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MK2 Promotes Colorectal Cancer Development, Invasion, and Growth Through MK2-Induced Cytokines

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MK2 PROMOTES COLORECTAL CANCER DEVELOPMENT, INVASION, AND GROWTH THROUGH MK2-INDUCED CYTOKINES

BY

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Abstract

Colorectal cancer (CRC) is the third most common malignancy in the United States for men and women. A major risk factor for CRC development and progression is inflammation, in particular the inflammatory cytokines IL-1, IL-6, and TNF-α. These proteins promote critical cancer functions, such as proliferation, invasion, survival, and migration. Control of inflammation is one potential area of therapeutic intervention for some cancers. Because signaling of these cytokines overlaps in many of the same pathways, targets upstream of protein production could prove an effective strategy to reduce inflammation. MAPK-activated protein kinase 2 (MK2) pathway controls production of these cytokines within the MAPK p38 pathway. By investigating the role of MK2 signaling in CRC, we demonstrate that MK2 is a critical protein in the initiation and progression of colitis-associated and spontaneous CRC. $MK2^{-/-}$ mice have much lower expression of IL-1, IL-6, and TNF-α than wild-type mice, indicating that MK2 is the primary regulator of these cytokines in a colitis-associated model of CRC. MK2 blockade also decreased cytokine-producing macrophage populations, which are an important

source of inflammatory cytokines that support tumor growth. $MK2^{-/-}$ mice are completely protected against neoplasms. MK2 is thus a crucial component of CRC development that regulates intestinal macrophage populations. Addition of wild-type macrophages to mice in the CRC model increased cytokines in both wild-type and MK2 mice, and tumor burden in wild-type mice. Pharmacological inhibition of MK2 in wild-type mice after neoplasms developed caused complete regression in the majority of mice. MK2 is thus implicated as a critical signaling pathway throughout tumor initiation and development in a colitis-associated model of CRC. To address MK2's participation in an invasive model of CRC, a syngeneic cell line was used to develop tumors. Cells treated with MK2 inhibitor developed smaller tumors and produced approximately 80% less MK2-induced cytokines than controls. Treatment of cells with MK2-induced cytokines induced MK2 signaling, and restoring MK2-induced cytokines to tumor cells treated with MK2 inhibitor restored tumor burden and cytokines. These data support the existence of an inflammatory feedback cycle that drives CRC. MK2 inhibition is effective in multiple models, and offers a potential therapeutic target.

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Chapter 1: Introduction

Background

Colorectal Cancer and Inflammation

Colorectal cancer (CRC) is one of the most common malignancies in both men and women (1). In the United States, an estimated 134,490 diagnoses and 49,190 deaths occurred in 2016 due to CRC (2). Most CRC patients are diagnosed between the ages of 55 and 84, and the majority of CRC are caught at an early stage (2). These diagnoses usually are a result of routine screening, including a variety of techniques including colonoscopies and fecal occult blood testing (3). Screening is relatively commonplace in the United States now and accounts for the increase in early detection of tumors and pre-cancerous polyps. The prognosis of patients who are diagnosed with early stage CRC is excellent, with an average 5-year survival of 90% (1). However, as CRC invades and spreads, survival probability decreases considerably. Once CRC reaches a local lymph node, the 5-year survival drops to 71%, then to 13% as the cancer metastasizes to distant sites (1). Over the last decade, the diagnosis of CRC has dropped by 2-3% per year (2). However, this rate of improvement is largely attributed to increased patient compliance with screening procedures, rather than decrease in risk factors. Screening procedures can lead to identification of pre-cancerous polyps; removal of these prevents their progression into CRC and reduces cancer diagnosis numbers. The improvements observed in diagnosis are expected to plateau as patient screening approaches 100%. A recent increase in young adult rates of CRC may indicate a potential

increase in CRC in younger populations (4). It isn't clear yet which factors have affected this increase, and whether it will continue in the future. Despite the improvements in recognition of early CRC, there are tumors with very poor prognoses. Survival rates for advanced CRC remain extremely low. Some cancers diagnosed early are extremely resistant to treatment, and progress despite current interventions. Strategies to target CRC are needed to improve outcomes in cases that are resistant to the current therapies.

The search for therapeutic targets is challenging, as colorectal tumors are genetically diverse. Even within a single tumor, different cancer cells can possess different mutations (5;6). A good target for therapeutic intervention is a pathway that is important throughout cancer development and progression, contributes to multiple pathological hallmarks, and is important in many tumors (7). Choosing a target that is important for cancers at many stages lessens the chance that a clinical intervention will miss a window of opportunity; this allows a particular therapy to be utilized at any point in diagnosis, and to be as universal as possible. Targets that participate in pathways that drive multiple characteristic cancer pathways, for example proliferation and invasion rather than just proliferation (7). CRC, like many cancers, has a diverse array of contributory factors, including a variety of genetic, environmental, and lifestyle factors. This can make it difficult to pinpoint a single target.

One extremely promising target in CRC is inflammation. Inflammation is best understood as a collection of responses to perceived harm. The classic gross signs of inflammation are redness, swelling, heat, pain, and loss of function. These symptoms

are a result of activated pathways that increase blood flow and vessel permeability to encourage immune cell infiltration into the tissue. The harm can take the form of damaged cells (injury) or recognition of pathogens (infection). Injury and infection may also be only perceived, as in the case of auto-immune responses, where the immune system is activated in response to the host. Cells that detect damage or infection produce cytokines. Cytokines are small signaling molecules that can act in autocrine, paracrine, or endocrine signaling, but are often secreted and bind to cytokine receptors on the outside of nearby cells. Inflammatory signaling activates many pathways in an attempt to activate immune cells, resolve infection, and initiate healing. Inflammation is part of the initial immune response, but the inflammatory response affects nearly every cell. Pro-inflammatory cytokines often promote production of more pro-inflammatory cytokines, increasing the inflammatory response until resolution of the harm. These responses can in themselves be harmful, and are carefully regulated by anti-inflammatory pathways. Unfortunately, many of the strategies to repair damage can also promote cancer growth.

Unresolved inflammation can, over time, cause significant pathology, including potential for initiation and progression of some cancers. For example, pro-inflammatory cytokines can activate proliferation of epithelial cells (8). In wound healing, this proliferation is necessary and beneficial, but in tumors, it can reinforce an already pathological proliferative response. Many other aspects of the inflammatory response – survival, angiogenesis, and migration in particular – are part of cancer pathology, and participate in making tumors thrive. In addition, some immune cells produce DNA-damaging

molecules, which can increase the risk of mutations, especially if coupled with pro-survival pathways in the damaged cells (9). Inflammation has been identified in many cancers as a contributory factor, but the association is especially strong in CRC. Inflammation is present throughout CRC, and positively correlated in advanced CRC. Inflammation naturally promotes many cell and systemic processes that have come to be associated with cancer: proliferation, survival, invasion, and angiogenesis are all classic characteristics of tumors and inflammatory environments. Unsurprisingly, Inflammation is associated with the onset and progression of many cancers (7). Pathways that drive inflammatory responses are a likely source of therapeutic targets in many diseases. CRC in particular has a rich literature connecting inflammation with tumor initiation, growth, spread, and mortality. Identification of common, overlapping aspects of inflammation is likely to provide a short list of targets for further investigation.

Inflammation in colitis-associated and spontaneous colorectal cancer

A healthy colon is not an inflammatory environment. The colon has extensive anti-inflammatory activity in order to prevent inflammation, even in the case of microbial detection (10). There is increased microbial contact in the mucosa compared to other tissues. If the gut reacts with a strong pro-inflammatory response every time there is microbial contact, inflammation will be unceasing. There are multiple strategies employed to regulate homeostasis, including changes to resident macrophage behavior (discussed later in this chapter), anti-inflammatory cytokine signaling, and constant sampling of the gut lumen to promote tolerance (10;11). Chronic inflammation in the

gut is present only in disease states, such as Crohn's disease or ulcerative colitis (referred to collectively as inflammatory bowel disease (IBD)). Crohn's disease and ulcerative colitis are each estimated to affect approximately 200 of every 100,000 adults in the United States (12). IBD is characterized by chronic bouts of inflammation in the gastrointestinal tract (13). IBD is a risk factor for CRC development and poor outcome.

The chronic, cyclical inflammation causes not only severe pathology from IBD disease, but also vastly increases the likelihood that its sufferers will develop CRC. The longer someone has IBD, the higher the risk of CRC (14). Reported risk for Crohn's and ulcerative colitis vary by study, as well as by disease severity. Patients with extensive colitis are 14.8-fold more likely to develop CRC, while those with distal colitis have a relative risk of 2.8-fold above the population level (15). Even the lower risk categories of IBD carry a much higher chance of CRC development than most lifestyle-associated risks, including meat consumption and obesity (16;17). As both the severity and length of illness affect CRC risk, chronic inflammation is considered to be an extremely important factor of tumorigenesis. The risk of chronic inflammation does not stop at increased tumor initiation. Cancers that are colitis-associated are more severe and aggressive than those that develop without chronic inflammation (18). Tumor growth and metastasis increase in colitis-associated CRC (19). As a result, the 5-year survival rates of colitis-associated CRC are much lower than spontaneous (non-colitis-associated) cancers (20).

The Roles of IL-1β, IL-6, and TNF-α in Cancer

The originating causes of IBD are not well understood, but the inflammation is mediated by pro-inflammatory cytokines. Three of these have emerged as promoting

inflammation in IBD and CRC. Interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) promote feedforward loops of inflammation (21). They are associated with colitis, cancer initiation, and cancer progression. Investigation of these associations in mouse models has indicated that they are important parts of disease pathology. These cytokines are implicated as key factors not only in IBD pathology, but also CRC, and indeed they are thought to be one of the reasons that the risk of CRC is greatly increased in IBD patients. Colitis-associated CRC has increased levels of these cytokines; however, these cytokines are elevated in both spontaneous and colitis-associated CRC, and drive pathology in both. IL-1, IL-6, and TNF-α have been investigated as biomarkers of CRC (22). Because basal levels of cytokines can vary among individuals, a single measurement of serum cytokine levels are not enough to diagnose potential CRC. However, once diagnosed, cytokine levels may predict outcome. Higher levels of serum IL-1, IL-6, and TNF-α are predictive of tumor progression and poor outcome (23). These cytokines regulate each other, reinforcing the tumor-promoting activities of each; however, each of these has been well-established to promote tumor formation and progression in their own right.

IL-1 in CRC

IL-1α and IL-1β are inflammatory cytokines that are similar enough in structure and function that they are considered to be two forms of IL-1 (24;25). Both IL-1α and IL-1β are produced as precursor proteins of approximately 31 kDa; post-translational cleavage turns these into their final form. Pro-IL-1α and IL-1α are both bioactive, however, while pro-IL-1β is not active (25). Production and cleavage of IL-1α is ubiquitous, and IL-1α

commonly secreted from many cell types in response to stress and inflammatory signals. The cleavage of pro-IL-1β occurs through the inflammasome. The inflammasome is cell-type restricted, so IL-1β production is limited by the cell type and activation. Inflammasomes are generally restricted to professional immune cells such as macrophages, dendritic cells, and T cells (26;27). IL-1α and IL-1β bind to the same receptors. Like many cytokines, IL-1 receptors include a membrane bound receptor that promotes signaling in response to agonist binding (IL-1R1), and a soluble "decoy" receptor, which binds cytokines without any resultant signaling (IL-1R2) (28). Thus, inflammation can be tuned by increasing or decreasing production and secretion of IL-1, or by increasing or decreasing overall quantity and proportions of IL-1 receptors.

In a healthy colon, IL-1β is barely detected, as the signals needed for inflammasome activity are present only upon inflammation (29). Pro-IL-1α is produced even without inflammation, but is usually present only in cell-bound forms under normal conditions. Low levels of intracellular pro-IL-1α are thought to promote homeostasis, and not to serve as pro-inflammatory alarm proteins unless cell damage occurs, releasing it extracellularly (30). During chronic inflammation, such as IBD, secreted IL-1 increases. An important source of secreted IL-1 is populations of inflammatory monocytes and macrophages. Monocytes recruited to the mucosa during inflammation differentiate into macrophages that actively produce IL-1β (31). IL-1 can promote DNA damage through nitric oxide elevation (29;32). A higher rate of DNA damage increases the likelihood of accumulating key mutations required for cancer initiation. IL-1α and IL-1β are also produced in response to DNA damage (33;34), meaning that the early stages of

CRC initiation may drive cytokine production that will result in more damage. DNA damage should halt proliferation and initiate DNA repair pathways, or, failing successful repair, cell death. IL-1 can reduce the initiation of DNA repair pathways in mutated cells. In combination with TNF-α, treatment with IL-1 reduces the amount of DNA repair initiated in biliary cancer cells by 70% (35). IL-1 is thus supportive of the accumulation of mutations required for initiation of CRC.

IL-1 is an important driver of proliferation, metastasis, and survival. Once a tumor is initiated, IL-1 promotes rapid growth. IL-1 has long been known increase proliferation in a wide variety of cells, including epithelial cells. Activation of IL-1 signaling induces rapid division (36). IL-1 activates transcription factors, such as NF-κB, that promote proliferation (37). Recently, IL-1 has been shown to mediate production of miR-181, which has been implicated in rapid growth in CRC as well as glioblastoma and leukemias (38). The presence of IL-1 increases early angiogenesis, increasing the supplies of nutrients and supporting rapid proliferation (39;39). Angiogenesis also reduces the distance potentially metastatic cancer cells need to travel in order to enter the bloodstream. The formation of new vascularization in cancer can predict metastasis (40;41). Cancer cells still need to invade in order to reach blood vessels, but IL-1 also promotes invasion (42). Because IL-1 can promote both invasion of cancer cells and angiogenesis of the surrounding tissue, it should not be a surprise that IL-1 abets metastasis in animal models, and is associated with metastasis in humans (43-45). IL-1 not only promotes cancer progression, but also helps tumors survive. IL-1 mediates prostaglandin signaling, which helps create a niche for cancer stem cells (46). Stem cell

niches allow for a reservoir of cells to survive radiation or chemotherapy. IL-1 promotes stem cell characteristics in CRC, resulting in reinitiated tumors and reduced remission rates (42).

IL-6

IL-6 is a pro-inflammatory cytokine produced by a wide variety of cells, including epithelial cells, fibroblasts, and immune cells like macrophages (47;48). IL-6 is produced in response to damage, infection, or inflammation, usually as a transient alarm signal until resolution of the problem (49). IL-6 mRNA is regulated through degradation and modification (50;51). The receptor for IL-6 can be either membrane bound or soluble. Membrane-bound IL-6R is widely expressed, and results in multifunctional effects of cells (52). Soluble IL-6R lacks the signaling domains present in the cytoplasmic region of membrane-bound receptors, but once it is bound to IL-6, the complex can activate signaling in cells through gp130 (53). Effects of IL-6 usually support classic inflammatory phenotypes, including proliferation, survival, and further inflammatory signaling.

IL-6 is critical in both chronic inflammation and cancer development. It is a contributing factor in multiple models of colitis (54-56). High levels of IL-6 decrease the junctions between epithelial cells, which increases permeability across the epithelial layer. With increased permeability, microbes are able to infiltrate into tissue. The immune response can cause further tissue damage. DNA damage, which can be brought on by inflammation, induces IL-6 production (57). IL-6 decreases apoptosis, encouraging survival of potentially cancerous cells, even as it promotes proliferation (58;59).

Increased chronic levels of IL-6 in serum is associated with a much higher risk of colorectal cancer in people (60).

