophages regulates calpain-2 driven IBD and CAC
3.1 Abstract

Calpain enzymes proteolytically modulate cellular function and have been implicated in inflammatory diseases. In this study, we found that calpain levels did not differ between intestinal tissues from IBD patients and healthy controls, but IBD tissues showed increased levels of the endogenous calpain inhibitor, CAST. To investigate the role of CAST in the immune system during IBD, mice were x-ray irradiated and reconstituted with either CAST knockout (KO) or wild-type (WT) bone marrow and subjected to DSS-induced colitis. CAST KO recipients with induced colitis exhibited more severe weight loss, bloody diarrhea, and anemia compared to WT controls. Histological evaluation of colons from KO recipients with colitis revealed increased inflammatory pathology.

Macrophages purified from the colons of KO recipients had higher IL-6, TNFα, and IFNγ mRNA levels compared to WT controls. Mechanistic investigations using siRNA and KO bone marrow to generate CAST deficient macrophages showed that CAST deficiency during activation with bacterial PAMPs including heat-killed E. faecalis or CpG DNA led to increased IκB cleavage, NF-κB nuclear localization, and IL-6 and TNFα secretion. Thus, CAST plays a central role in regulating macrophage activation and limiting pathology during inflammatory disorders like IBD.

CAST regulates the function of all calpain enzymes, but because calpain-1 and -2 are the predominant isoforms expressed in most tissues (including the immune system), we focused our attention on these two calpain enzymes for their role in inflammatory activation of macrophages during IBD. In order to
determine which calpain enzyme contributes to the activation of BMDMs, we stimulated these cells with TNFα and *E. faecalis* in the presence of calpain-1 or -2 inhibitors. We found that calpain-2 inhibitors limited the production of the inflammatory cytokines IFNγ, TNFα, IL-6, and MCP-1.

Using a single treatment of DSS to generate acute colitis we determined that 0.75 mg/kg daily treatment of calpain-2 inhibitor delivered i.p. was the optimum dosage for limiting calpain-2 activity in the colon. We proceeded to use this dosage in a mouse model of chronic DSS-induced colitis and AOM induced colon carcinoma to examine the effects of a calpain-2 inhibitor on inflammatory disease and inflammation associated cancer. Calpain-2 inhibitor treatment limited the duration of inflammation associated weight loss in the chronic IBD model and allowed mice to recover body weight after the discontinuation of DSS treatment. The mice treated with the inhibitor also showed significantly decreased colonic inflammatory cytokine mRNA. This reduced inflammation was concurrent with a reduction in colon pathology in the calpain-2 inhibitor treated mice for both IBD and CAC. The colorectal tumor volumes of the treated mice were also significantly reduced by the calpain-2 inhibitor treatment. Our findings support the potential of calpain-2 inhibition as an anti-inflammatory treatment for IBD that also limits the progression of inflammation associated colorectal carcinoma.

### 3.2 Introduction

Calpain enzymes are calcium activated cysteine proteases. These enzymes are dynamically regulated by the expression of their endogenous
inhibitor CAST during macrophage activation. We have recently reported that Toll-like receptor (TLR)-stimulation of macrophages leads to the upregulation of CAST, an effect that was not observed in other immune cells. This implies that the use of a calpain inhibitor may allow us to specifically target and inactivate inflammatory macrophages. This is important in the context of IBD given that the gut mucosa represents the most extensive interface between the body and the external environment and it contains the largest population of resident macrophages.

Inflamed tissues from IBD patients exhibit increased recruitment of blood derived CD14+ monocytes into the lesions and an upregulation of CD40+ and CD80+ activated macrophages, particularly adjacent to the epithelium. These invading macrophages play a major role in the development or severity of IBD. A previous study in rats suggested that a pharmacological inhibitor of calpains may be useful in treating acute colitis, although the experimental approach taken in this study limited the interpretation of the results. The calpain/CAST system may represent an effective target for modulating inflammatory diseases. Understanding the role of the endogenous inhibitor, CAST, in regulating the inflammatory processes that drive IBD may facilitate a more effective use of pharmacological calpain inhibitors. Also, there is a need to better understand whether CAST deficiency, or an impairment in its function, may predispose individuals to increased susceptibility or severity of IBD. We used a mouse bone marrow transplant model to investigate the role of CAST in the immune system during colitis and found CAST deficiency in immune cells.
increased the severity of colitis. CAST was upregulated by intestinal macrophages during colitis, and *in vitro* experiments demonstrated a key role for this inhibitor of calpain activity in preventing hyperactivation of macrophages upon exposure to commensal bacteria or bacterial CpG DNA. These results present novel insight into how impaired CAST expression may lead to heightened susceptibility to IBD or increase disease severity from chronic inflammatory disorders in general. It also supports the idea that the use of a calpain inhibitor might prove to be an effective approach to limit inflammation.

Calpain-1 and-2 are both expressed in macrophages, although calpain-2 is found at much higher levels. Calpain-1 is activated by μM concentrations of Ca^{2+} while calpain-2 is activated by mM Ca^{2+}. These differing activation thresholds suggest that calpain-2 is more likely to dynamically respond to large Ca^{2+} fluctuations during macrophage activation. Our laboratory has previously demonstrated that selenoprotein K is specifically cleaved by calpain-2 during macrophage activation by LPS. Selenoprotein K has been linked to Ca^{2+} flux in T cells, neutrophils and macrophages, and has also been shown to be involved in the palmitoylation of proteins. These and other calpain-2 specific functions in activated macrophages point to this enzyme as an important mediator of activation.

Calpain-2 is also expressed in various cancers including colorectal cancer. Importantly, the expression of calpain-2 also negatively correlates with survival outcomes in ovarian cancer, breast cancer, prostate cancer, and potentially colon cancer.
3.3 Materials and methods

3.3.1 Human tissues and cells

De-identified human samples were purchased from BioServe Biotechnologies (Beltsville, MD) and Bio-Options, Inc. (Brea, CA). Also, human biopsy tissues were generously provided by Dr. William Faubion (Mayo Clinic, Rochester, MN) and Dr. Zhenyou Jiang (Jinan University Medical School, Guangzhou, China). For Crohn's disease and ulcerative colitis tissues, diagnoses were confirmed for each patient sample and normal tissues from equivalent intestinal regions from healthy donors were used as controls. Blood for human monocyte-derived macrophages (HMDM) was obtained from healthy volunteers under an approved protocol (CHS#19345). Monocytes were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and cultured in X-Vivo-10 culture media (BioWhittaker, Walkersville, MD) containing 10% autologous serum. Media was replaced with fresh media on day 3 of culture and fully differentiated human macrophages used for experiments on day 6 of culture. All human samples were provided in a de-identified manner under IRB-approved human protocols (CHS#19345).

3.3.2 Bone marrow transplants and colitis model

The CAST knockout (KO) mice with more than ten generations of back-crossing onto a C57BL/6 background have been previously described. A colony generated from C57BL/6J mice (Jackson Laboratories) were used as
wild-type (WT) controls. The CAST KO mice express a smaller CAST protein generated from the targeted allele generated by exon 6-skipping, which results in defective CAST activity and a lighter, more rapidly migrating band in Western blots. Male 5-wk-old C57BL/6J mice were used as bone marrow transplant recipients. Bone marrow transplantation was performed as previously described\textsuperscript{130} \textsuperscript{122}. Briefly, bone marrow cells (2 x 10\textsuperscript{6} in PBS) from donor mice were administered to recipient mice by intravenous tail vein injection 24 hr after an ablative dose of whole-body x-ray irradiation (10.5 Gy). Mice were allowed to recover for 4 weeks, bone marrow reconstitution of the immune system confirmed, and then were subjected to an established model of chronic, relapsing and remitting colitis\textsuperscript{131}. For this model, mice were given 1.0% dextran sulfate sodium (DSS; CAS#9011-18-1; MP Biomedicals, Santa Ana, CA) in drinking water alternated weekly with regular drinking water for 7 d, for total of 35 d and weight loss typically peaks around d 10. Control mice received normal drinking water throughout the 35 d period. Importantly, the effect of irradiation on intestinal macrophages was assessed by irradiating Ly5.2 mice (C57BL/6) followed by transplantation with Ly5.1 congenic bone marrow and transplantation with Ly5.2 bone marrow as a control. Flow cytometric analyses demonstrated that macrophages in both the colons and spleens of mice receiving Ly5.1 bone marrow were 90-95\% derived from the donor marrow, with small contributions (5-10\%) from recipient sources (data not shown). All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.
3.3.3 Mouse IBD and CAC

The mouse DSS induced colitis with AOM induced CAC model was adapted from a previously described protocol \(^{132}\). In brief, mice were injected on d 0 with AOM (Sigma) (12mg/kg) and were then treated with three 5 d cycles of 2% DSS over 63 d. Intervention with inhibitor occurred on d 8 and was injected i.p. (0.75mg/kg) 5 days a week throughout the entire protocol.

3.3.4 Assessment of colitis in mice

Weights of mice were monitored over the 35 d or 63 d period to assess effectiveness of DSS. To evaluate blood in the feces of the mice, cecums were excised and homogenized in water to extract hemoglobin (Hb), and Hb quantified using ELISA (ICL, Inc., Portland, OR). In the 35 d model the colons were removed, and homogenized into single cell suspensions using a GentleMacs (Miltenyi, San Diego, CA), of which \(10^6\) cells were removed and analyzed for different immune cell markers by flow cytometry as previously described \(^{133}\).

3.3.5 Histology

Colon tissues were washed, cut open on Kimwipes \(^{\text{TM}}\) (Kimberly-Clark, Rosewill, GA) and fixed in 10% buffered formalin. Standard H&E staining of paraffin-embedded tissue samples was conducted as previously described \(^{134}\). Five H&E stained sections from each mouse were blindly scored for IBD pathology as previously described \(^{135}\). Colon cancer pathology was assessed on
a four point system as follows: 1 = no tumor or dysplasia present; 2 = basally oriented nuclei, mild nuclear enlargement, nuclear crowding and hyperchromasia, decreased or loss of intracellular Mucin; 3 = prominent nuclear stratification, more severe hyperchromasia and pleomorphism, marked architectural distortion; 4 = back to back glands with no intervening stroma, dysplastic epithelial cells and invasion of the colonic basement membrane. Images captured using a Zeiss Axioskop 2 Plus upright light microscope and camera.

3.3.6 Visualization of whole mouse colons and volume calculation from images

Mouse colons were dissected out on d 63 of the colitis and colon cancer mouse model. The colons were cut longitudinally and spread out on clear plastic. The colons were washed with PBS using squeeze bulb pipettes. Images were taken using an Infinity 2 microscope mounted camera (Lumenara, Madrid, Spain) on a Stemi 2000-C dissecting scope (Zeiss, Oberkochen, Germany). Separate images were assembled into continuous colon images using Photoshop software (Adobe Systems Inc., San Jose, CA) Colon tumor volumes were assessed by a combination of images and H & E slides. Not all H & E slides allowed for tumor volume calculation due to tissue compression, or loss. In order to determine the z-axis measurement for all tissues, the tumors with measured z-axes were used to generate a correlation between tumor area and volume. The correlation equation was 

\[ y = -0.00015x^2 + 0.033x + 0.121 \]  

where \( x \) is the tumor area and \( y \) is
the z-axis measurement. This was then used to estimate the z-axes of the remaining tumors.

### 3.3.7 Isolation of macrophages from mouse colons and realtime PCR

Colons were rinsed with PBS and incubated in DMEM + 5% FBS containing 1 mM DTT for 30 min at room temperature to remove mucus, then the tissue was digested in DMEM media with 5% FBS, 2 mg/mL collagenase IV (Sigma-Aldrich), and 30 µg/mL DNase I (Roche Applied Sciences, Indianapolis, IN) for 2 hr at 37ºC on a shaking incubator. The digested tissue was then filtered through 70 µm cell separators, cells from 2 mice within each group were pooled and washed with DMEM with 5% FBS and counted using a Scepter (Millipore-GE Healthcare, Billerica, MA). Cells were then incubated with magnetic beads in the ratio of 5 X 10^6 cells per 25 µL of Dynabeads (Life Technologies-Invitrogen) pre-coupled with 5 µg anti-CD68 (BioLegend, San Diego, CA). These cell and bead mixtures were incubated in eppendorf tubes at 4ºC for 30 min and tubes added to a magnetic stand (Life Technologies/Invitrogen) for 2 min. The non-bound cells were removed, then magnetically bound cells washed with complete media, and the process repeated two more times and tubes removed from magnetic stand and resuspended in PBS. Cells were enumerated using a Scepter 2.0 cell counter (Millipore). Purity was confirmed using flow cytometric detection of CD68 using PE-anti-C68 (BioLegend), PE-anti-F4/80 and PE-anti-CD11b (Ebioscience, San Diego, CA) using a FACScaliber flow cytometer (BD Biosciences, San Diego, CA). Total RNA extraction from purified CD68-positive cell pellets was
performed using RNeasy (Qiagen, Valencia, CA), cDNA synthesized using a High Capacity cDNA Synthesis Kit (ABI), and real-time PCR used to evaluate levels of inflammatory mRNA using protocols previously described \(^{136}\).

### 3.3.8 Flow cytometry

Single cell suspensions of colons and spleens were prepared using a GentleMacs cell dissociator (Miltenyi, Inc.). Cells (5 X 10\(^6\)) were stained with recommended dilutions of fluorochrome-conjugated antibodies in 100 µL FACS Buffer (PBS with 2% FBS) after pre-incubation with FcBlock (BD Pharmingen). Antibodies included PE-anti-CD45.1, PE-CD45.2, APC-conjugated anti-CD4, anti-CD8, anti-CD11b, anti-Gr-1 (all from Ebioscience, Inc., San Diego, CA). For intracellular staining, cells were permeabilized using Ebioscience Permeabilization Buffer and intracellular CAST detected with PE-anti-CAST or PE-conjugated isotype IgG as a control (Bioss, Inc., Woburn, MA). Cells were evaluated on a FACScaliber flow cytometer (BD Biosciences). All flow data were analyzed using FlowJo software.

### 3.3.9 Mouse macrophages from bone marrow

Preparation of bone marrow-derived macrophages (BMDM) was performed as previously described (14). In brief, bone marrow was flushed from femurs and tibiae with HBSS using a syringe with a 25-gauge needle. Cells were released from clumps by drawing the suspension through a syringe with an 18-gauge needle and cell suspensions were then passed through a 70 µm pore cell
strainer (BD Falcon, San Diego, CA) to remove tissue debris. The cells were
plated in DMEM containing 10% FBS, 1% penicillin-streptomycin-L-glutamine
(Life Technologies-GIBCO, Grand Island, NY), and 10% L929 conditioned media.
On day 5 the cells were removed with Cellstripper™ solution (Mediatech, Inc.,
Manassas, VA), replated with fresh media in plates needed for each experiment,
which were conducted on day 6 of culture.