As tumors form and grow, IL-6 continues to promote survival of cancerous epithelium through STAT3-mediated pathways, protecting against DNA damage from nitric oxide and radiation (38;61-63). The pro-survival effects of IL-6 support chemoresistance, and blockade of IL-6 has been shown to increase sensitivity of tumor cells to chemotherapy (64). Like IL-1, IL-6 is angiogenic, increasing the access to nutrients through increased blood flow (65). It also increases invasion, which in combination with the increased blood vessel formation, is thought to drive metastasis (66). In a metastatic model of CRC, IL-6 promotes spread and invasion throughout lung. Metastasis is driven by signaling through STAT3, a recognized promoter of cancer (67). As tumors progress and produce more inflammatory cytokines, infiltrating immune cells increase and also take on pro-inflammatory phenotypes. One of the cytokines made is IL-6. The IL-6 from immune cells has been linked to increases in metastasis and invasion in CRC (68). Because IL-6 promotes many facets of aggression in CRC, it is considered a potential therapeutic target. There are several strategies under development, including antibodies against IL-6 or IL-6R. IL-6 blockade has shown promise in other cancers, but clinical data on CRC has yet to be released (69).

TNF-α

Like IL-6 and IL-1, TNF-α is a pleiotropic cytokine produced in response to damage, infection, or inflammation. TNF-α was originally identified as a cytokine that promoted necrosis in tumors. However, TNF-α activity does not always induce cell death; signaling

can also promote important cancer pathways, including survival, proliferation and invasion. TNF-α comes in two forms. Initially, it produced as a membrane-bound protein which is cleaved into its soluble form by ADAM17 (70). Blocking this cleavage has been shown to be protective in TNF-driven endotoxic shock and intestinal inflammation, suggesting that the soluble form may be the critical driver of the widespread inflammatory signaling that is associated with TNF (70;71). Soluble TNF-α promotes a systemic response to inflammatory events, acting as an alarm protein that activates responses on cells that express TNF receptors. There are two receptors for TNF-α, TNFR1 and TNFR2. TNFR1 has different distribution and function than TNFR2. TNFR1 is ubiquitous, and the cytosolic portion of the protein contains death-domain motifs. As the name implies, these death-domain motifs promote programmed cell death via activation of caspase-8, leading to apoptosis, and mixed lineage kinase domain-like protein (MLKL), which promotes necrosis (72-74). TNF-α signaling thus induces direct cell death via TNFR1, and in this capacity can help reduce tumor cell numbers. However, TNFR1 signaling also activates signaling pathways that promote cell survival and proliferation, including NF-κB, JNK, MAP/ERK, and p38 pathways (75). Each of these can promote cell survival, so there is a tension between the cell death and cell survival pathways initiated by TNF-α (76-78). Unlike TNFR1, TNFR2 does not contain death-domain motifs, and is not ubiquitous. TNFR2 is expressed primarily on endothelial and immune cells, but can be induced in intestinal epithelium (79). Under most models, TNFR2 is thought to promote homeostasis and tissue regeneration (80). However, TNFR2 is upregulated in the epithelium of IBD patients and after inflammation in mouse

models, and promotes proliferation in an inflammation-associated CRC mouse model (79). Thus, both TNFR1 and TNFR2 signaling may participate in CRC pathology.

TNF-α is usually present in extremely small quantities in normal colons. TNFR2 signaling helps maintain populations of anti-inflammatory T cells, which maintain gut homeostasis (81). Sizeable amounts of TNF-α are produced only during injury or disease. TNFR1 signaling can cause death of intestinal epithelial cells, causing further wounding of the colon (82). TNF signaling encourages migration and proliferation of surviving cells (76;83). At the same time, TNF induces mitochondrial reactive oxygen species (ROS) production, which contributes to DNA damage (84;85). ROS damage can contribute to TNF-related cell death, but cells are protected by the inflammatory p38 pathway. This pathways is activated by TNF-α or other inflammatory cytokines. Active p38 signaling reduces apoptosis and increases survival of cells with DNA damage. p38 signaling reduces apoptosis and increases survival at the G2 DNA damage checkpoint (86). During initiation of CRC, TNF-α assists by promoting inflammation, proliferation and survival of DNA damaged cells.

As tumors are established and begin to grow, TNF-α continues to support proliferation of malignant cells. Proliferation pathways are multiple, including NF-kB driven proliferation, and supports proliferation of dysplasic populations (37;79). Critically, TNF-α supports chronic inflammation. IL-1, IL-6 and TNF-α can all induce each other, but TNF-α drives IL-6 production in fibroblasts, which can help support tumor growth and progression (87). TNF signaling also induces MCP-1, a cytokine that attracts and activates inflammatory macrophage populations that contribute to cytokine production

within tumor tissue. As the tumor grows, TNF supports invasion through the basal lamina (88). Both TNF receptors support migration. The receptor that is the primary signal for migratory behavior depends on the concentration of TNF-α. Normal levels of TNF signaling promotes TNFR2-dependent migration, while higher levels of TNF signaling promotes TNFR1-dependent migration. These signals are mediated by NFκB and SNAIL, which have been implicated in progression of many cancer types (83;89). In addition to cell transformation, TNF-α increases the production of proteolytic enzymes that promotes invasion and migration of melanoma *in vivo* (90). These enzymes help remove barriers to invasion, allowing migratory cells to invade more deeply through basal lamina, so TNF-α "clears the way" for metastasis and tumor progression. The importance of TNF-α is supported by a wealth of literature concerned with TNF-α's contribution to cancer initiation, proliferation, invasion, and metastasis. In CRC, TNF-α is increased in advanced tumors and considered a potential intervention to reduce inflammation and improve outcomes (88).

The p38 family as a target to control inflammation

As IL-1β, IL-6, and TNF-α are extensively implicated in CRC initiation and progression, pathways that affect these cytokines have been considered for therapeutic intervention. One pathway that contributes to pro-inflammatory cytokine production occurs through p38 mitogen-activated protein kinase (MAPK) signaling (91). There are four members of the p38 family: p38α, p38β, p38γ and p38δ. p38α, referred to as p38, promotes survival and inflammation, including IL-1β, IL-6, and TNF-α production. p38 is activated primarily through extracellular signaling pathways as a result of stress or inflammation. Stress

can be induced by heat, osmotic changes, and UV light (92-94). A wide variety of inflammatory signals can activate p38; activating signals include IL-1, IL-6 and TNF-α (95;96). Growth factors can also activate p38 signaling (97;98). In response to these signals, MKK3 and MKK6 dually-phosphorylate p38, causing activation that drives inflammation, survival, cell cycle checkpoints and DNA repair, among other pathways (99-101).

p38 has been considered as a target for anti-inflammatory therapy for several diseases, primarily rheumatoid arthritis, but also chronic obstructive pulmonary disease, cardiovascular disease, and cancer (102-106). In multiple animal cancer models, p38 inhibition reduced tumor growth (107). Multiple small molecule inhibitors have been developed, and some showed efficacy in Phase II clinical trials (108-110). However, in many cases, initial improvements reversed, or were not statistically significant (111). A reason for observed ineffectiveness of inhibitors may be due to the activation of compensatory pathways. One concern with using p38 as a therapeutic target is that it promotes anti-tumorigenic as well as tumor-promoting pathways. For example, p38 can suppress initiation of tumors, but once cancer is present, promotes the survival of cancer cells (112). Another problem has also been the high rate of side effects observed in p38 inhibitors. These issues have led to a re-examination of p38 as an appropriate pathway to target (108;113). One option that has been considered to reduce off-target effects and improve targeting of inflammatory pathways has been moving downstream in the p38 pathway. p38 has many substrates, but IL-1, IL-6, and TNF-α production is not

evenly distributed among these; activation of a single kinase immediately downstream of p38 accounts for much of the inflammatory cytokine production (91).

The MK2 signaling pathway

Downstream of p38 is the mitogen-activated protein kinase-activated protein kinase 2 (MK2) is regulated by, and in turn regulates, p38 (Figure 1). MK2 lies directly downstream from p38. A nuclear localization signal lies at the C-terminus of the protein. While inactive, p38 and MK2 form a complex, and MK2 localizes the complex to the nucleus (114). This inactivated complex blocks binding grooves that would allow docking of substrates of either p38 or MK2 (115). In response to inflammatory or stress-related signals, p38 is phosphorylated to an active form, and then phosphorylates MK2 at up to 3 sites: Thr222, Ser272, and Thr334 (116). These phosphorylations change the conformation of the complex, allowing for binding to substrates for both p38 and MK2 (115;117). Phosphorylation of the Thr334 site creates a nuclear export signal, allowing for export of MK2 and complexed p38 into the cytoplasm (118). Both p38 and MK2 have substrates in the nucleus and the cytoplasm, so MK2's control of localization has an effect on which substrates are activated by p38 as well as MK2 (114).

MK2 regulates production of IL-1, TNF-α, and IL-6. Part of this production is regulated through increases in mRNA stabilization. The regulation of TNF-α is well characterized; IL-1 and IL-6 may be regulated through the same pathway, but the mechanisms have not been fully explored. For TNF-α, MK2 functions by promoting stabilization of mRNA

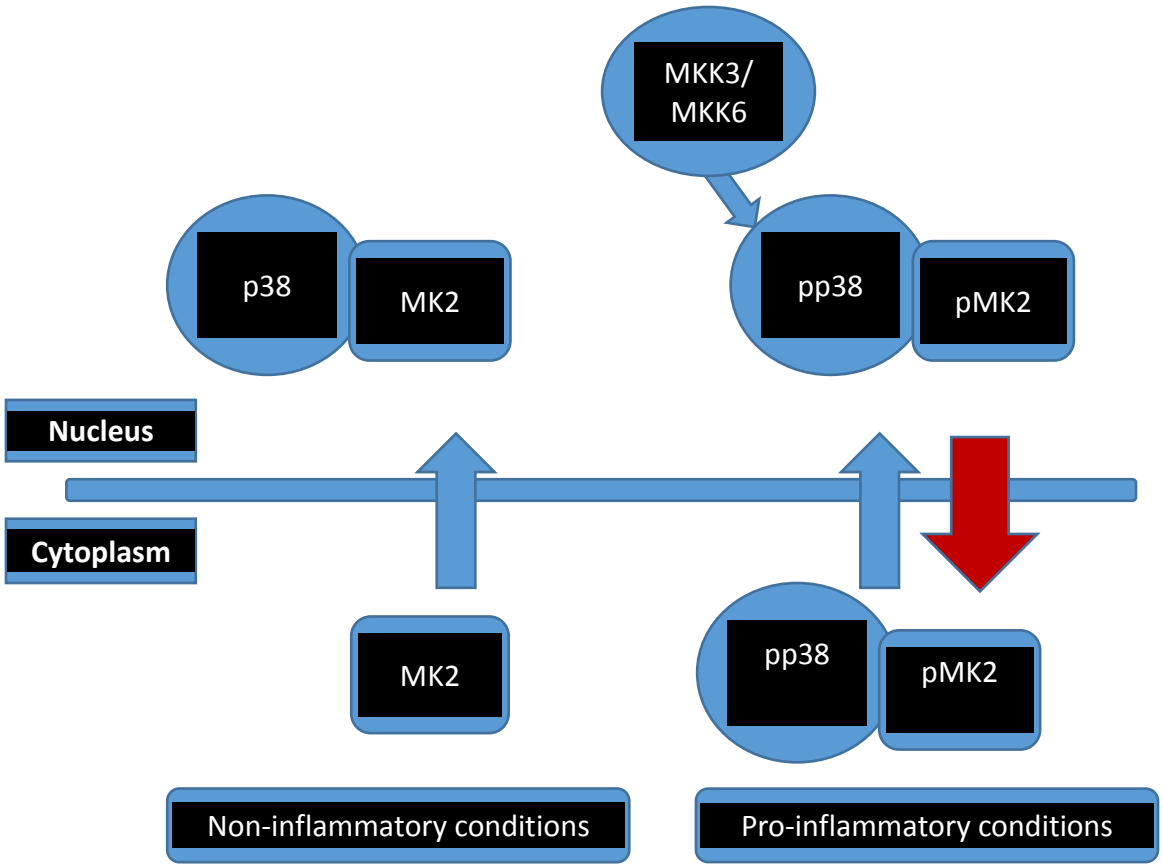

Figure 1. p38 and MK2 activation and trafficking. p38 and MK2 co-localize when inactive. Upon activation, p38 phosphorylates MK2, which exposes a nuclear export motif, allowing trafficking of p38 and MK2 into the cytoplasm. This process is important for cytokine production.

(194). TNF-α has AU-rich elements (ARE) in the 3'-UTR of its mRNA. Under normal conditions, tristetraprolin (TTP) binds to TNF's ARE, and recruits enzymes to form a complex that degrade mRNA (119;120). Active MK2 phosphorylates TTP in two places, which reduces its affinity for ARE (119). The reduced affinity allows for increased translation of TNF-α mRNA into protein. Simultaneously, MK2 increases stabilization by changing localization of protein human antigen R (HuR) and phosphorylation of heterogeneous nuclear ribonucleoprotein A0 (hnRNP A0), which bind ARE of TNF-α and block degradation (121;122). MK2 may affect IL-1 and IL-6 in similar ways. IL-6 is at least partially regulated by MK2 through its ARE, and IL-1 can be regulated by TTP (123;124). However, in macrophages, TNF-α and IL-6 are regulated independently, suggesting that there are additional mechanisms in MK2-driven cytokine production (125).

MK2 is an important regulator of IL-1β, IL-6, and TNF-α, and in turn they cross-regulate and can activate the p38 pathway, suggesting the existence of a positive feedback loop between MK2 and MK2-induced cytokines. p38 has been shown to be activated by IL-1α, IL-1β, IL-6, and TNF-α (95;96;126). As p38, mediated by MK2, promotes production of more IL-1, IL-6, and TNF-α, any one of these cytokines has the potential to induce the others. Extensive cross-regulation between these cytokines is confirmed by experimental observations, where IL-6 can induce and be induced by either IL-1 or TNF-α (127;128). IL-1 signaling increases TNF-α production, and in turn, TNF-α regulates IL-1α and IL-1β (129-131). Thus, the activities of MK2-induced cytokines are difficult to separate from one another, as the presence of one strongly upregulates production of

them all. The forward feedback loop in cytokine signaling and production means that factors that regulate all three together are especially attractive targets.

MK2 and Cancer

There has recently been an increasing interest in MK2 as a target for treating cancer. Our lab produced the first evidence for MK2's importance within CRC, but investigation into other cancers has increased, and evidence showing pro-cancer roles for MK2 in other tissues is emerging. In human non-small cell lung cancer, different studies have shown both increases and decreases in DNA copy number (132;133). Upregulation of MK2 protein has been observed in chemoresistant pancreatic cancer cells, suggesting that MK2 may support survival (134). MK2 mutations that increase the quantity of MK2 present in cells increase the risk of developing lung cancer, and worsens the prognosis once it develops (132;135). MK2 shows pro-survival activity in other tissue types, where MK2 promotes survival of tumor cells and relieves cell death stemming from oxidative stress (136;137). In response to DNA damage, which can be caused by any of the MK2-induced cytokines, MK2 signaling results in higher levels of Khsrp, a protein which modulates cell cycle in response to DNA damage (138). In addition to cell survival, MK2 promotes immune infiltration; active MK2 is associated with increased ICAM-1 expression on endothelial cells, which is a critical aspect of immune cell extravasation from the bloodstream into tumor tissue (121). MK2-sourced inflammation has not been studied as much as survival, but MK2 has been shown to support IL-6 production in glioblastoma cells (139). MK2 knockout also reduces skin tumors in a mouse model where inflammation is a major component of tumor formation (140). Investigation into

MK2-induced inflammation in cancer is limited, and this dissertation, with the experiments within, provides the first look at MK2's role in the development of CRC.

Macrophages and Inflammation in the Tumor Microenvironment

Infiltrating immune cells are an important source of inflammatory cytokines, including MK2-induced cytokines. Macrophages are cells that are some of the first responders to inflammatory events, and have been implicated in many cancers. Like other immune cells, macrophages have some pehnotypical plasticity, and their phenotype depends on the manner of activation, cytokine milieu, and other signals. Macrophages are implicated in pro-inflammatory behavior during IBD and CRC; their activity can alter the environment for potential or existent tumors.

Intestinal macrophages are frequently being replenished by monocytes as a normal part of intestinal homeostasis and during inflammation (141). Under homeostatic conditions, these monocytes give rise to intestinal macrophages that display an anti-inflammatory phenotype. They have reduced responsiveness to TLR stimulation and produce IL-10. During inflammation, monocytes differentiate into pro-inflammatory macrophages that are highly responsive to pathogen-associated molecular patterns (PAMPs) (141-143). Intestinal macrophages are abundant in the colon and are vital to gut homeostasis (144). Gut macrophages shape helper and regulatory T cell responses. These tissue specific macrophages are imprinted with an anti-inflammatory gene signature, allowing them to produce robust amounts of IL-10, which assists in the maintenance of regulatory T cells (Tregs) (141). This anti-inflammatory population of macrophages is crucial to withstand acute insults in the gut that may otherwise initiate a lethal

inflammatory response (144;145). The resolution of inflammation is in part dependent on resident macrophages producing IL-10 to inhibit pro-inflammatory responses of other innate (including newly infiltrating macrophages) and adaptive immune cells via an IL-10-STAT3-SOCS3 axis (146). The IL-10 pathway is critical for gut homeostasis as IL-10-deficient mice or a deficiency in downstream signaling proteins (STAT3 and SOCS3) results in spontaneous gut inflammation (146).

During gut inflammation, monocytes traffic to the gut and differentiate into macrophages with a pro-inflammatory gene signature (IL-1, IL-6, TNF-α). Homing to the gut is dependent on MCP-1, MIP-1α, and MIP-2 secretion by the gut, primarily from epithelial cells (141;147). These three cytokines are upregulated in colitis-associated CRC models (148). Recently, MCP-1 was also shown to enhance LPS-induced IL-10 production from macrophages (149). MCP-1 appears to coordinate two vital events involved in intestinal macrophage generation: i) directing monocytes to gut and ii) imprinting newly differentiated macrophages into IL-10-producing cells. However, there is also evidence that indicates that MCP-1 can prevent a normal anti-inflammatory phenotype in macrophages (150). The circumstances that dictate MCP-1's influence on phenotype are not fully understood. However, MIP-1α, MIP-2, and MCP-1 recruit macrophages, which participate in cytokine production, arteriogenesis, and inflammation (151-153).

Hypothesis

Inflammatory cytokines, in particular IL-1, IL-6, and TNF-α, participate in initiating and advancing CRC (24-88). These cytokines increase the production of each other and all contribute to proliferation, survival, and invasive pathways. Targeting any one of them without the others is likely to be inadequate to fully address inflammation-expedited CRC. Regulating MK2 activity offers the opportunity to modulate inflammation, which may relieve inflammation-related proliferation and survival of cancer cells. MK2 signaling results in increased production and secretion of these three cytokines within the p38 pathway, providing an opportunity to investigate MK2 as a potential therapeutic target. The hypothesis for this project is that **MK2 promotes production of the MK2-induced cytokines IL-1, IL-6, and TNF-α in colorectal cancer, promoting CRC growth** (Figure 2).

Specific Aims

Aim 1.

To determine the extent that MK2 drives neoplasm development and cytokine production in a colitis-associated model of colorectal cancer. For this aim, we hypothesize that $MK2^{-/-}$ mice will have decreased IL-1, IL-6, and TNF-α and reduced neoplasm development. We further hypothesize that one important source of the MK2-induced cytokines is macrophage populations.

Aim 2.

To examine MK2's role in CRC growth as mediated by MK2-induced cytokines, and to evaluate the use of MK2 inhibitors as therapeutic intervention in CRC models. We hypothesize that interruption of MK2 activity in a tumor will result in decreased IL-1β, IL-6, and TNF-α, and a lower tumor burden.

Rationale

MK2 signaling drives production of IL-1, IL-6, and TNF-α, cytokines known to participate in CRC development and growth. One source of MK2-induced cytokines is tumor cells, but also other cells in the microenvironment, including infiltrating macrophages. Blockade of MK2 signaling, either during CRC initiation or growth, could reduce cytokine levels, reducing the speed of tumor growth and thus tumor burden at harvest. Restoration of MK2 signaling and MK2-induced cytokines, either through direct injection of cytokines or by restoring cytokine-producing cells to the area, would increase tumor burden in response.

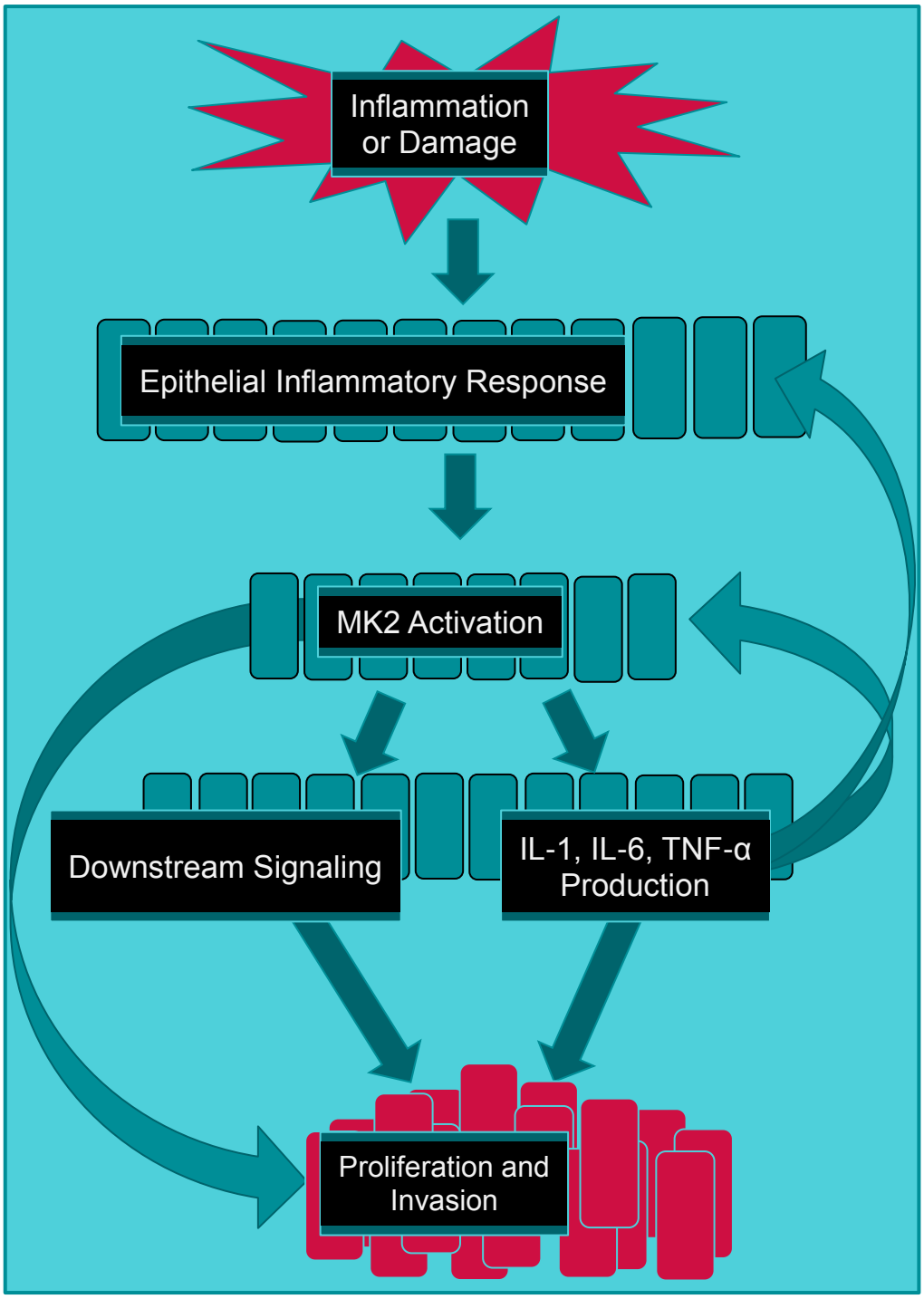

Figure 2. Proposed effects of MK2 signaling during CRC.

Chapter 2: Methods and Materials

Mice

C57Bl/6 mice from Harlan Laboratories and the MK2$^{-/-tm1Mgl}$ mouse strain (154) were bred under pathogen free conditions. Balb/c mice used were from Harlan. All mice used were 6-10 weeks old. Animal procedures were approved by the University of New Mexico IACUC.

AOM/DSS Model of CRC

To model colitis-associated cancer, the AOM/DSS model begins first by initiating key mutations in the gut. The model is initiated with a single injection of AOM, a DNA alkylating agent. Repeated injections of AOM result in development of non-inflammation-associated adenocarcinomas (with number varying between mouse strains), but a single injection without further treatment is not sufficient to cause neoplasm development (155-157). AOM induces mutations in human cells that are characteristically associated with CRC (158). β-catenin, K-ras, Cox-2, and prostaglandin receptors are all commonly mutated in both AOM-induced tumors and human CRC (158-161). AOM can also cause mutations in gut microflora, although the extent to which these changes persist or influence outcome in disease is unclear (162).

Once mutations are initiated in the gut, a model of colitis is implemented, using dextran sodium sulfate (DSS) to model pro-inflammatory flares. DSS is a sulfated polysaccharide. When dissolved in drinking water, it creates one of the most widely used murine models of colitis (163). Metabolism of DSS begins in the stomach, where it is depolymerized

(164). In the cecum, anaerobic microflora desulfate and begin degradation of DSS as it passes on to the colon (165). Although the mechanism of DSS-caused colitis is not fully understood, once it reaches the colon, DSS forms complexes with existing fatty acids in the gut. These complexes disrupt intestinal epithelial cells (166). Addition of antibiotics alleviates DSS colitis in both mice and rats (165;167). Data suggests that after initial epithelial cell disruption, the intestinal flora further metabolizes DSS. Infiltration of gram-positive bacteria through the disrupted epithelium contributes to colitis by initiating inflammatory responses and infiltration of monocyte and macrophage populations (168). The AOM/DSS model has been found to be highly clinically relevant to human CRC (169). AOM/DSS tumors show many of the markers associated with colitis-associated cancer, including p53 loss, β-catenin alterations, and inflammation that follows human CRC patterns, both grossly and through cytokine increase (169).

Timelines for the application of AOM and DSS vary between labs and strains of mice (170). We followed a commonly used protocol in terms of DSS concentration and timing after AOM administration, summarized in Figure 3 (171;172). A single dose of AOM (Sigma Aldrich, St. Louis, MO) was injected intraperitoneally (IP) into 6-8 week old female mice at 12.5mg/kg. For this project, all AOM/DSS mice were C57Bl/6 background (either $MK2^{+/+}$ or $MK2^{-/-}$). DSS (MP Biomedicals, MW 36,000-50,000) was added to drinking water at 2.5% on days 5 and 26 and at 2.0% at day 47. DSS treatments were

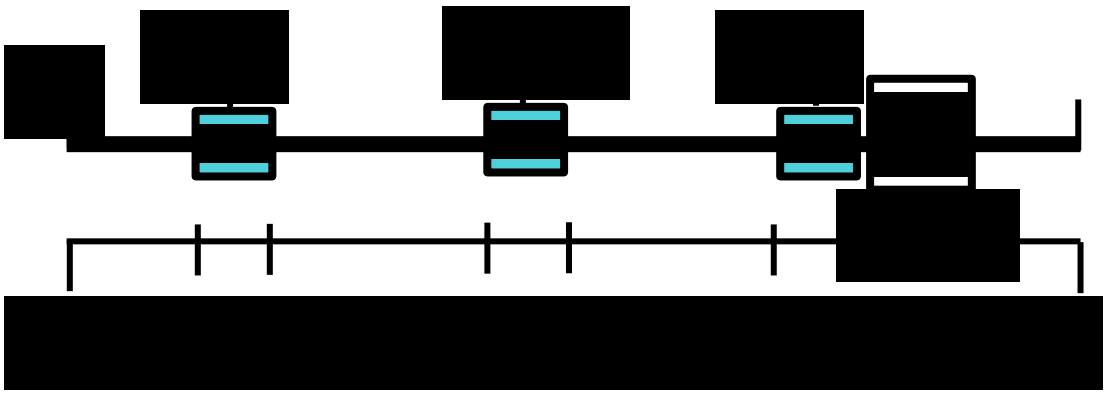

Figure 3. AOM/DSS model. This model of colitis-associated CRC begins with an intraperitoneal injection of mutagen azoxymethane (AOM), which causes critical mutations in the colon epithelium. Dextran sodium sulfate (DSS) is added to the drinking water for 5 days, beginning day 5, 26, and 47. DSS treatment causes damage to the colon lining, modeling colitis. Neoplasms develop by harvest at day 80. For mice treated with MK2 inhibitor, treatment began after the third DSS treatment and was administered three times/week for three weeks.

continued for five consecutive days with each treatment. On day 80, mice were sacrificed. Control mice received PBS IP and no DSS in water.

MK2 Inhibitors

Two specific MK2 inhibitors were used for *in vitro* and *in vivo* work. For mice that were treated with MK2 inhibitors under the AOM/DSS protocol, treatment began at day 52. Mice were injected IP three times per week, at 52 hour intervals, with 2 mg/kg of one of two specific MK2 inhibitors, either PF-364402 (Calbiochem, San Diego, CA) or MK2 inhibitor III (Calbiochem). Vehicle control mice received an injection with the same volume of 5% DMSO in calcium/magnesium-free DPBS. See Figure 3. For CT26 cell pre-treatment with MK2 inhibitors, cells were incubated for 48 hours before injection with 50 µM of MK2 inhibitor PF-364402 (Calbiochem) or a like amount of sterile DMSO. For *in vitro* cultures using MK2 inhibitors, inhibitors were used at a concentration of 50 µM unless otherwise stated. Control wells received an equal amount of DMSO.

Tissue Dissociation

To prepare tissues for flow cytometry, mouse colon tissues were washed once in Ca/Mg-free HBSS, then immersed in Ca++/Mg++ HBSS with 25U/ml of collagenases I, II, and IV (Sigma Aldrich) and dispersed using the gentleMACs tissue dissociator per manufacturer's protocol (Miltenyi Biotech, Cologne, Germany). Tissues were placed on a rotator for a 20 minute incubation at 37° C, 5% CO2. After this incubation, tissues were once again dissociated, and 90U/ml DNAse I was added, and the tissue incubation step

repeated. Cells were passed through a 40 µm filter. Single cells were washed twice in HBSS, counted, and collected for further use.

Macrophage Culture and Treatment

Bone marrow was collected from 6-8 week old female mice per standard protocol (173). In brief, femurs and tibia were harvested and cleaned of all muscle tissue. Bones were dipped in ethanol before being flushed with 3 mL of complete RPMI to remove the marrow. The marrow cells were stored in freeze media at -80°C until ready for use.

Macrophages were extracted and cultured as previously described (174). For macrophage-treated mice in Chapter 3, $1x10^6$ macrophages were injected IP on the first day of each DSS treatment (days 5, 26, 47) and on day 68. For macrophage-treated mice in Chapter 4, $1x10^6$ macrophages were injected intratumorally (IT) on day 1 and day 8. The macrophages injected on day 1 were first stained with 1 uM of CSFE (Thermo Fisher), and the day 8 macrophages were stained with e670 proliferation dye (eBioscience) per manufacturer's protocol.