3.3.10 Stimulation of macrophages, Western blots, and calpain activity
assays

BMDM or HMDM were plated at a density of 2 X 10^6 cells per well in 6-
well plates in 2 mL of DMEM with 5% FBS overnight at 37°C and 5% CO2. In
some cases, siRNA for CAST or non-targeting control siRNA (Santa Cruz
Biological, Santa Cruz, CA) were transfected using a Neon electroporator
(Invitrogen). The next day the cells were stimulated for 20 hr with LPS (0111:B4;
100 ng/mL; Sigma), or BioLegend cytokines including TNFα (20 ng/mL), IFNγ
(100 ng/mL), IL-17A (5 ng/mL), IL-10 (10 ng/mL), and IL-4 (10 ng/mL). Other
reagents used for stimulation included CpG (5 ng/mL, TriLink Biotechnologies,
San Diego, CA) and heat-killed Enterococcus faecalis (1 µg/mL; ATCC,
Manassas, VA). Cell pellets were then harvested and lysed in 10 mM Tris-HCl
pH 7.5, 1% Triton X-100, 5 mM EDTA, 1X proteinase inhibitors (Calbiochem-GE
Healthcare, Pittsburgh, PA), and 5 mM NaCl using a probe sonicator. In some
cases, nuclear lysates were separated from cytosolic lysates using the NF-κB
Activation Assay Kit (FIVEphoton Biochemicals, San Diego, CA). Protein
concentration in the lysates was measured by Bradford assay reagent (Bio-Rad) and 15 µg total protein was combined with reduced Laemeli buffer, boiled at 95°C for 10 min, cooled on ice, and loaded into wells of 10 - 14.5% polyacrylamide gels (Bio-Rad).

After gel electrophoresis, protein was transferred to PVDF membranes, which were blocked for 1 hr with low fluorescence blocking agent (Li-Cor) and incubated with primary antibodies including anti-CAST, anti-IκB (detects both IκBα and IκBβ), anti-NF-κB (all from Cell Signaling, Danvers, MA), anti-μ-calpain and antim- calpain (Millipore), or anti-β-actin (Sigma). After washing with PBS, membranes were incubated with secondary antibodies from Li-Cor for 1 h, membranes washed with PBS, and signals detected with densitometry conducted using the Li-Cor Odyssey imaging system. Calpain activity was measured using a calpain activity assay kit (BioVision, Inc., Milpitas, CA). This fluorometric assay is based on the detection of cleavage of calpain substrate Ac-LLY-AFC and each sample was measured for fluorescence in triplicate using a SpectraMax M3 fluorometer (Molecular Devices, Inc.).

3.3.11 Cytokine analysis

Media was removed directly from treated macrophages, spun down at 300 x g for 5 minutes to remove cell debris. Media cytokine analysis was done using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences). Analysis of cytokine data was done using Flowjo software.
3.3.12 Statistical analyses

Comparison of means was carried out using an unpaired Student's $t$ test using GraphPad Prism version 4.0 (GraphPad, La Jolla, CA). Standard curves and regression analyses were also conducted using GraphPad Prism version 4.0. Weight curves and experiments with 3 or more groups were compared by one way ANOVA followed by Tukey post test. All comparisons were considered significant at $P < 0.05$.

3.4 Results

3.4.1 CAST is increased at chronic stages of IBD, while calpain levels remain constant

While emerging evidence suggests that calpain cleavage may be involved in regulating inflammation \(^{137}\), little is known regarding how levels of calpains or their inhibitor, CAST, may be altered in inflamed versus healthy intestinal tissues. Based on the conventional notion that calpain activity promotes inflammation, we expected higher levels in inflamed intestinal tissues from human IBD patients. However, analyses of human tissues revealed that calpain activity was decreased in tissues from both ulcerative colitis and Crohn's disease patients compared to healthy controls (Figure 9A). This decreased calpain activity coincided with increased CAST, but not with any changes in levels of calpain-1 or -2 (Figure 9B-E).
The relationship between IBD and the calpain/CAST system was further investigated using an established mouse model of colitis in which mice were given 1.0% DSS-treated water alternated weekly with normal drinking water for 35 days to induce a remitting, relapsing colitis. Induction of colitis was confirmed in mice receiving DSS-treated/regular water compared to control mice that only received regular water by evaluating weight loss, macroscopic and microscopic analyses of colon tissues (data not shown). Calpain activity was increased at early stages of acute colitis, but was decreased by day 10 and remained lower than healthy tissues at the chronic stage (day 35) (Figure 10A). In this sense, the chronically inflamed intestinal tissues exhibited lower calpain activity compared to healthy controls similar to human IBD tissues. Also similar to human IBD, CAST was increased in chronic colitis tissues while calpain levels did not differ between colitis mice and healthy controls (Figure 10B-C). Thus, decreased calpain activity during chronic colitis correlates with increases in the endogenous inhibitor, CAST, while no changes in calpain-1 or -2 levels are found.
Figure 9 Calpain activity is decreased and CAST is increased in human intestinal tissue during IBD.

(a) Intestinal biopsy tissues from healthy controls (n = 7), ulcerative colitis patients (n = 5), or Crohn’s disease patients (n = 6) were analyzed for calpain activity, with relative fluorescent units/mg total protein measured using a fluorometer. Data are mean ± SE. *p < 0.05, one-way ANOVA with Tukey post test. (b) Western blot analysis of CAST and calpain levels in intestinal tissue from ulcerative colitis patients and five healthy individuals (15 μg/well). β-actin was used as a loading control. (c) Densitometry was performed to determine the intensity of Western blot signals for CAST relative to β-actin for each lane. Data are mean ± SE. *p < 0.05, Student t test. (d-e) Western blot and densitometry were used to analyze CAST and calpain levels in six healthy intestinal tissues and six tissues from Crohn’s disease patients, similar as described above for ulcerative colitis. Each experiment was repeated in two independent experiments. *p < 0.05; Student t test.
Chronic colitis results in decreased calpain activity and increased CAST in colons of mice.

(a) A mouse model of colitis involving DSS-treated water was performed, as described in Materials and Methods. At different days of the colitis model, colon tissues (n = 5 each) were analyzed for calpain activity and relative fluorescent units/mg total protein was measured using a fluorometer. Data are mean ± SE. (b) Western blot was used to analyze CAST and calpain levels in lysates prepared from colons of mice subjected to DSS-induced chronic colitis (day 35) and healthy controls (15 μg/well). β-actin was used as a loading control. (c) Densitometry was performed to determine the intensity of Western blot signals for CAST relative to β-actin for each lane. Data represent mean ± SE. Each experiment was repeated in two independent experiments. *p < 0.05, Student t test.
3.4.2 CAST deficiency in the whole animal or only in immune cells leads to more severe colitis

To investigate the role of CAST in the immune system during colitis, we performed DSS-induced colitis experiments using two different models. The first model involved wild-type (WT) and whole animal CAST knockout (KO) mice and the second model focused on CAST-deficiency only in the immune system. For the latter model, irradiated C57BL/6 mice were transplanted with bone marrow from WT (WT→WT) or CAST KO mice (KO→WT). After 4 wk of recovery, blood samples were evaluated to confirm equivalent immune system reconstitution in WT→WT and KO→WT mice as well as effective KO of CAST in immune cells, and the mice were then subjected to the DSS-induced model of chronic colitis.

With the first model, inducing colitis in the mice with CAST-deficiency in all tissues resulted in one death (at day 21) and the remaining KO mice exhibited more severe weight loss compared to WT mice with colitis (Figure A). Similar results were obtained using the second model in which two deaths (days 21 and 24) occurred in the KO→WT mice subjected to colitis, and the surviving KO→WT mice with colitis had more severe weight loss compared to WT→WT mice with colitis. CAST deficiency in the immune system alone or in the whole animal were similar in producing more severe bloody diarrhea (Figure 11B) and inflammatory pathology in colon tissue compared to controls (Figure 11C-D).

Histological evaluation revealed that CAST deficiency in the immune system alone or in the whole animal led to more abundant hallmarks of inflammation such as disrupted epithelial crypts and cellular infiltration and,
some cases, serosal ulceration. Both CAST deficiency in the immune system and whole animal resulted in increased calpain activity during colitis, whereas WT→WT and WT controls both showed lower calpain activity in colitis tissues consistent with results described above. Importantly, KO and KO→WT mice given normal drinking water showed no signs of illness or pathology, suggesting CAST deficiency in the immune system is not sufficient for triggering the inflammatory pathology or symptoms of colitis. Overall, CAST deficiency only in the immune system was similar to CAST deficiency throughout all tissues in causing more severe colitis compared to controls.
CAST deficiency in the whole animal and in the immune system alone similarly lead to more severe colitis.
(a) Weights of mice with CAST deficiency in the whole animal compared with WT controls using a model of DSS-induced chronic colitis involving DSS-treated water alternated with untreated water (n = 10 WT and KO) or untreated water alone (n = 8 WT and 9 KO) (left panel). WT mice were x-ray irradiated and reconstituted with WT bone marrow (WT→WT) or CAST-KO bone marrow (KO→WT) and then subjected to DSS-treated water or untreated water (right panel). For both graphs, weights of mice were measured over a 35-d period, with decreased weights indicating colitis. (b) For colitis models involving CAST deficiency in the whole animal (left panel) or in the immune system alone (right panel), mice were sacrificed on day 35, and ELISA measurement of hemoglobin in ceca was used for determining the level of blood in feces. (c-d) For colitis models involving CAST deficiency in the whole animal (left panels) or in the immune system alone (right panels), tissue sections from colons of mice were analyzed by H&E staining for features of inflammation and pathology. Scores for levels of inflammatory pathology were derived as described in Materials and Methods. The KO and KO→WT mice with induced colitis showed a higher degree of cellular infiltration into the submucosa and muscularis propria (large arrows) and highly disrupted crypt architecture (small arrows) compared with WT and WT→WT controls, respectively. Scale bar, 100 μm. (e) Macrophages isolated from DSS treated mouse colons demonstrated increased production of 3 inflammatory cytokine mRNAs. *p < 0.05, Student t test.
3.4.3 Impaired CAST response during macrophage activation results in increased activation of the NF-κB pathway

Our *in vivo* data above showed that CAST deficiency in intestinal macrophages led to increases in mRNA levels for TNFα, IL-6, and IFNγ, which are induced by the transcription factor NF-κB. NF-κB is sequestered in the cytosol and translocation to the nucleus is prevented by IκB, which has been shown in other cell-types to be a target of calpain cleavage (31). Thus, we used BMDMs to investigate the possibility that CAST deficiency in macrophages leads to increased IκB cleavage and hyperactivation of the NF-κB pathway. Two independent methods were used to generate CAST deficiency in macrophages under conditions of TNFα priming: siRNA was used to knockdown CAST in WT BMDM with nontargeting siRNA serving as a negative control, or in separate experiments WT and CAST KO BMDMs were used. After 24 hr of TNFα priming, the BMDM were then stimulated with bacterial PAMPs including heat-killed *E. faecalis* or CpG DNA for different times. Western blot analyses of total IκB demonstrated that CAST deficiency induced by either knockdown or KO of CAST led to decreased levels of IκB with either bacterial PAMP (Figure 12A). Consistent with these results, levels of nuclear NF-κB induced by the PAMPs were increased under conditions of CAST deficiency (Figure 12B). These experiments were replicated in three independent experiments and densitometry performed showing that CAST deficiency resulted in significantly lower IκB levels and higher nuclear NF-κB.
Figure 12 CAST deficiency in macrophages leads to decreased IκB and increased NF-κB translocation to the nucleus.

(a) CAST deficiency in BMDMs was accomplished by two methods: pretreating WT BMDMs with CAST siRNA and/or nontargeting siRNA as a negative control (left panel) or WT versus CAST-KO BMDMs (right panels). These macrophages were then primed with TNF-α (20 ng/ml) for 24 h, followed by stimulation for different time periods with two bacterial PAMPs: heat-killed E. faecalis (1 μg/ml) or CpG DNA (5 μg/ml). Levels of IκB were decreased to a greater extent under conditions of CAST deficiency. β-actin served as a loading control, and CAST levels were measured to confirm deficiency. As explained in Materials and Methods, CAST-KO mice are characterized by low levels of a functionally impaired truncated CAST. (b) BMDMs with CAST deficiency, induced using either siRNA or KO, as described above, were stimulated with TNF-α (20 ng/ml) for 24 h, followed by either heat-killed E. faecalis or CpG DNA stimulation for 120 min. Nuclei were isolated, and levels of NF-κB were detected by Western blot, with nuclear TATA-binding protein (TBP)-1 serving as a loading control. Results are representative of three independent experiments.
3.4.4 Calpain-2 inhibition limits macrophage inflammatory activation

In macrophages, calpain cleaves IκB and releases NF-κB to translocate to the nucleus and activate production of inflammatory cytokine mRNA as shown above \(^{128}\). We set out to determine if increased regulation of calpain activity by calpain inhibitors could further limit inflammatory response of macrophages beyond the endogenous inhibition by CAST. Macrophages were primed and activated in the presence of calpain-1 or -2 inhibitors and a bead based immunoassay (CBA, BD Biosciences) kit was used to measure the expression of extracellular inflammatory cytokines. Results showed that calpain-2 inhibitor treatment during macrophage priming and activation led to a significant decrease in the production of the inflammatory cytokines IFNγ, TNFα, IL-6, and MCP-1 (Figure 13). Calpain-1 inhibitor had no effect on the production of cytokines by stimulated and activated macrophages. There was no significant effect on the production of IL-10 or IL12p70.
Figure 13 Calpain-2 inhibition in bone marrow derived macrophages reduces inflammatory cytokine expression

Bone marrow derived macrophages were primed 24 hr with TNFα (20 ng/ml) and activated with *E faecalis* (1 μg/ml) 24h in the presence or absence of calpain-1 or -2 inhibitor. Media was collected from wells and cytokines assessed by CBA kit (BD Biosciences). Data are mean ± SE (n = 3) and represent results from three independent experiments. *p < 0.05; one way ANOVA followed by Tukey post test.
3.4.5 Calpain-2 inhibition reduces long term weight loss in a mouse model of chronic IBD

In order to determine the proper concentration of calpain-2 inhibitor to use *in vivo* we decided to use a short 4 d DSS treatment in combination with calpain-2 treatment. Mice were treated with 2% DSS in combination with 0, 0.25, 0.50, 0.75, 1.00, or 1.25 mg/kg calpain-2 inhibitor for 4 days, which was consistent with our previously observation that DSS induced calpain activity peaks at 3-5 days of treatment (Figure 10A). At the end of this treatment the mice were sacrificed and colons extracted, frozen, homogenized by mortar and pestle, and calpain activity determined. The data indicated that at 0.75 mg/kg the maximum amount of calpain activity inhibition was achieved (Figure 14A). This dose was selected for further *in vivo* use. It should be noted that total calpain activity was measured and only reduced by 50%, which suggests that other calpain enzymes may be functionally active in this tissue or that calpain-2 activity was not fully inhibited in the mouse colons.