CT26 Culture and Tumors

CT26 cell were purchased from ATCC (Manassas, VA). Cells were cultured in complete RPMI, which contains 5% FBS, 1% penicillin/streptomycin (Gibco, Grand Island, NY), and 1% L-glutamine (Gibco). CT26 cells were pretreated for 48 hours before injection with either 50uM of MK2 inhibitor PF-364402 or DMSO vehicle control. Cells were injected at 10^5 cells in 50µl of PBS into the flank of 6-8 week old Balb/c mice. Some mice injected with cells exposed to MK2 inhibitors were treated with recombinant IL-1β, IL-6, and

TNFα to replenish cytokine production in amounts of 25 ng IL-1β (Biolegend, San Diego, CA), 25 ng TNFα (Biolegend), and 1 µg IL-6 (Shenandoah, Warwick, PA). Treatments continued 3 times/week from day 2 until day 13. At Day 13 or Day 19, tumors were excised. Length, width, depth, and weight were recorded. Volume was calculated as the product of length, width, and depth.

CT26 Tumor Cytokine Treatments

For mice treated with MK2-induced cytokines, after CT26 injection (day 0), each tumor was injected with PBS control or MK2-induced recombinant murine cytokines (25 ng IL-1β, 25 ng TNFα, and 1 ug IL-6). Treatments continued 3 times/week from day 2 until harvest at day 13. IL-1β groups received 25 ng of IL-1β per injection. Recombinant cytokines were obtained from Shenandoah (IL-6) or Biolegend (TNFα, IL-1β).

Mice treated with recombinant murine MIP-1α, MIP2, and MCP-1 followed the same schedule as the MK2-induced cytokine mice. Each tumor received 50 ng of MIP-1α, 700 ng of MIP-2, and 800 ng of MCP-1. These quantities were based on calculations made by looking at the cytokines produced by a control tumor on d19 over the course of 16 hours via Luminex bead-based array.

Transfection of CT26 cell line

CT26 cells were transfected with copGFP plasmid (sc-108083) from Santa Cruz Biotechnology (Dallas, TX) per manufacturer's directions. Success of transfection was tested by looking at GFP+ cells by flow cytometry via a Guava easyCyte 8HT flow

cytometer. Selection was maintained in cell culture by maintaining on 10 ug/ml puromycin (Santa Cruz).

Isolation of CT26 Tumor Cells

Tumor tissue was dissociated in HBSS with Ca++/Mg++, 25U/ml of collagenase I, II, and IV (Sigma) with a GentleMACS dissociator (Miltenyi Biotech, Cologne, Germany), then incubated at 37°C and 5% CO2 for 20 min while rotating. 90U/ml DNAse I (Sigma Aldrich) was added and tissues were incubated at 37°C and 5% CO2 for 20 min while rotating for another 20 min. Cells were filtered through a 40uM filter, washed, and resuspended in complete RPMI.

Invasion Assay

Tumor cells isolated from CT26 tumors were plated in 96-well plates coated overnight with 10 ug/ml of human fibronectin (R&D Systems, Minneapolis, MN). Excess fibronectin was removed, and 10^5 dissociated tumor cells were added to each well. After 30 minutes, the wells were washed once with sterile DPBS and complete RPMI was added.

Upon confluency, cells were incubated in serum-free RPMI for 24 hrs, then scratched with a 10ul pipette tip. 50 ul of matrigel (Corning, Corning, NY) was added to each scratch, and matrigel was allowed to set for 5 minutes. Wells were incubated were vehicle control, MK2 inhibitor (50 uM PF-364402), 10 ng/ml of IL-1β (Biolegend), TNFα (Biolegend), and/or IL-6 (Shenandoah). Images were taken at initial scratch and at 12

hrs. The mean scratch width (5 measurements per well) at 12 hrs was compared to the means scratch width at 0 hrs to calculate the percent closure of the scratch.

Cytokine Arrays

Tumor tissues were rinsed in sterile PBS and weighed. 8 mg (± 0.5 mg) of tissues were incubated in complete RPMI with antibiotics for 12 hours (for experiments in Chapter 3) or 16 hours (for experiments in Chapter 4). Supernatants were stored at -80, and then analyzed for cytokines (IL-1β, IL-6, TNF-α, IL-10, MCP-1, MIP-1α, and MIP-2), by multiplex bead array (Millipore, Billerica, MA) according to manufacturer's instructions. All samples were analyzed on a Luminex 200 machine (Bio-Rad, Hercules, CA).

Flow Cytometry

Tissues were dissociated per above. For experiments in Chapter 3, cell suspensions were incubated overnight in media before staining. Flow cytometry was carried out according to standard Biolegend protocols (Biolegend, San Diego, CA). Macrophages were stained with anti-F4/80-PE or FITC (Biolegend, BM8), anti-CD11b-APC or PE (eBioscience M1/70), anti-IL-1α-PE (eBioscience, ALF-161), anti-IL-1β-APC (eBioscience NJTEN3), anti-IL6 (eBioscience MP5-20F3), anti-TNF-α-PEcy5 (MP6-XT22 eBioscience), anti-IL-10-APC (eBioscience JES5-16E3), anti-Arg1-FITC (R&D Systems IC5868F) or isotype controls. All samples were run on a Guava easyCyte 8HT flow cytometer (Millipore). Cells were gated on the forward and side scatter plot to remove debris. Macrophage plots were gated next on the $F4/80^{+}CD11b^{+}$ population and examined for cytokines. Macrophage numbers per colon were calculated by the percent of gated cells in relation to the

overall number of cells per mouse colon. Viability staining for cell lines was performed via FACS staining with e660 viability dye (eBioscience) per standard surface staining protocol (Biolegend, San Diego, CA).

Quantitative Real Time PCR

RNA was isolated using Ribozol (Amresco, Solon, OH) according to the manufacturer's instructions. RNA concentrations were measured using a Nanodrop (Thermo Scientific, Wilmington, DE, USA). The RT reaction mixture includes random 2.5 µM hexamers, 500 µM dNTPs, 0.4 U/µL of the RNase inhibitors, 5.5 mM $MgCl_2$, MultiScribe Reverse Transcriptase (3.125 U/µL) and its buffer, and 1 µg of cellular RNA. The RT step was performed according to the following protocol: 10 min at 25°C, 60 min at 37°C, 5 min at 95°C. Obtained cDNA samples were stored at -80°C and used for the PCR reaction step. The PCR reaction mix was prepared using the Assays-on-Demand™ gene expression assay mix (Applied Biosystems) for mouse β-actin, IL-1α, IL-1β, IL-6, and TNF-α (a 20X mix of unlabeled PCR primers and TaqMan® MGB probe, FAM dye-labeled) and 2 µL of cDNA were added to the PCR reaction mix. The reaction was carried out according to the following protocol: 2 min at 50°C, 10 min at 95°C (1 cycle), and 15 sec at 95°C and one min at 60°C (45 cycles) on Applied Biosystem's StepOnePlus instrument. The endpoint used in real-time PCR quantification, CT, was defined as the PCR cycle number that crossed the signal threshold. Quantification of cytokine gene expression was performed using the comparative CT method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to the mouse housekeeping gene, β-actin mRNA.

Proliferation

10^3 CT26 cells were seeded in a 96 well plate in complete RPMI. After 4 hours, wells were washed and incubated in serum-free RPMI containing 10 ng/ml of recombinant mouse IL-1β (Shenandoah), IL-6 (Biolegend), TNF-α (Shenandoah), or all three cytokines. Controls received an equal quantity of sterile PBS. For time course experiments, plates were imaged every 2 hours on an IncuCyte Zoom (Essen Bioscience, Ann Arbor, MI) and analyzed by IncuCyte Zoom 2016A software by standard manufacturer's program for analyzing confluence. For non-timecourse experiments, cells were plated as above, grown for 96 hours, and they were counted via hemocytometer. Samples were diluted in 50% Trypan blue (Sigma) and dead cells were excluded.

H&E Staining

Tumor samples were fixed in zinc formalin for 24 hours before being transferred to 50% ethanol, 50% DI water for longer storage. Samples were then paraffin embedded, sectioned, and stained by the Human Tissue Repository at UNM.

Western Blot

Cell line and tumor samples were lysed in RIPA buffer (Thermo Scientific, Rockford, IL). Protein was quantified using BCA protein assay compatible with reducing agents (Thermo Scientific). 20 µg protein for cell lines and 50 µg for tumors was loaded into each lane of a 10% bis-acrylamide gel. The gel was electrophoresed at 120v for 5 minutes, then voltage lowered to 60-80v. Proteins were transferred using an iBlot2 (Invitrogen, Carlsbad, CA) at 45 min at 100V. The membrane was blocked overnight with

5% dry non-fat milk in TBS with 0.1% Tween-20. pMK2 antibody (Santa Cruz, Santa Cruz, CA) was added at 1:1000 for 2 hours at room temperature. Secondary antibody donkey anti-mouse (Santa Cruz) was added at 1:10000 for one hour in blocking buffer. Samples were visualized using SuperSignal West Femto substrate.

Statistics

All results are expressed as the mean $\pm$ standard error of the mean. Differences between means were evaluated by student's t-test or one-way ANOVA in GraphPad Prism 5. *p* values of 0.05 or less were considered statistically significant. Power analysis was performed to determine the sample size of the experimental and control groups to ensure that any effect, if one is in fact present, is statistically detectable.

Chapter 3: Blockade of MK2 is protective in inflammation-associated colorectal cancer development

This chapter is modified from:

Blockade of MK2 is protective in inflammation-associated colorectal cancer development.

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Abstract

Chronic inflammation is a risk factor for colorectal cancer. The MAPK-activated protein kinase 2 (MK2) pathway controls multiple cellular processes including p38-dependent inflammation. This is the first study to investigate the role of MK2 in development of colitis-associated colon cancer (CAC) using the AOM/DSS model. Herein, we demonstrate that $MK2^{-/-}$ mice are highly resistant to neoplasm development when exposed to AOM/DSS, while wild type C57BL/6 mice develop multiple neoplasms with the same treatment. MK2-specific cytokines IL-1, IL-6 and TNF-α were substantially decreased in AOM/DSS treated $MK2^{-/-}$ mouse colon tissues compared to wild type mice which coincided with a marked decrease in macrophage influx. Restoring MK2-competent macrophages by injecting wild type (WT) bone marrow derived macrophages into $MK2^{-/-}$ mice led to partial restoration of inflammatory cytokine production with AOM/DSS treatment; however, was not sufficient to induce neoplasm development. Our results indicate that MK2 functions as an inflammatory regulator to promote colonic neoplasm development and may be a potential target for CAC.

Introduction

Colorectal cancer will kill approximately 50,000 people every year in the US.(175) Patients with chronic colonic inflammation develop colorectal cancer (CRC) at a rate 2-6 times above normal and CAC is more difficult to treat than other CRCs (176). Although the mechanisms of inflammation promoting cancer development are not fully understood, tissue damage and pro-survival mechanisms likely contribute to the growth of colon cancer. The protein kinase $p38^{MAPK}$ pathway has known pathogenic

contributions to inflammatory bowel disease (175(177). However, p38 ^{MAPK} inhibition has resulted in poor outcomes; side effects of treatment can be severe and affect multiple organ systems, such as infection, dangerous increases in liver enzymes, and skin disorders, in humans (102;111;178;179). In animal models utilizing p38 inhibitors, gastrointestinal toxicity, destruction of lymphoid tissues, and promotion of cardiac plaques have been observed (180;181).

MK2 is downstream of p38 ^{MAPK} and stabilizes the mRNAs of IL-1, IL-6, and TNF-α. Due to its stringent regulation of rather few genes compared to p38, it may be a better target. In LPS induction of inflammation in MK2$^{-/-}$ mice, IL-1, IL-6, and TNF-α were greatly reduced (154), indicating a major role of this pathway in production of these cytokines. Additionally, these cytokines have been implicated in CAC development (182;183). This is likely due to recruitment of immune cells to the site of inflammation and promotion of cell survival and proliferation.(184) MK2 is constitutively expressed at steady-state in multiple cell-types, both hematopoietic and non-hematopoietic origin. Recent evidence shows that intestinal macrophages maintain intestinal homeostasis, but are also major contributors to chronic inflammatory conditions in the colon.(185) Given this plasticity in function, it is crucial to understand the role of macrophages in CAC development. This functional plasticity may depend on MK2 activation and subsequent conditioning of the microenvironment to an inflammatory state. Pro-survival and pro-growth signals driven by inflammatory cytokine production from macrophages may be at the apex of inflammation and cancer development.

In this study, we set out to uncover the role of MK2 in CRC development as well as understand MK2 signaling in macrophages. Herein, we demonstrate $MK2^{-/-}$ mice were resistant to neoplasm development in the AOM/DSS CAC model. The complete loss of MK2 led to stunted production of cytokines and reduced macrophage infiltration into the colon. Transfer of MK2-proficient macrophages into $MK2^{-/-}$ mice ignited an inflammatory response, but was insufficient to restore tumorigenesis. Taken together, these results highlight the importance of MK2 in the inflammatory response and CAC development and highlight the importance of MK2 in multiple cell types is required to establish tumor development.

Results and Discussion

MK2 is essential for CAC development:

Activation of MK2 leads to IL-1, IL-6, and TNF-α production. These cytokines are known to induce multiple cell survival and invasion pathways.(182-184) However, a specific role in cancer development has only been examined for MK2 in one study of skin cancer (186). Thus, we set out to investigate pro-tumorigenic effects of MK2 in CAC. WT and $MK2^{-/-}$ mice were subjected to AOM and chronic treatments of DSS through three treatments and examined at day 80 as the commonly examined endpoint for this model (157). Remarkably, $MK2^{-/-}$ mice did not develop neoplasms (Figure 4A), while wild type mice had 100% incidence. Also, upon H&E staining, AOM/DSS treated WT mice developed well defined neoplasms with dysplastic proliferation of the colonic epithelium (Figure 4B) and with DSS treatment had mild architectural disarray consistent with chronic injury (Figure 4C). In contrast, $MK2^{-/-}$ mice treated with

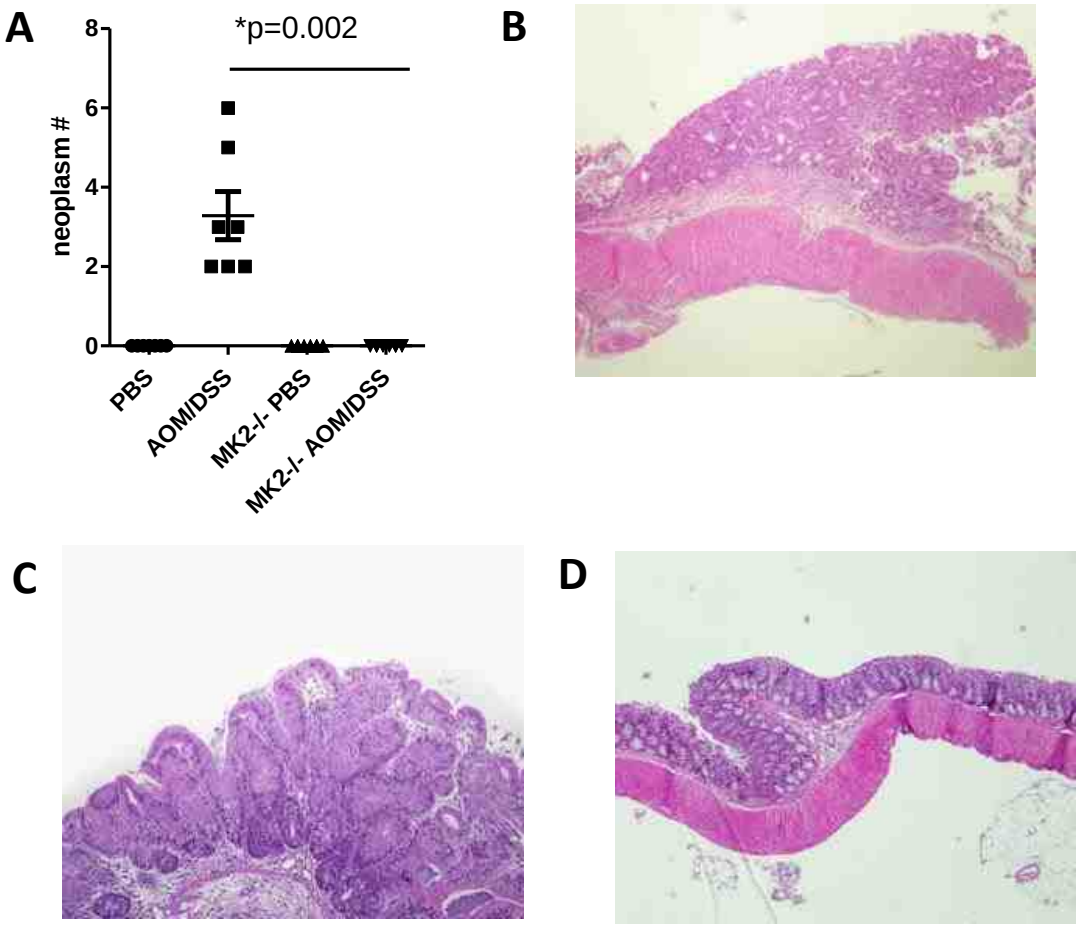

Figure 4. MK2$^{-/-}$ mice exposed to AOM/DSS do not develop neoplasms and have substantially decreased dysplasia production compared with WT mice. AOM/DSS treated mice develop (a) multiple neoplasms, while MK2$^{-/-}$ mice do not. H&E staining indicates that (b) AOM/DSS treated WT mice developed defined neoplasms with dysplastic proliferation of the colonic epithelium compared with (c) architectural disarray of mucosal tissue consistent with chronic injury from multiple DSS treatments, while (d) AOM/DSS treated MK2$^{-/-}$ mice displayed no visible signs of dysplasia or mucosal damage. N = 7 for WT mice and 8 for MK2$^{-/-}$ mice in duplicate experiments.

AOM/DSS displayed no signs of dysplasia (Figure 4D). The complete absence of neoplasms in MK2-deficient mice after AOM and DSS-induced colitis indicate that MK2 is an important player in neoplasm development in CAC although the possibility exists that development could be delayed.

Cytokine response is substantially reduced in MK2$^{-/-}$ mice exposed to AOM/DSS compared to wild type mice:

IL-1, IL-6 and TNF-α are major factors in the establishment of the IBD promoting chronic inflammation, and are also known as tumor promoting cytokines in CRC (187;188). Interestingly, MK2$^{-/-}$ mice display decreased IL-1, IL-6 and TNF-α production in multiple models (189-191). Since these cytokines may be the major inflammatory mediators driving inflammation and neoplasm development, we hypothesized the lack of neoplasms in MK2$^{-/-}$ mice could be the result of a dampened inflammatory response. Conditioned media collected from AOM/DSS treated wild type mouse colon organ cultures in a previously described tissue explant approach used by multiple groups (192;193) displayed a marked increase in IL-1α, IL-1β, IL-6 and TNF-α compared to control mice receiving one PBS injection and regular water (Figure 5A-D). To further support the induction of MK2-induced cytokines in mouse colons, we found a similar pattern of increase in IL-1α, IL-1β, IL-6 and TNF-α gene expression in WT AOM/DSS treated mice, but minimal induction in MK2$^{-/-}$ mice (Figure 5E). These high levels (compared to PBS groups) of cytokines indicate a chronic inflammatory response in the colon due to multiple DSS treatments. Conversely, supernatant from colon tissues of MK2$^{-/-}$ mice treated with AOM/DSS displayed a significant reduction in

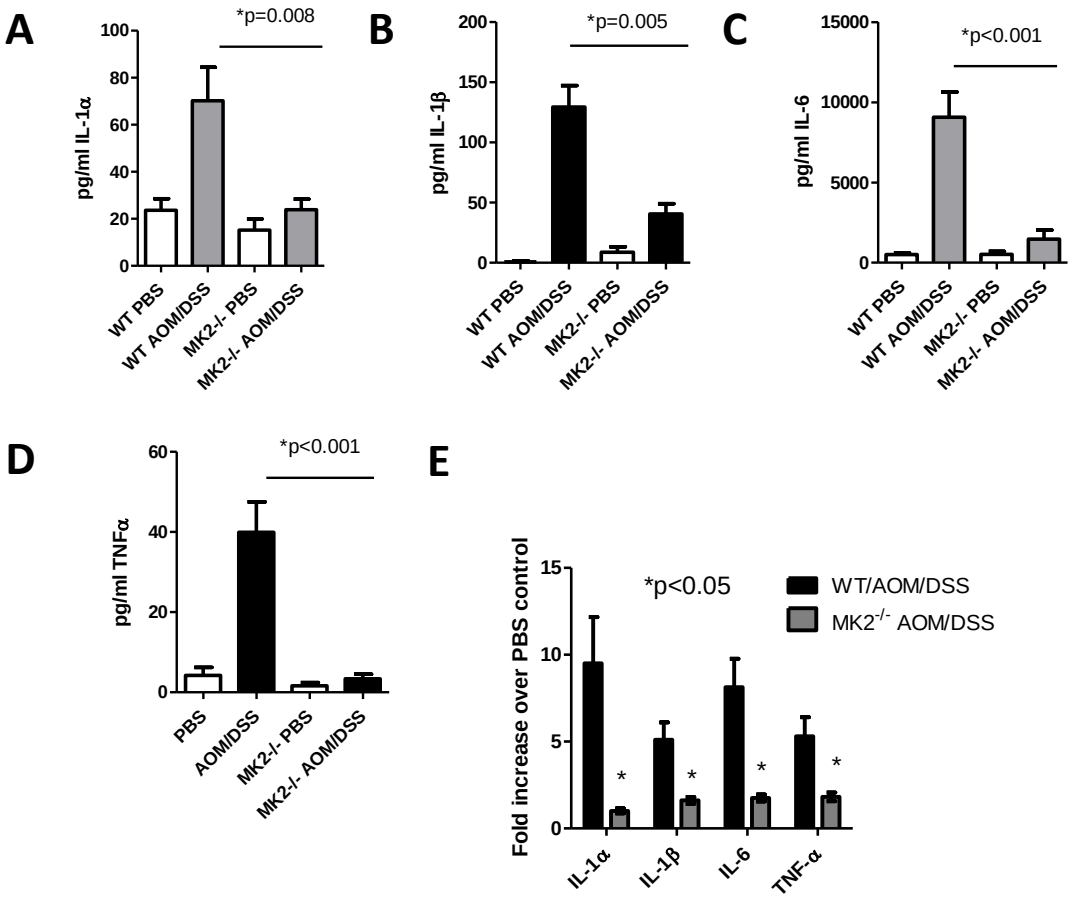

Figure 5. MK2$^{-/-}$ mice exposed to AOM/DSS have substantially decreased cytokine production compared with WT mice. AOM/DSS treated MK2$^{-/-}$mice have significantly decreased (a) IL-1α, (b) IL-1β, (c) IL-6 and (d) TNF-α in organ culture supernatants compared to WT mice by multiplex bead array. N = 7 for WT mice and 8 for MK2$^{-/-}$ mice in duplicate experiments. (e) WT mice treated with AOM/DSS have an increase in colonic gene expression of MK2 downstream cytokines compared to control mice, but MK2$^{-/-}$ mice have markedly less induction of cytokine gene expression. N = 6.

these cytokines compared to WT AOM/DSS treated mice. These findings emphasize the importance of MK2 in regulating the production of inflammatory mediators that promote colon neoplasm development.

MK2 deficiency reduces colonic macrophage accumulation and cytokine production in AOM/DSS treated mice:

The MK2 downstream cytokines IL-1, IL-6 and TNF-α are produced by multiple cell types, including macrophages, in the AOM/DSS model (174). To examine the impact of macrophages in MK2-dependent inflammation and neoplasm development, we first examined factors related to macrophage accumulation and activation in mouse colon supernatants. GM-CSF and MCP-1 were substantially decreased in $MK2^{-/-}$ mice compared to WT mice administered AOM/DSS (Figure 6A and B). The expression of these factors is important for macrophage development and accumulation.(194;195) Due to the role of GM-CSF, MCP-1, IL-1, IL-6 and TNF-α in macrophage accumulation and activation, we assessed the number of colonic macrophages by staining single cell colon suspensions for F4/80 and CD11b. The number of $F4/80^{+}$ $CD11b^{+}$ cells found in the colon of AOM/DSS treated WT mice at day 80 was drastically increased in AOM/DSS treated WT mice compared to control groups, but markedly decreased in $MK2^{-/-}$ mice (Figure 6C). To further understand the role of MK2 in macrophages localized in the colon of mice that have developed neoplasms, intracellular cytokines were examined after isolation and ex vivo stimulation. A substantial number of macrophages isolated from colon preps of AOM/DSS treated WT mice expressed IL-1α, IL-1β, IL-6 and TNF-α

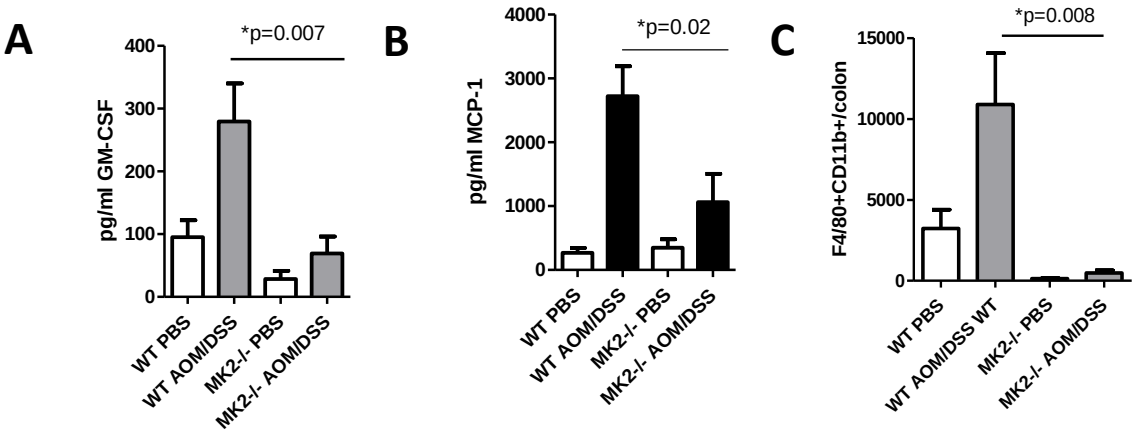

Figure 6. Macrophages are decreased in MK2$^{-/-}$ mice. Cytokines related to macrophage influx and function, (a) GM-CSF and (b) MCP-1 are substantially increased in AOM/DSS treated mice, but significantly decreased in MK2$^{-/-}$ mice. WT mice treated with AOM/DSS have increased (c) F4/80^{+}CD11b^{+} macrophage influx, which is substantially decreased in MK2$^{-/-}$ mice treated with AOM/DSS. N=6

compared to macrophages isolated from AOM/DSS treated MK2$^{-/-}$ mice (Figure 7A -D). The considerable decrease in colonic macrophages during CAC development in MK2$^{-/-}$ mice indicate the proper cells are not present in the colon to mount an inflammatory response to contribute to neoplasm development. We also found an increase in IL-10-expressing macrophages in WT AOM/DSS treated mice (Figure 7E) and arginase-1 expressing macrophages (Figure 7F), both of which were significantly decreased in MK2$^{-/-}$ mice. These data suggest that MK2$^{-/-}$ mice not only show a decrease in macrophages expressing MK2 downstream mediators, but are also decreased in macrophages producing M2-like pro-tumorigenic factors. In addition to macrophages, there are also other myeloid-derived cells that are attracted to the mouse colon during inflammation. Myeloid-derived suppressor cells (MDSC) have been found to contribute to colitis and colitis-associated tumor development and growth. Suppressing trafficking to the colon, or knocking out MDSC-associated activity reduces inflammation and tumor burden in the AOM/DSS model (196;197). Thus, we also stained for MDSC (CD11b^{+}Gr1^{+} cells). These cells were increased in WT mice treated with AOM/DSS compared to PBS and present at higher levels in WT than MK2$^{-/-}$ mice, but the difference did not achieve significance (Figure 8).

Macrophages enhance pro-inflammatory cytokine production, but not neoplasm development in AOM/DSS treated mice:

Macrophages harbor both anti- and pro-tumorigenic features that can hinder or enhance tumor formation. Our data above clearly indicate that macrophages

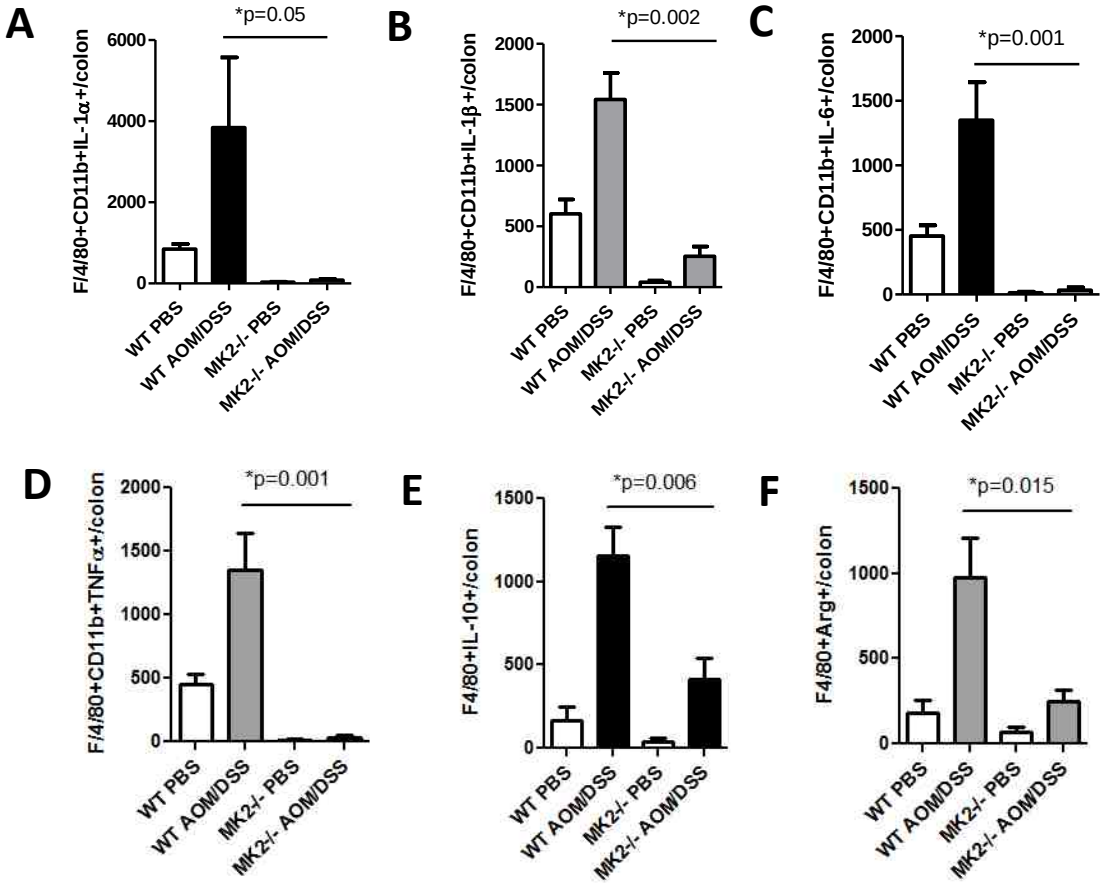

Figure 7. Macrophages are a major source of MK2 downstream cytokines. $F4/80^{+}CD11b^{+}$ cells in $MK2^{-/-}$ mice displayed significantly less intracellular staining of (a) IL-1α, (b) IL-1β, (c) IL-6, (d) TNF-α, (e) IL-10, and (f) ARG1 compared with WT mice. N=7 for WT mice and 8 for $MK2^{-/-}$ in duplicate experiments.