The AOM/DSS model was used in order to determine if a calpain-2 inhibitor could be used as an intervention for colitis and inflammation induced colorectal cancer. The mice were i.p. injected on day 0 with AOM followed by three 5 d treatments of DSS (Figure 14B). The weight of the mice was measured every other day. The mice treated with the calpain-2 inhibitor showed a significant increase in weight recovery after DSS disruption of the Mucin layer of the colon when compared to the weights of the mice injected with vehicle control (Figure 14C).
Figure 14 Calpain-2 inhibitor treatment reduces long term weight loss associated with chronic IBD mouse model

(a) Mice were treated with 2% DSS and calpain-2 inhibitor at concentrations varying from 0 to 1.25 mg/kg intraperitoneally injected for 4 days. On the 4th day the colons were harvested and calpain activity measured with a calpain activity assay. (b) The experimental setup for the IBD CAC model starts with day 0 injection of AOM carcinogen followed by 3, 5 day DSS treatments with IP inhibitor treatment beginning on day 8 for a total of 63 days. (c) Mice were weighed every other day for the entirety of the 63 day protocol (n=20). Weight curves were analyzed by repeated measures one way ANOVA followed by Tukey post test. Data represent mean ± SEM.
3.4.6 Inhibition of calpain-2 improves IBD pathology and lowers the expression of colonic cytokines

Colon histology in mice treated with a calpain-2 inhibitor showed a significant decrease in inflammatory bowel pathology (Figure 13A,B). The scores were not completely reduced to the levels of the negative control mice but the residual tissue damage may be simply due to the physical damage caused by the treatment with the DSS detergent itself and not the resulting inflammation. Hemoglobin ELISA of cecum samples was used as another measure of the health of the colons (Figure 15C). The mock treated mice showed elevated cecum hemoglobin indicative of bloody diarrhea while the calpain-2 inhibitor treated mice showed no increase over the hemoglobin levels of the negative control group. Mouse colons were harvested at the end of the 63 d chronic colitis model and samples taken for mRNA analysis. Real-time PCR showed that calpain-2 inhibitor treatment lowered colonic inflammatory cytokine mRNA levels to that of the untreated control mice relative to the mock injected mice for TNFα, IL-6, and MCP-1 (Figure 15D)
Figure 15 Calpain-2 inhibitor treatment improves IBD pathology and decreases colonic inflammatory cytokines

Mice subjected to the colitis model were sacrificed on day 63 and colons were removed and embedded in paraffin. Paraffin embedded sections were H & E stained (a) and blindly scored for colitis by assessment of 5 sections from each colon (b). (c) Cecum hemoglobin ELISA was performed on cecum samples to assess blood loss in feces. (d) Real-time PCR was performed on total RNA extracted from colons to assess expression of cytokine mRNAs. Groups were analyzed using one way Anova followed by Tukey post test. Data represent mean ± SEM.
3.4.7 Calpain-2 inhibitor treatment improves inflammation associated cancer outcomes

On day 63 of the chronic colitis model the mice were sacrificed and the colons removed and imaged to determine tumor volumes as a result of the combination of the AOM carcinogen and inflammation. Calpain-2 inhibitor treated mice had significantly reduced total tumor volume compared to mock treated mice (Figure 16). Histological assessment of colons was preformed blinded using a 4 point system with a higher number representing more severe disease as described in the Methods section. The calpain-2 inhibitor treated mice showed significantly less pathology than the mock injected mice, although those mice treated with the inhibitor were not completely protected from disease (Figure 17). The inhibitor treated group had less general tissue disruption, pleiomorphism, and hyperchromasia. These histological analyses were consistent with the macroscopic assessment of the colons using a dissecting microscope.
Figure 16 Calpain-2 inhibitor treatment results in smaller colorectal tumor volumes

Mice subjected to the AOM/DSS model of colitis and associated colorectal cancer were assessed for tumor volume. (a) Large colon tumors are visible in the distal regions near the rectum and calpain-2 inhibitor treatment significantly reduced the size of these tumors. (b) The measurements of colon tumors from mice (n=20) shows a statistically significant reduction in tumor volume p<0.0001 by Tukey post-test. Data represent mean ± SEM.
Figure 17 Calpain-2 inhibitor treatment reduces colon cancer pathology

Mice subjected to the AOM/DSS model of colitis and associated colorectal cancer were assessed by histology. (a) Colon cancer pathology was evident in AOM/DSS mice and calpain-2 inhibitor treatment significantly alleviated the pathology. (b) Slides were blindly scored by an independent Pathologist (Dr. Chrisy Mafnas). Data represent mean ± SEM (n=20) and means were compared by one way ANOVA followed by Tukey post-test, p<0.05.
Chapter 4: Discussion
4.1 Mesothelioma cells require antioxidants to limit reactive oxygen species

Selenium has been a topic of great interest in the field of cancer research, but the role of this essential micronutrient in mesothelioma disease onset or progression has not been fully elucidated. Due to its antioxidant and cancer-cytotoxic properties, super nutritional levels of selenium are thought to limit the pro-oxidant conditions that may initiate carcinogenesis or fuel progression of established tumors. The role that higher selenium intake plays in promoting DNA damage repair is very different from the role selenium and selenoproteins play in redox homeostasis in established, proliferating cancer cells. While our study does not address MM carcinogenesis, our findings suggest that higher selenium intake is not beneficial for individuals with established MM tumors. In fact, depending on the manner in which increasing selenium is utilized for reducing capacity by the MM cells comprising the tumor, selenium supplementation may even promote tumor progression.

Mesothelioma is a relatively rare form of cancer, but significant numbers of asbestos exposed individuals in the U.S. are still at risk of developing this deadly disease and the incidence of mesothelioma is on the rise in many parts of the world. It is important to fully characterize the relationship between dietary selenium and mesothelioma given that dietary selenium intake widely varies compared to other micronutrients, with mean values of 40 μg per day in Europe and 93 μg per day (in women) to 134 μg per day (in men) in the U.S. Furthermore, an estimated 18-19% of adults in the U.S. use supplements...
containing selenium \(^6\) and selenium-fortification of foods is being pursued in many parts of the world to boost sub-optimal populations and as a supernutritional means of nutritional cancer prevention \(^7\)-\(^9\). The minimum selenium intake and supernutritional levels by rodents has been established to be 0.1 ppm and approximately 0.8 ppm, respectively \(^10\). Thus, our study was designed to include moderately low (0.08 ppm), adequate (0.25 ppm) and supernutritional (1.0 ppm) levels of selenium intake. Some rodent anticancer studies use diets with 2.0 ppm selenium \(^11\), which is likely to further increase the levels of potential anti-cancer selenium metabolites \(^12\). It is possible that increasing our mouse diets to 2.0 ppm may have induced some toxic effects on the MM tumors, but given that this level is 20-fold higher than the minimum intake and the recommended 200 \(\mu g/day\) selenium supplemented levels in humans is 4-fold higher than the minimum intake \(^10\), we chose a more practical use of 1.0 ppm as a supernutritional selenium diet for this study.

The specific mechanisms by which increased selenium affects cancer cells are complex, with both selenoproteins and selenium metabolites playing important roles \(^1\). In fact, selenoproteins themselves may play roles in both preventing and promoting different types of cancer \(^13\). Our findings suggesting that some MM cells utilize increasing selenium to increase reducing capacity reflected by increased GSH stores are difficult to explain through a simple model of increased selenoprotein activity. There is no evidence to date showing that selenoproteins directly contribute to increased synthesis or stability of GSH, though other studies have shown interactions between increasing selenium and
GSH levels\textsuperscript{14, 15}. Comparing CRH5 to AB12 MM cell lines, both exhibited increased GPx1 activity with increasing selenium intake but only CRH5 demonstrated increased GSH levels as well. This endowed CRH5 MM cells with the ability to detoxify H\textsubscript{2}O\textsubscript{2} more effectively with increasing selenium, presumably through GPx1 activity. Thus, GSH appears to be the limiting factor in mediating the effects of increased selenium on redox status in MM cells and this further affects the activation of pro-growth effector molecules like ERK. ERK is a crucial MAPK that has been previously identified as a ROS-sensitive survival signaling factor\textsuperscript{16}. It has also been shown that ERK activation is an essential step in the progression of mesothelioma tumors\textsuperscript{17}. Thus, we have established an important linkage between selenium intake, GSH reserves, and ERK activation that may reflect a different metabolism that is established in cancer cells arising from the inflammation driven by the original exposure to asbestos or erionite.