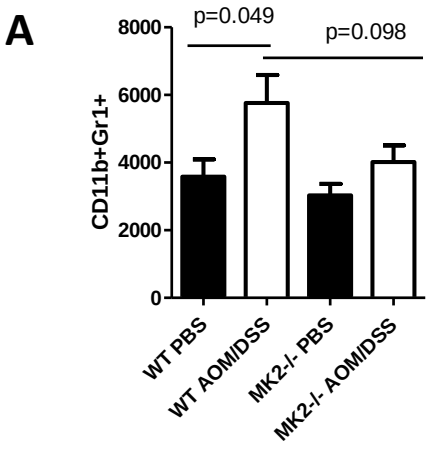

Figure 8. WT mice treated with AOM/DSS have a significant increase in colon CD11b+Gr1+ myeloid cells compared to PBS treated mice. These cells are decreased in MK2$^{-/-}$ mice compared to WT, but the decrease does not reach significance. N=6.

accumulate and are producing MK2 downstream pro-inflammatory cytokines in tumor burden colons (Figure 5). To assess the contribution of macrophage to CAC, bone marrow-derived macrophages (BMM) from WT mice were adoptively transferred into WT and $MK2^{-/-}$ AOM/DSS treated mice at days 5, 26, 47, and 68. Interestingly, AOM/DSS treated $MK2^{-/-}$ mice receiving WT BMM displayed no neoplasms, while an increased number of neoplasms were found in WT mice injected with WT BMM (Figure 9A). WT mice receiving WT BMM had a mean of 6 neoplasms per mouse, while the WT mice had a mean of 3 neoplasms per mouse, which is a significant increase in number ($p=0.006$). These data indicate that macrophages promote neoplasm development in this system.

Furthermore, addition of WT macrophages restored a proportion of the MK2 downstream cytokine production in $MK2^{-/-}$ mouse colons and also enhanced the levels in WT mice receiving cells (Figure 9B-E). We further found an increase in the macrophage related cytokines GM-CSF and MCP-1 (Figure 9F and G). When comparing AOM/DSS treated $MK2^{-/-}$ mice supplemented with WT macrophages to AOM/DSS treated WT mice in Figures 5, 6, 7, and 9, IL-6, GM-CSF, and MCP-1 levels were drastically increased to similar levels, indicating the role of MK2 in promoting these responses. These data suggest that the protocol of BMM transfer into $MK2^{-/-}$ mice was adequate to mount a similar level of these cytokines as in wild type mice. IL-1α, IL-1β, and TNF-α were also increased upon WT BMM transfer into $MK2^{-/-}$ mice, but not to the same levels as WT mice suggesting that MK2 signaling in other cells may be responsible for production of these cytokines in WT mice. Introduction of WT macrophages

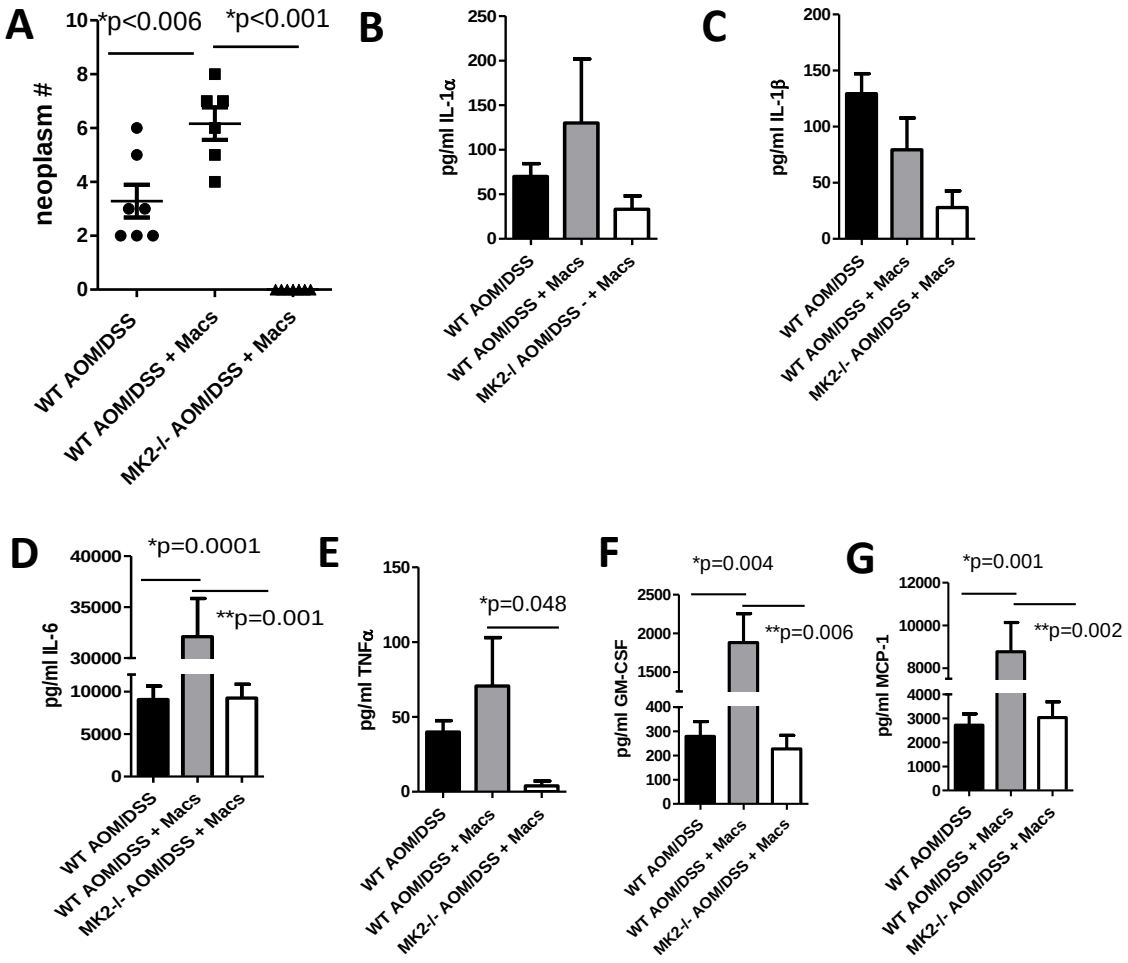

Figure 9. WT BMM injection in MK2$^{-/-}$ mice restores some cytokine production, but not neoplasm development. BMM injections into WT and MK2$^{-/-}$ mice led to (a) increased neoplasm development for WT mice, but not MK2$^{-/-}$ with AOM/DSS treatments. In organ culture, (b) IL-1α, (c) IL-1β, (d) IL-6, (e) TNF-α, (f) GM-CSF and (g) MCP-1 production were found at higher levels in WT mice, but were also increased in MK2$^{-/-}$ mice supplemented with WT macrophages. N=6 for BMM supplementation experiments in duplicate experiments.

into MK2$^{-/-}$ mice was not sufficient to restore neoplasm development indicating that MK2 is critical in other cells, perhaps epithelial cells, for neoplasm development. Furthermore, the significantly increased neoplasm development upon addition of extra macrophages into WT mice highlights the importance of MK2 signaling in macrophages in promoting tumor growth. Nevertheless, in our model, we have eliminated macrophages as being the primary determinant driving CAC development.

Our data highlight the importance of MK2 in CAC development and provide a target to hinder the inflammatory response. We demonstrate that restored MK2 signaling in a single cell type (macrophages) can restore the inflammatory response in MK2$^{-/-}$ mice and hence, this further substantiates the robustness of MK2 signaling pathway as a highly inflammatory event. Given the insurgence of reports demonstrating macrophages promote inflammation and cancer, we set out to the test hypothesis that MK2 in macrophages drives CAC development. The transfer of these cells was sufficient to restore the inflammatory response as determined by cytokine output (Figure 9B-G). Nonetheless, restoration of MK2 in these cells (as well as the pro-inflammatory cytokines detected) was not sufficient to re-establish tumor development in MK2$^{-/-}$ mice. This suggests a more complex role for MK2 in CAC development where restoration of the inflammatory cytokines is only one part of the equation. Upon supplementation of macrophages to WT mice, neoplasm number was significantly increased (Figure 9A). These data indicate that MK2-induced cytokines from macrophages promote increased tumor growth, but MK2 signaling is also needed in other cells, such as epithelial cells for

tumor development. Thus, these studies raise the possibility that MK2 is a potential therapeutic target for patients with colitis or CAC that could prove beneficial.

Acknowledgements:

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The authors have no financial conflicts to disclose.

Chapter 4: MK2 inhibition reduces tumor growth in a cytokine-dependent manner in two mouse models of colorectal cancer.

Portions of this chapter are in preparation for submission to International Journal of Cancer for publication.

Impact of Work

This is to evaluate MK2 inhibition effects on inflammation and tumor burden within two mouse models of colorectal cancer (CRC). MK2 is identified as an important signaling pathway during CRC progression in both spontaneous and colitis-associated models. These data demonstrate that MK2 is essential for inducing pro-tumorigenic cytokines. Inhibition of MK2 reduced tumor burden and cytokine production. Restoring MK2 downstream cytokines to the tumor restored tumor growth. Therapeutic intervention relieves inflammation, reduces tumor growth and invasion in a syngeneic invasive model of CRC, and causes tumor regression in a colitis-associated model of CRC.

Abstract

Colorectal cancer (CRC) development and progression is associated with chronic inflammation. MAPK-activated protein kinase 2 (MK2) drives inflammatory signaling in response to stress and inflammation signals. MK2 signaling promotes production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. These cytokines have been implicated in tumor growth and invasion. In Chapter 3, we found that MK2 is essential for colitis-

associated CRC development in a mouse model, and is a primary regulator of pro-inflammatory cytokines and inflammatory macrophage populations in the gut. We investigate whether MK2 inhibition can improve outcome in two mouse models of CRC. In the AOM/DSS model of colitis-associated CRC, MK2 inhibitor treatment eliminated neoplasms in the majority of the mice. In a model using syngeneic murine CRC cell line CT26, treatment with MK2 inhibitors dramatically reduced tumor volume. Tumor cells treated with MK2 inhibitors produced an average of 80% less IL-1β, IL-6, and TNF-α. They also showed slower invasion. Addition of MK2-induced cytokines to CT26 cells increased proliferation, and activated MK2, suggesting a positive feedback loop between MK2 and its associated inflammatory cytokines. Mice treated intratumorally with murine recombinant IL-1β, IL-6, and TNF-α developed tumor burdens equivalent to control mice. These results demonstrate the importance of MK2 in multiple models of CRC through a cytokine-driven mechanism and suggest MK2 has potential as a target for therapeutic intervention.

Acknowledgements

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the US, with over 135,000 new diagnoses and 50,000 deaths from CRC expected (1). However, as CRC progresses, the prognosis becomes worse; tumors that have spread to distant sites have a 5-year survival rate of only 13.5% (2). The number of CRC cases in the United States is expected to rise in the next decade, due to an increase of people aged 55-84 years old, which comprises 67% of CRC diagnoses (2). New treatments and approaches to therapy are needed, particularly to address advanced CRC that is diagnosed at later stages or has proven resistant to surgical and chemical interventions.

Chronic inflammation is a major risk factor for cancer development and progression (198). For example, inflammatory bowel diseases are correlated with a sharply increased risk of CRC (199;200), which increase the longer chronic inflammation continues (201). Patients with inflammatory bowel disease who develop colorectal cancer also have a poorer prognosis than patients without chronic inflammation (202). Beyond inflammatory bowel disease, inflammation is generally present in CRC (18). As tumors advance, inflammatory cytokines are often present in high levels. In advanced CRC, measuring inflammatory cytokines in plasma can be used to predict outcome. Higher cytokine levels are associated with worse prognosis (203). Because of the risks that accompany inflammation, inflammatory pathways present potential therapeutic targets.

Among the potential therapeutic targets that have been investigated for CRC inhibition, the p38 pathway has been of interest as a source of inflammatory signaling. p38 MAPK is activated by inflammatory signaling or cell stress (204). Once phosphorylated, p38 initiates multiple pathways, including inflammatory pathways, metabolic pathways, and cytoskeletal changes (205-207). Inhibitors of p38 have entered clinical trials for treatment of multiple inflammatory diseases, including rheumatoid arthritis, Crohn's disease, and psoriasis (208). However, inhibition of p38 has resulted in off-target effects in multiple animal species (103). One potential therapeutic avenue is to target effectors downstream in the p38 pathway to try to prevent inflammation while potentially lessening off-target effects.

In this study, MK2, a kinase directly downstream of p38, is investigated as a potential therapeutic target. MK2 is phosphorylated by p38 and the p38-MK2 complex is transported to the nucleus, where MK2 induces the mRNA stabilization of three characteristic inflammatory cytokines associated with CRC: IL-1β, IL-6, and TNF-α (24;209;210). Additionally, MK2 promotes TNF-α production by activating the mRNA stabilizer HuR (211;212). Unlike p38, which activates a number of other pathways, MK2 inhibition targets far fewer pathways while decreasing production of tumor-promoting inflammation. The MK2 downstream cytokines are particularly significant in inflammation and are associated with multiple aspects of cancer. These cytokines have been shown to increase as both colitis-associated and spontaneous CRC tumors progress (213;214). By controlling MK2 activity, it may be possible to affect three of the most common cytokines associated with CRC, and slow or reduce inflammation. IL-1β,

IL-6, and TNF-α are strongly associated with inflammation-driven cancers, including CRC. They are also known to contribute to tumor growth and invasion in multiple types of cancers, particularly CRC (24;209;210).

IL-1β is an inflammasome-dependent cytokine, which is produced mostly by pro-inflammatory immune cells. IL-1β initiates a cascade of pro-inflammatory activity that results in amplification of the inflammatory response. In a mouse model of colitis, the severity of disease was greatly alleviated by blocking inflammasome activity, and thus IL-1β release (215). In CRC development and progression, IL-1β increases invasion of the tumor (216). Blocking IL-1β signaling in a colitis-associated model of CRC resulted in a reduction in tumor burden, indicating that it may be a good therapeutic target (217). Because IL-1β is produced by infiltrating cells, reducing widespread inflammation within the tumor microenvironment may also reduce IL-1β levels (218).

IL-6 has been shown to be an important regulator in CRC. In human samples, elevated IL-6 levels is associated with increased risk of CRC development (60). IL-6 production is stimulated in response to antigen recognition by immune cells, recognition of microbial patterns, or inflammatory cytokine signaling (219). IL-1β signaling in many cell types, including epithelial cells, fibroblasts, and infiltrating immune cells, results in high quantities of IL-6 (220-222). IL-6 levels are correlated with poor survival, increased invasion, increased distant metastasis, and more advanced human tumors (223). Some pro-tumorigenic mechanisms of IL-6 include activation of STAT3, a transcription factor for genes associated with cancer cell proliferation and invasion (224;225). IL-6 also contributes to the progression of CRC through hypoxia-inducible pathways, which

promote survival of tumor cells (226). In mouse models of CRC, ablation of IL-6 reduces tumor burden and increases survival time suggesting it is a critical tumor-promoting factor (227).

The other documented cytokine regulated directly by MK2 activity is TNF-α. This cytokine is produced by a wide variety of cells in response to pro-inflammatory signaling, including in response to IL-1 (228). Increased TNF-α predisposes mice to development of CRC (229). Some of the suggested pro-tumorigenic activities of TNF-α are promoting migration and invasion in CRC (230) and pro-tumorigenic activity in infiltrating immune cells (231). The combination of TNF-α and IL-6 produces a synergistic response in CRC that promotes proliferation, survival, and further cytokine production through the activation of STAT3 (232). Preventing TNF-α also signaling reduces colitis-associated CRC in a mouse model (233). Because of its role in inflammation and tumor promotion, TNF-α control is an important area of interest in CRC therapies.