Interestingly, our findings suggest that MM cells that more efficiently utilize bioavailable selenium for higher reducing capacity may better adapt to the oxidative stress associated with the dense environment of tumors. This was demonstrated by the data showing that CRH5 and EKKH5 cells benefitted most from higher selenium conditions as cells approached confluency. The need of cancer cells to detoxify ROS increases as the density of cells increases has been demonstrated\textsuperscript{18}, and our data support this concept. Whether MM cells utilize increasing selenium for increasing antioxidant capacity may depend on their location within a tumor, availability to the vasculature, or other factors. Overall, our data suggest that increasing intake of selenium is not beneficial for MM
patients and provide important insight into the use of this micronutrient by certain MM cells for increasing proliferative capacity as cells approach conditions of higher cell density.

### 4.1.1 Future Directions for dietary selenium and mesothelioma

Our laboratory has demonstrated potentially harmful aspects in the assumption of the beneficial effects of increased dietary selenium. This does not suggest that a high level of dietary selenium is detrimental for human health as a whole but instead suggests that a more nuanced approach to the consumption of selenium as a dietary supplement is needed as we move forward.

We did not completely elucidate the mechanistic underpinnings that cause these cell lines to behave so differently when exposed to differing concentrations of selenium. A more complete picture of the selenium sensitivity of mesothelioma cells may also potentially lead us to a biomarker to differentiate selenium sensitive cell lines from insensitive cell lines in cancer patients. In order to determine if selenoproteins might serve as a diagnostic or prognostic biomarker we could investigate with real-time PCR and Western blotting in selenium sensitive and insensitive cell lines and look for consistent selenoprotein expression differences.

We suspect that a contributing factor in the redox metabolism of these cell lines may be the age or number of passages in culture. There has been some suggestion that cancer cell lines adapt to their conditions over time and it has been established that they continue to accumulate genomic mutations over time.
It is possible to imagine that the cells may adapt to thrive in a nutrient rich, crowded environment. This may indicate the accumulation of mutations that regulate redox stress may be particularly favored by rapid proliferation.

In the future we would like to further characterize these cells to determine the number and type of mutations, to characterize telomere length as an indicator of number of passages, and to also work with newly derived (within 5 years) human cell lines. In this way it would be possible to demonstrate whether there is a selective pressure that favors selenium sensitive or insensitive mesotheliomas independent of other factors such as number of passages in vitro.

4.2 The calpain/CAST system controls macrophage inflammatory activation

Inflammation is at the root of the carcinogenesis of MM and colon cancer. We have discussed the dangers of selenium supplementation in the case of MM and the possibility of cancers taking advantage of biological mechanisms to limit reactive oxygen species and promote their own proliferation. We have not discussed addressing the inflammation that leads to the resulting cancer. Here we will discuss one of the signaling mechanisms in inflammatory macrophages that leads to inflammation induced colon cancer.

The calpain/CAST system regulates cellular function through the proteolytic modulation of structural and signaling proteins. Importantly, Ca$^{2+}$ induces activation of calpain in an irreversible manner, and thus the endogenous calpain inhibitor CAST is crucial in the regulation of calpain activity. Our recent publication suggested a unique role for CAST in regulating macrophage
activation \cite{113}, and the results presented here extend those findings to suggest a
dynamic, regulatory role for CAST in macrophages during IBD.

Calpains have been suggested to promote inflammatory conditions for
different disease states \cite{119,139,79}, and we predicted IBD tissues would exhibit
higher levels of calpain activity. However, our data show that calpain activity
initially increased during acute inflammation and then decreased in chronically
infamed intestinal tissue compared to healthy controls. Despite decreases in
calpain activity in the colon during chronic IBD, levels of both calpain-1 and
calpain-2 remained unchanged. In contrast, the endogenous inhibitor of calpains,
CAST, was increased during chronic IBD and this appears to contribute to the
decreased calpain activity observed in chronically inflamed intestinal tissues.
Thus, the regulation of calpain activity during inflammatory conditions arising
during colitis largely relies on the protein dynamics of CAST, mainly upregulated
in macrophages.

This highlights the crucial role this endogenous inhibitor plays in limiting
the level of inflammation during IBD and perhaps other macrophage-driven
inflammatory diseases. A key finding in this study is that CAST deficiency in the
immune system alone is similar to CAST deficiency in the whole animal in
causing more severe colitis compared to controls. This suggests that CAST is
particularly important in the immune system for regulating the level of
inflammatory response during colitis. There were some minor differences in the
whole animal and immune system CAST KO models. In particular, the bone
marrow chimeric mice with induced colitis exhibited more persistent weight loss
compared to the mice included in the whole animal knockout model. This may be due to effects on the gut resulting from the x-ray irradiation such as chronic epithelial cell shedding, differences in appetites, or some other factor included in the bone marrow reconstitution process.

Regardless, the similar patterns of weight loss combined with similar levels of colitis pathology suggest that CAST deficiency in the immune system is sufficient to reveal an important regulatory role for CAST in limiting the severity of this disease. One previous study showed that the type-I calpain inhibitor, which inhibits both µ- and m-calpain isoforms, significantly reduced the severity of DNBS-induced acute colitis in rats. Daily treatment of the rats with the calpain inhibitor reduced colon injury and clinical symptoms such as haemorrhagic diarrhea and weight loss, and also reduced inflammation as indicated by lower levels of mucosal myeloperoxidase activity and colonic expression of ICAM-1 and Pselectin. This suggests that calpain inhibitors may be useful in the treatment of IBD\textsuperscript{120}, although the lack of information regarding mechanisms by which the calpain/CAST system modulates inflammatory processes is a major impediment to the development of drugs targeting these proteins. Our data from the mouse model of colitis demonstrated a much higher level of CAST in intestinal macrophages compared to other cell types, with CAST levels mainly upregulated in macrophages in the colon during colitis. Importantly, Western blot analysis showed a higher degree of increased CAST (83% increased) compared to results from intracellular flow cytometric analyses of CASTβ (38%), which highlights how these two very different approaches may give different results.
This may be due to differences in recognition of CAST in the fixed/permeabilized cells involved in the flow cytometric assay or other factors. These results suggest that macrophages may serve as a more specific target of pharmacological modulation of the calpain/CAST system.

We found that the total number of macrophages in colons from mice with induced colitis was increased compared to healthy controls, which is consistent with other studies \(^{116, 117, 118}\). However, enumeration of other immune cell types was not performed and despite our data suggesting that CAST was largely expressed in macrophages from colitis tissues, other immune cell types are likely to be involved in the inflammatory processes underlying colitis. In fact, emerging evidence suggest that calpains likely play important roles in other immune cell types. Levels of calpain mRNA and, in some cases, protein have been shown to increase during activation of T and B cells \(^{55}\). This same study suggested increased calpain mRNA and protein results from IFNγ-induced activation of the human monocytic cell lines. Migrating T cell adhesion via LFA-1 is also regulated by calpain \(^{57}\). Thus, different components of inflammatory responses contributing to the pathology of IBD likely depend on CAST and these mechanisms are currently under investigation.

Our data show that TNFα priming of macrophages increases CAST, and without this increased CAST a subsequent exposure to PAMPs like \textit{E. fecaelis} and CpG results in hyperactivation. While our data reveal a critical role for CAST in limiting NF-κB activation in macrophage-driven inflammation, the notion of NF-κB as a critical regulator of IBD is not novel. NF-κB activation has been shown to
be markedly induced in IBD patients, particularly in macrophages and epithelial cells isolated from inflamed intestinal tissues from these patients. Our data in macrophages place CAST upstream of NF-κB activation through its inhibitory actions on calpain-cleavage of IκB, suggesting CAST may serve as a master regulator of hyperactivation of these cells. The multiple cellular functions regulated by the calpain/CAST system pose significant challenges to determining how treatment with calpain inhibitors may interrupt the development of IBD.

Targeting macrophages with calpain inhibitors to reduce NF-κB pathway activity may more specifically attenuate inflammation in IBD patients. CAST appears to be critical in determining the extent to which IκB is cleaved by calpain and serves to limit the activation state of macrophages during IBD. Interestingly, despite both iNOS and MCP-1 also being transcriptional targets of NF-κB regulation, our data suggest these are not influenced by CAST deficiency. Also, several other cellular targets of calpain cleavage are likely affected by the dynamic regulation of CAST in macrophages. We have focused primarily on NF-κB due to its known involvement with macrophage inflammation. We have also looked for alterations in AP-1 signaling through ERK, p38, and JNK. We did not find any evidence that phosphorylation of these kinases was altered in the CAST KO inflammatory BMDM relative to the WT.

Calpains cleave a large subset of cellular proteins, including cytoskeletal proteins, membrane receptors, calmodulin binding proteins, G proteins, protein kinase C, other enzymes involved in signal transduction, and many transcription factors. Some of the signaling molecules that have been shown to be regulated
by calpain cleavage in other cell types include Stat3, Stat5 and Stat6 in mast cells as well as SHP-1 and -2 in T cells. Cell motility and adhesion are important immune cell functions that are also regulated by the actions of calpain. Thus, in addition to IκB, other calpain targets are likely involved in macrophage activation and the role CAST plays in the activation and differentiation process is currently under investigation.

Another possible interpretation of the findings in the present study is that CAST deficiency may increase susceptibility to severe IBD in humans. This raises the question of whether single nucleotide polymorphisms (SNPs) in the CAST gene or regions that regulate mRNA expression for CAST may increase susceptibility for onset or severity of human IBD. In fact, 48 CAST SNPs have been identified, 20 within the CAST gene itself. None of these SNPs have to date been associated with either Crohn's disease or ulcerative colitis through genome-wide association studies. This may reflect a role for CAST in regulating the severity of IBD, not necessarily triggering the onset of disease. The lack of data associating CAST SNPs with IBD is not surprising, given the complex genetic architecture of these diseases in humans. One disease that has been linked to CAST SNPs is Parkinson's Disease, which is a very different inflammatory disease. In fact, the study of the calpain/CAST system in disease pathogenesis has to a large extent focused on its role in apoptosis related to ischemia/reperfusion injury, cancer, muscular dystrophy, or neurological disease. However, the novel findings presented in this study as
well as recently published data from our laboratory suggest an additional and critical role for CAST in regulating macrophage activation and colitis.

We observed a dynamic role for CAST during macrophage activation that was not found in other immune cells such as T cells or B cells. These findings suggest that CAST levels in macrophages are particularly important during \textit{in vivo} inflammation. Indeed, the present study showed that macrophages purified from inflamed colons exhibited very high levels of CAST compared to the non-macrophage population including other immune cells. The combination of the increased numbers of macrophages in the inflamed intestines together with the increased CAST in these cells highlight the particularly important role of CAST in regulating macrophage-driven inflammation.

Based on these findings, we propose that, in addition to its widely studied roles in apoptosis-related pathologies, the calpain/CAST system plays an important but underappreciated role in chronic inflammatory diseases, with CAST acting as a crucial factor in limiting macrophage activation and attenuating inflammation. Studies are ongoing to determine the exact role that CAST plays in attenuating activation of macrophages and the development of IBD.

4.2.1 Future directions for CAST investigation

While it is clear that CAST is dynamically regulated in order to regulate calpain activity it is currently unclear exactly how CAST itself is regulated. As mentioned above there have been many diseases linked to NF-κB in general and calpain activity more specifically. Our ongoing research is focused on the use of
calpain inhibitors to treat some of these diseases but it may also be advantageous to investigate therapeutic targets that regulate the expression of the endogenous inhibitor.

With this end in mind it is important that research insights be made that further our knowledge of the cellular mechanisms that regulate CAST expression. The literature is currently not clear on the source of the upregulated CAST protein\textsuperscript{148 149}. We have seen that CAST protein levels increase within 30 minutes of macrophage activation implying that the regulatory mechanism is not based in mRNA upregulation and translation. It may be that protein degradation processes are alleviated allowing the concentration of CAST to rise rapidly. It is also possible that CAST is regulated by release of monomeric CAST from oligermized stores of the protein, which allows a rapid inhibition of calpains that we observe\textit{ in vitro}. In any event it would be extremely beneficial to understand the mechanisms by which CAST is regulated in different cell types so that we can potentially use the mechanisms to intervene in more physiologically relevant ways.

4.3 Calpain-2 is a promising future anti-inflammatory target

The current focus for the treatment of inflammatory diseases is cytokine inhibitors, specifically TNF\textsubscript{α} inhibitors, which are a primary example of a general immune suppression treatment. This sort of treatment focuses on the soluble mediators of inflammation such as cytokines. There are many reasons why this might not work for every patient. It is important to keep in mind that different cells
produce different mediators at various points in time for many purposes and often these purposes are locally oriented. A good example of this is the fact that gut epithelial cells produce TNFα in response to gram-positive bacteria. This TNFα in turn causes goblet cells to upregulate the production of Mucin in order to restore local protective barriers. If TNFα production is systemically targeted then this localized production for anti-inflammatory purposes would also be affected.

Our studies are focused on the cellular factors that act as master regulators of inflammatory cytokine production. In this manner, our approach involves therapeutically targeting cellular factors that regulate multiple inflammatory mediators instead of individual mediators and this will provide a more effective treatment strategy. Emerging evidence published by our laboratory and others suggests that calpains play an important role in regulating inflammation and immune responses. The calpain protease system and its dynamic role in macrophage activation represent a promising target of potential drug intervention for inflammation driven disease. Calpain-2 inhibition with the small molecule inhibitor zLLY-CH2F shows great potential for the inhibition of inflammatory cytokines produced by activated macrophages and the attenuation of colorectal cancer growth through the attenuation of the production of inflammatory cytokines.

We stimulated macrophages with TNFα in the presence or absence of calpain-2 inhibitor to determine if excess calpain inhibition beyond the normal endogenous CAST could further reduce inflammatory response. We found that treatment with a calpain-2 inhibitor reduced the production of the inflammatory
cytokines IFNγ, TNFα, IL-6, and MCP-1. This suggests that at least part of the inflammatory response in macrophages is directly controlled by calpain-2 specific proteolytic activity and implies that a calpain-2 inhibitor may serve as a macrophage specific anti-inflammatory drug intervention. The lack of inhibition of IL-10 supports the idea that this is an inflammatory activation specific process.

The DSS induced model of IBD leads to significant weight loss in mice due to the detergent removing the Mucin layer that protects the underlying epithelium. The removal of this Mucin layer leaves the tissue unprotected from the commensal bacteria populations and allows invasion by the bacteria that leads to an immune cell reaction causing the symptoms and pathology of colitis. Once the DSS treatment is removed, the gut can begin to recover and the mouse can regain lost water weight. It is continuing inflammation that might limit this process.

Our data show that mice treated with a calpain-2-specific inhibitor regain weight much more rapidly after treatment with DSS and regain weight up to the weight of the untreated negative control mice. We believe this indicates that the calpain-2 inhibitor treated mice have dramatically reduced chronic inflammation resulting from the inflammatory stimulation caused by the DSS treatments. This is an important distinction. If calpain-2 inhibitor treatment totally blocked weight loss then we would suspect that events that trigger colitis and weight loss were eliminated. It is unlikely that an anti-inflammatory agent like calpain-2 inhibitor would interfere with the direct effects of DSS on the epithelial barrier, and our
data suggest that the inhibitor is acting to limit the inflammatory response induced by the effects of DSS.