Since MK2 signaling is responsible for production of these three critical cytokines, it may be a novel tumor target. Blocking MK2-induced cytokines, either with antibodies to block cytokine signaling activity or by using knockout models, has resulted in reduced tumor size and invasion (224;234;235). We have also shown that majority of IL-1β, IL-6, and TNF-α produced during a model of colitis-associated CRC is MK2-dependent (236). Here, the potential for MK2 inhibition as a treatment during tumor development is explored in two models of CRC. We found that that inhibition of MK2 can drastically reduce growth in an IL-1β, IL-6, and TNF-α dependent mechanism. These data suggest that MK2 may be an attractive target for therapeutic intervention.

Results

MK2 inhibition reduces CRC tumor growth

We previously showed that MK2 signaling is required for CRC development in the AOM/DSS model, which is a model for colitis-associated CRC (Chapter 3). To investigate the importance of this pathway in tumors progression, the syngeneic CT26 model was examined. The CT26 model is a model of spontaneous CRC. CT26 cells are fast growing and invasive, similar to advanced CRC tumor cells in humans. By using a cell line, we had the opportunity to selectively target tumor cells before injection into mice, allowing a better understanding of the cell-specific behavior of MK2.

CT26 cells in culture were found to have a constitutive level of phosphorylated MK2 (Figure 10A). To prevent initial MK2 signaling during tumor growth, CT26 cells were treated with 50 µM of specific MK2 inhibitor PF-364402 for 48 hours before injection into the mice. When injected into mice, at day 13 the mean tumor volume for vehicle control treated cells was 113 mm^3, while the mean tumor volume from cells treated with MK2 inhibitor was 49 mm^3 (Figure 10B). A similar effect was seen a week later at day 19 where control tumors had a mean tumor volume of 2260 mm^3 and those treated with MK2 inhibitor had a mean of 957 mm^3 (Figure 10C). The observed decrease in tumor burden suggest a lasting effect of MK2 inhibition where a 60% decrease in tumor size; measurements at d13 and d19 had a similar ratio of tumor size, suggesting that the MK2 inhibition effect is long-lasting (Figure 11A). A picture comparing the relative size

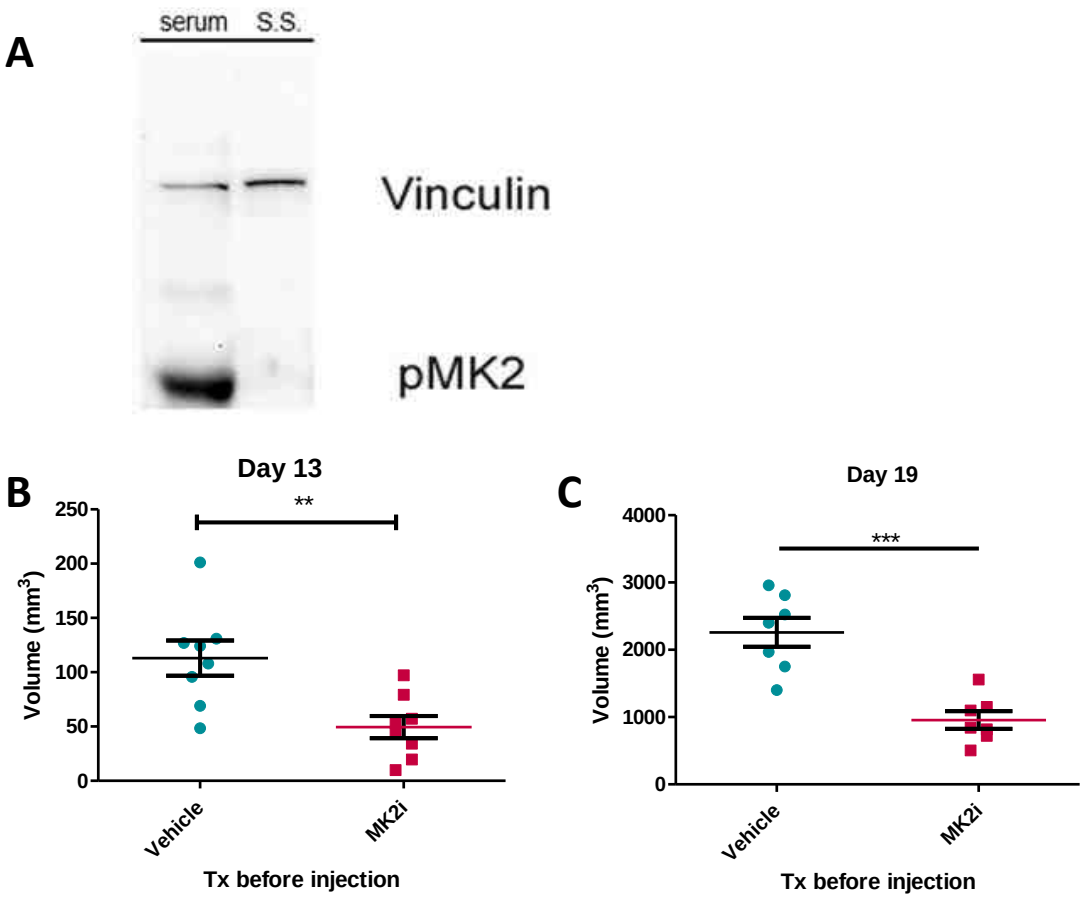

Figure 10. MK2 inhibition reduces CT26 tumor size. A) Western blot of CT26 cells shows that MK2 is active in CT26 tumors and signaling stops when serum-starved (S.S.). CT26 cells that were MK2-inhibited make smaller tumors at B) day 13 and C) day 19. n=8 for d13 and n=7 for d19. ** $p < 0.01$, *** $p < 0.001$

of tumors grown from MK2-inhibited CT26 to those grown from vehicle-treated CT26 is visible in Figure 11B and C. MK2-treated cells made smaller tumors without any decrease in viability in culture (Figure 11D).

To further investigate the potential for MK2 inhibition as a therapeutic approach, a second model of CRC was examined. The AOM/DSS model is a clinically relevant model of colitis-associated cancer, where neoplasms develop in mice after administration of a mutagen and then recurrent colitis bouts. This model is initiated with an IP injection of the DNA alkylating agent (AOM), followed by three treatments of DSS added to drinking water. Previously, our lab found that neoplasms were grossly visible by day 52 (237), thus MK2 inhibitor treatments were administered starting at day 52, three times a week for 3 weeks (Figure 12A). Two different, specific MK2 inhibitors were tested. At day 80, the majority of mice treated with MK2 inhibitor were completely neoplasm-free (Figure 12B). Control mice had an average of 2.6 neoplasms, while mice treated with MK2 inhibitors had a mean of less than 1 neoplasm per mouse. The observed decrease in tumor growth in the CT26 tumor model and regression of tumors in the AOM/DSS model suggests that MK2 activity is critical for tumor growth in multiple models of CRC.

MK2 inhibition reduces pro-inflammatory cytokines associated with CRC

Systemic MK2 deletion has been shown to reduce production of pro-inflammatory cytokines in pancreatitis and LPS-induced inflammation (191;238) To examine the

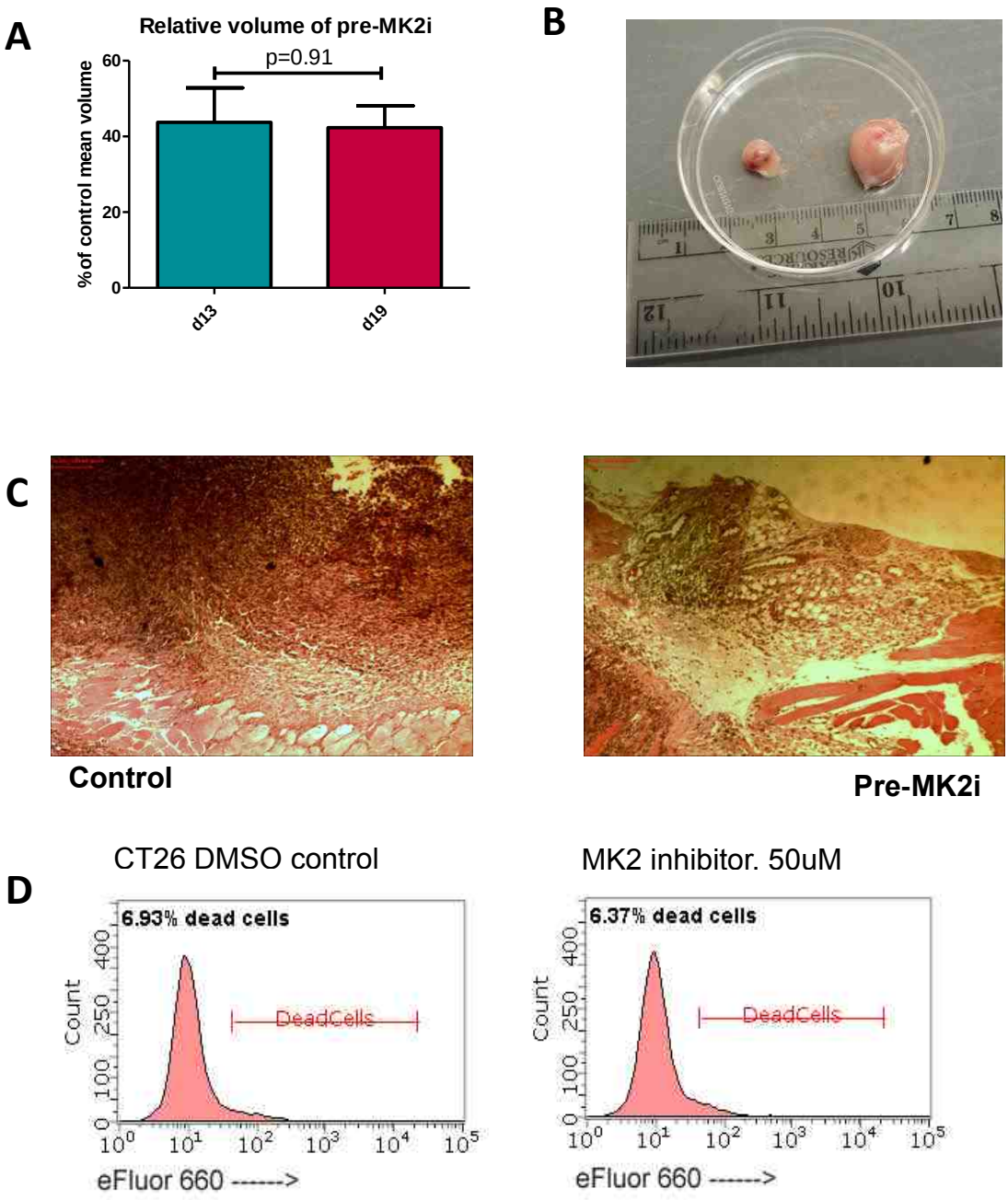

Figure 11. MK2 inhibition reduces CT26 tumor size. A) MK2-inhibited tumor volume as a percent of the mean of control tumor volume stays the same between day 13 and day 19 harvests. B) Representative picture of inhibited and control tumors at day 19. C) H&E staining shows increased size on control (left) compared to tumors from MK2 inhibited cells (right), but D) MK2 inhibition doesn't decrease viability of CT26 cells in culture. N=7 for d19 and 8 for d13.

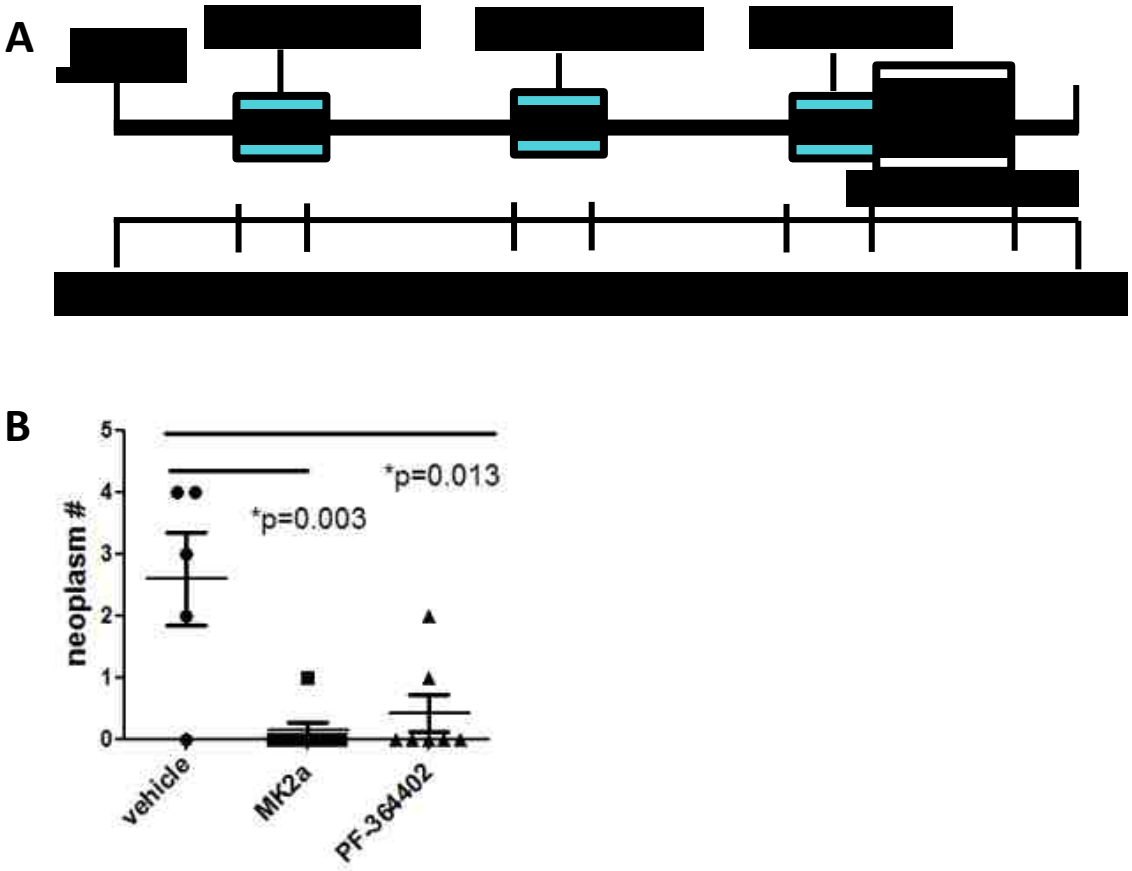

Figure 12. MK2 inhibitors decrease neoplasm number in AOM/DSS model. In A), a colitis-associated model of CRC, treatment with either of two MK2 inhibitors, MK2a or PF-364402, for 3 weeks results in B) fewer neoplasms in inhibited groups. N=5 for vehicle control, N=7 for MK2 inhibitor groups.

impact of the MK2 pathway in cytokine production in CRC, organ culture supernatants were examined from two models of CRC. These were produced by whole tumor pieces, incubated to collect the secreted cytokines. Cytokines were measured via multiplex bead-based array. In the CT26 model, MK2 inhibition led to dramatically reduced levels of IL-1β, IL-6, and TNF-α when compared with control tumors (Figure 13A-C). These cytokines have many potential cellular sources, including tumor cells, infiltrating immune cells, and stromal cells. To distinguish the tumor cell-specific differences between the inhibited and control tumor cells, tumor cells were isolated and cultured. Tumors were dissociated and cultured in fibronectin plates. CT26 cells bind to fibronectin, so tumor cells adhered to the plates; nonadherent cells were removed. To test the purity of the fibronectin-adherent population, we transfected CT26 cells with GFP. Tumors from these cells were dissociated and cultured; measuring these cells by flow indicated these cells were 98% GFP+ (Appendix A). In these cultures, CT26 cells cultured from tumors that had been treated with MK2 inhibitors before injection into the mice produced extremely reduced levels of IL-6 and did not produce detectable levels of TNF-α (Figures 14A and B). Neither the untreated control nor the inhibited tumor cells produced detectable levels of IL-1β, suggesting that other cells in the tumor microenvironment are the source of IL-1β in this model. The initial MK2 inhibitor treatment may have caused a decrease in inflammatory cytokine production