These results were not completely surprising given that we have previously demonstrated that macrophages are the predominant immune cells that dynamically regulate calpain activity in the DSS induced mouse model of IBD with the endogenous inhibitor CAST. Dendritic cells and T cells also become activated in an inflammatory response to the invading bacteria. This activation contributes to inflammation. The inflammatory response in turn causes damage to the surrounding gut tissue that leads to weight loss, replacing DSS-water with regular drinking water allows the mice to immediately begin to regain weight and continue to gain weight until they match the negative control group. We suspect that the anti inflammatory effect is primarily due to the inhibition of NF-κB signaling in macrophages and reduces their ability to promote chronic inflammation, but we have not ruled out effects of calpain-2 inhibition on other signaling pathways.

Blinded scoring of H&E-stained sections from the mouse colons showed significantly reduced colitis as measured by the infiltration of leukocytes, disruption of epithelial crypts, and ulcerations in the epithelial tissue. The results clearly indicated that treatment with a calpain-2 inhibitor reduces the pathology resulting from chronic colitis. We also used a hemoglobin ELISA of the cecum as another measure of the health of the mouse colons. Under chronic colitis conditions ulcerations of the gut lead to both water and blood loss from the tissue. The hemoglobin ELISA showed that the levels of hemoglobin in the DSS
treated mock injected mouse cecums were significantly increased while the calpain-2 inhibitor treated mice had levels similar to the untreated mice. All of this taken together indicates that the calpain-2 inhibitor was protective against the inflammatory conditions created by DSS induced colitis in mice.

Analyses of the mouse colons for cancer pathology demonstrated a reduction in inflammation from calpain-2 inhibition as indicated by smaller colon tumors and lower levels of colon cancer pathology. Histological evaluation of mouse colons revealed a marked increase in intracellular Mucin. The cells of the crypts appeared more intact with less stratification and overall architectural distortion. It is evident that the anti-inflammatory effects of the calpain-2 inhibitor reduced pathology, but it remains unclear how this treatment may reduce the size of tumors. We need to consider the carcinogenesis events along with the tumor growth itself. Effects on either or both of these factors may contribute to reduced tumor size by the calpain-2 inhibitor. This question is complicated by the fact that tumor-associated macrophages have a significant role in the development of a tumor and that the calpain-2 inhibitor may affect these cells or could affect the colon cancer cells themselves.

The possibility of a calpain-2 inhibitor acting directly on the colon cancer cells themselves is supported by the fact that calpains are involved in processes such as cytoskeletal remodeling, cellular signaling, apoptosis, and survival. Calpains can also be phosphorylated by ERK and this phosphorylation has been shown to be related to changes in mobility and tissue invasion\textsuperscript{150}. Calpain activity has been linked to chemotherapeutic resistance in colorectal cancer,
esophageal cancer, and melanoma\textsuperscript{151 152 153}. This resistance has been specifically correlated with calpain-2 activity. In melanoma, the correlation has been extended to survival outcomes with tumors expressing high levels of calpain-2 resulting in shorter survival for patients\textsuperscript{152 154}. Importantly, calpain proteolytic activity has been shown to increase NF-κB signaling in melanoma cancer cells and treatment with calpain inhibitor was able to attenuate this signaling in cisplatin resistant cells \textsuperscript{154}. Other researchers have demonstrated a correlation between calpain expression and drug resistant esophageal cancer \textsuperscript{155}. These correlations are clear evidence pointing to the need for further investigation of the involvement of the calpain/CAST system in carcinogenesis and tumor progression.

These data taken together indicate that calpain-2 inhibition is a viable treatment for IBD and may also reduce the risk of IBD patients to inflammation associated colorectal cancer. Calpain-2 inhibition protects from chronic inflammation in IBD and can prevent pathology of the gut arising from this chronic inflammation. Secondarily, calpain-2 inhibition may directly regulate growth of the colon cancer cells themselves. The combination of effects on the inflammation and the colorectal cancer suggest this approach could prove to be an effective inflammation treatment.

The effects of the calpain-2 inhibitor on IBD were extremely significant and a partial elimination of this disease is a success. In the case of the cancer though, this is not necessarily the case. We were able to reduce the resulting sizes of the tumors in the colons but not the incidence of cancer in these mice.
This distinction means that this treatment is not a complete solution for colon cancer resulting from inflammation. It has been previously mentioned that calpain-2 activity is involved in chemotherapeutic resistance. This may mean that the use of a calpain-2 inhibitor, while not enough to completely eliminate colon cancer, may be able to act in conjunction with chemotherapy to eradicate growing colon cancer.

4.3.1 Future Directions for calpain-2 inhibition

Calpain-2 is a fairly ubiquitously expressed enzyme. This means that the potential for off target effects when treating with a calpain-2 inhibitor are significant. We plan to examine a sampling of tissues from calpain-2 inhibitor treated mice including the lungs, brain, liver, and eyes. We will run calpain activity assays on the treated and untreated control organs to determine if we are lowering the calpain activity in these organs with our treatments. We will also use H & E staining of tissues to determine if any pathologies are present with long term treatment. We will also explore the possibility of colon targeted delivery of the calpain-2 inhibitor. IBD is unique in that the organ of interest can be easily targeted through oral or rectal administration. Rectal drug administration is useful in case of cancers given that the orientation of colon cancer is often nearest the rectum. However, this sort of drug delivery is not effective for the treatment of the rest of the colon or small intestine. Oral administration is still the preferred route for treatment of IBD. This delivery poses problems for stability of the treatment given the harsh conditions of the upper gastrointestinal tract. One method that
has been previously used successfully is the addition of 5-aminosalicylic acid by azo bond which makes the drug less permeable and more stable\textsuperscript{156}. In this way we may be able to limit potential side effects to the colon specifically for our future work.

Inhibiting calpain-2 activity limited macrophage inflammatory activation. This is a positive outcome in the case of aberrant inflammation but in the case of normal inflammatory processes this could mean a weakened immune system and greater likelihood of dangerous infection. In order to address these concerns we will infect mice with viruses and bacteria while treating with calpain-2 inhibitor. This will allow us to assess the immune system function in the context of long term treatment with a calpain-2 inhibitor.

Given the macrophage specific nature of the dynamic regulation of the calpain-2 for cellular activation, calpain-2 inhibitor treatment may be viable for IBD or other macrophage driven inflammatory disease. Macrophages have roles in various diseases including osteoporosis, atherosclerosis, fibrosis, cancer, obesity and diabetes, and arthritis. Our future goals involving this inhibitor include the examination of the effects in other mouse models of human inflammatory diseases to determine the extent of its possible use as a treatment. If the drug is demonstrated to be safe for use in humans it will be useful to know the range of inflammatory diseases that it can possibly alleviate.

We will also expand our research to human cells and tissues in other diseases. We have demonstrated that CAST, the endogenous inhibitor of calpain activity, is upregulated in human IBD. Tissues will be acquired pertaining to other
human inflammatory diseases to determine if CAST is limiting inflammation responses in other diseases as a first step to determine if calpain-2 inhibition might also serve to increase upon the normal endogenous calpain inhibition to limit other inflammatory disease.

It is also important that we revisit the question of how calpain-2 inhibition is limiting the size of colon tumors in the mouse model of CAC. We will look at the direct affect of calpain-2 inhibition on colon cancer proliferation but also on carcinogenesis. If we can answer these questions then we will have a better picture of the potential anti cancer aspects of calpain-2 inhibition.
Bibliography


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