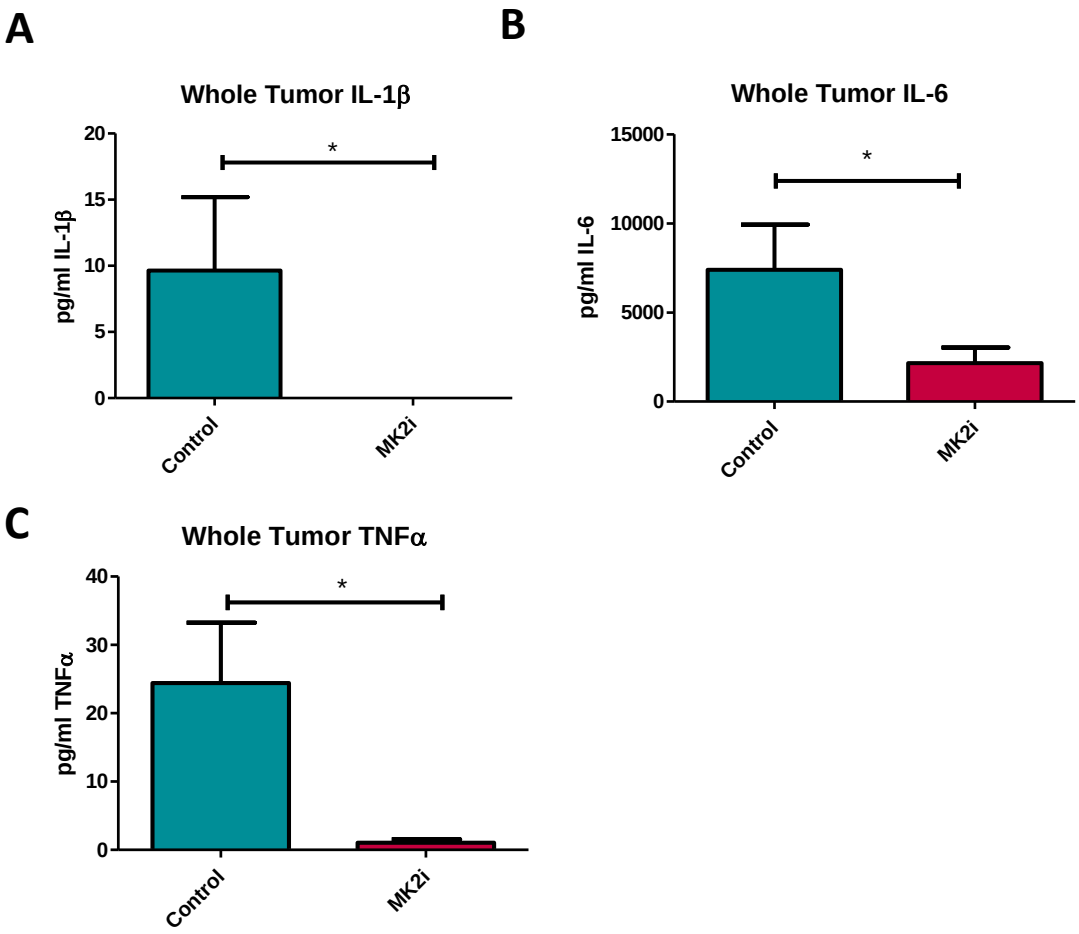

Figure 13. MK2 inhibitor treatment reduces cytokine production of CT26 tumors. Organ cultures from CT26 tumors grown from MK2-inhibited cells shows tested via Luminex multiplex array shows reduced A) IL-6, B) TNF-α, C) IL-1β. N=7 MK2i and 8 for controls.

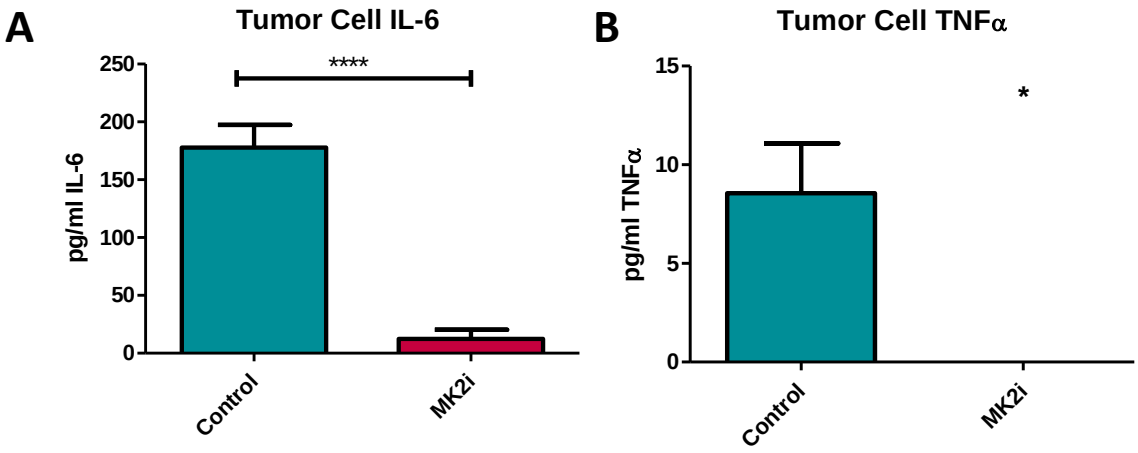

Figure 14. MK2 inhibitor treatment reduces cytokine production of cultured tumor cells. Tumor cells isolated from tumors grown from MK2-inhibited CT26 produce no detectable A) IL-6 and B) TNF-α. N=6. **** = $p \leq 0.0001$. *Undetectable.

that reduced the overall inflammation in the tumor microenvironment. The long-lasting anti-inflammatory phenotype supports the central role of MK2 in CRC-related inflammation.

Cytokines were also measured in the AOM/DSS mouse colon tissues. In this model, therapeutic administration of MK2 inhibitors resulted in colons that showed no visible signs of inflammation, redness or thickening as seen in control mice. Similar to the CT26 model, AOM/DSS treated mice administered MK2 inhibitors showed drastically reduced IL-1β, IL-6, and TNFα (Figure 15A-D). IL-6 was also reduced compared to the normal tissue from the controls (Figure 15C). The ability of CT26 tumors to establish inflammation via MK2-mediated signaling is an important component of their rapid growth. Since MK2-induced cytokines are known to promote growth, invasion, and metastasis, MK2 could be a critical component in pathogenesis of CRC.

Cytokines produced downstream of MK2 induce proliferation and invasion of tumor cells

Since MK2 inhibition led to both decreased tumor burden and reduced downstream cytokine production, the role of MK2 inhibitors and these cytokines on tumor cell proliferation was examined. To first assess the potential direct effect of MK2 inhibition on CT26 cell proliferation, cells were treated for 48 hours with MK2 inhibitor. Cells were grown for 96 hours and then counted. MK2 inhibition was found to have no ability to directly reduce proliferation (Figure 16A). Conversely, when cells were treated with 10 μg/ml of IL-1β, IL-6, or TNF-α, significant increases in cell numbers were observed at 96 hours (Figure 16B). Furthermore, combining all three cytokines led to an increased

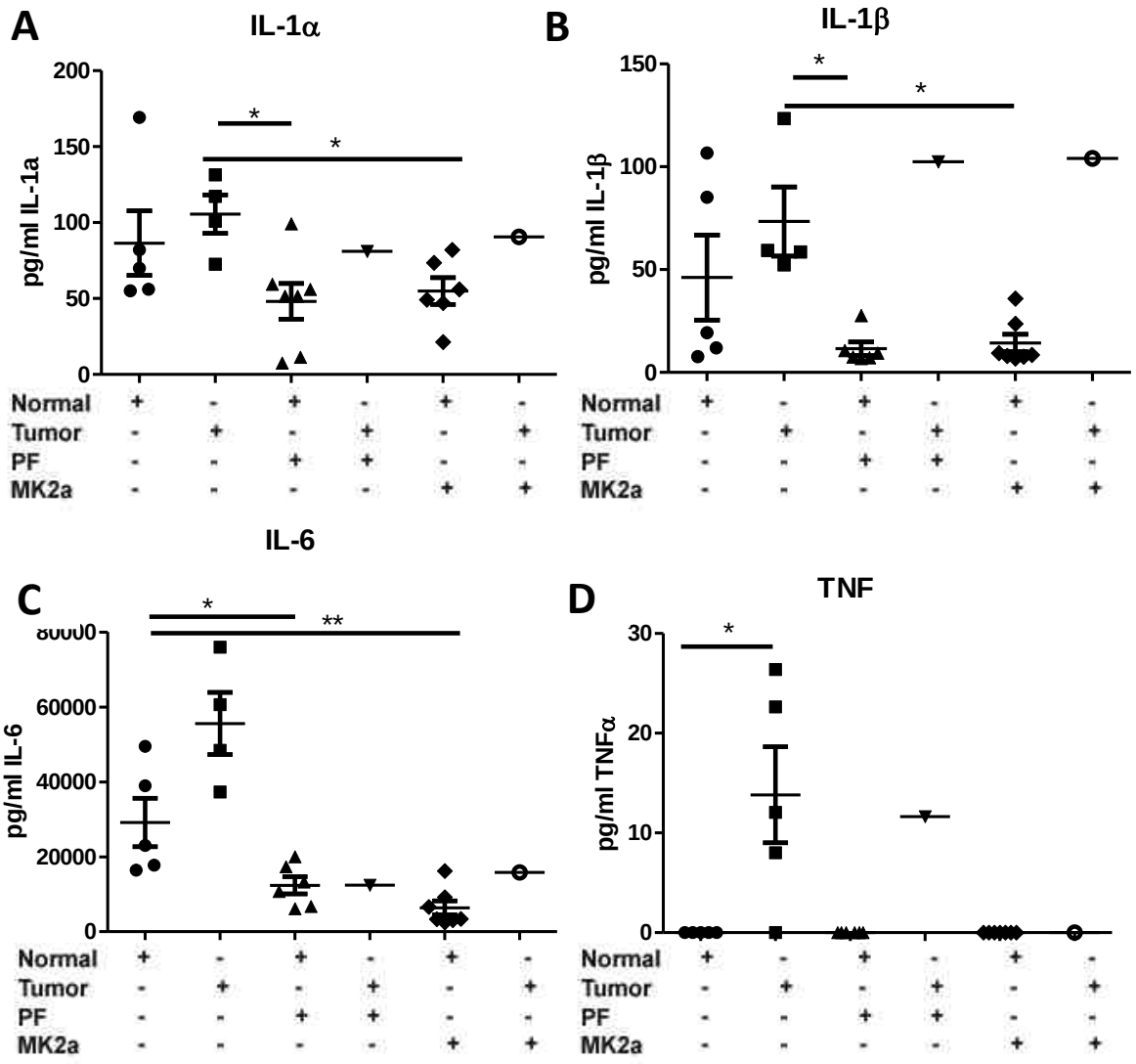

Figure 15. MK2 inhibitor treatment reduces cytokine production in AOM/DSS model. Organ cultures from normal and tumor tissues of AOM/DSS show increases in MK2-induced cytokines in tumor tissue, including A) IL-1α, B) IL-1β, C) IL-6, and D) TNF-α. N=5 for control, 7 for MK2-inhibited normal, and 1 MK2-inhibited tumor.

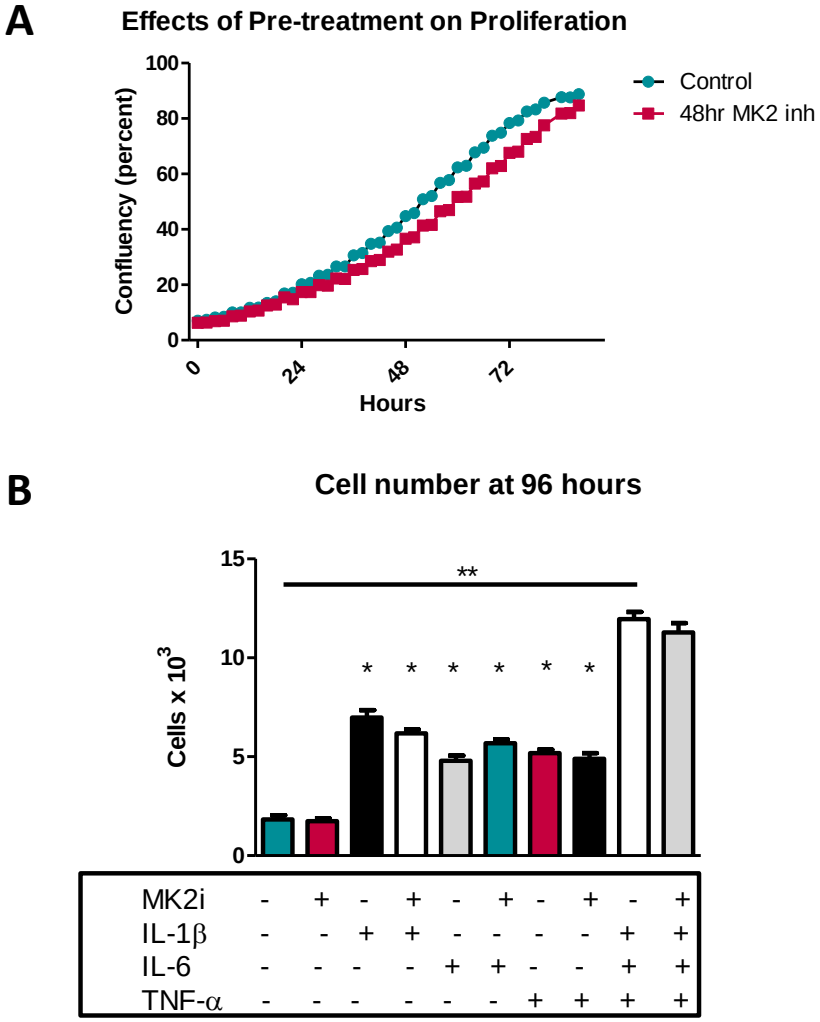

Figure 16. MK2-induced cytokines increase proliferation in CT26 cells. CT26 cells A) inhibited for 48 hours with MK2 inhibitor show no decrease in proliferation. Addition of B) IL-1β, IL-6, TNF-α increases proliferation, and a cocktail of those cytokines increases proliferation further over serum-starved controls. N = 9 in triplicate experiments. *= $p \leq 0.05$, **=$p \leq 0.01$.

effect on control cells, and had a similar effect on cells treated with MK2 inhibitor. These data support the role of each of the MK2-induced cytokines in promoting tumor cell proliferation.

In addition to proliferation, MK2-induced cytokines can drive invasion of tumor cells through the lamina propria (42;67;90). Thus, the role of these cytokines in CT26 invasion and migration was analyzed in scratch-wound assays where matrigel was added to the scratched wound. As seen in example images in Figure 17A, CT26 cells invaded the matrigel filled scratch by migrating into protrusions, which bridged the scratch within 72 hours. In contrast, wells treated with MK2 inhibitor developed very few protrusions and demonstrated vastly decreased invasion (Figure 17B and C). To assess the impact of MK2-induced cytokines on invasion, a mixture of IL-1β, IL-6, and TNF-α significantly increased invasion (Figure 17D). Furthermore, addition of these cytokines to cells treated with MK2 inhibitor rescued the effects of the inhibitor on invasion. The ability of cytokines induced by MK2 to restore proliferation and invasion of cells treated with MK2 inhibitor suggests the mechanism by which MK2 signaling promotes colorectal tumors.

Addition of MK2-induced cytokines to tumors increases tumor size and cytokine production

Given the effects of MK2 downstream cytokines on tumor cell proliferation and invasion, the effects of these cytokines on tumors were examined *in vivo*. Control CT26 cells or cells treated with MK2 inhibitors were injected into mice. On d1, a cocktail of recombinant IL-1β, IL-6, and TNF-α (in amounts similar to those produced by tumors in

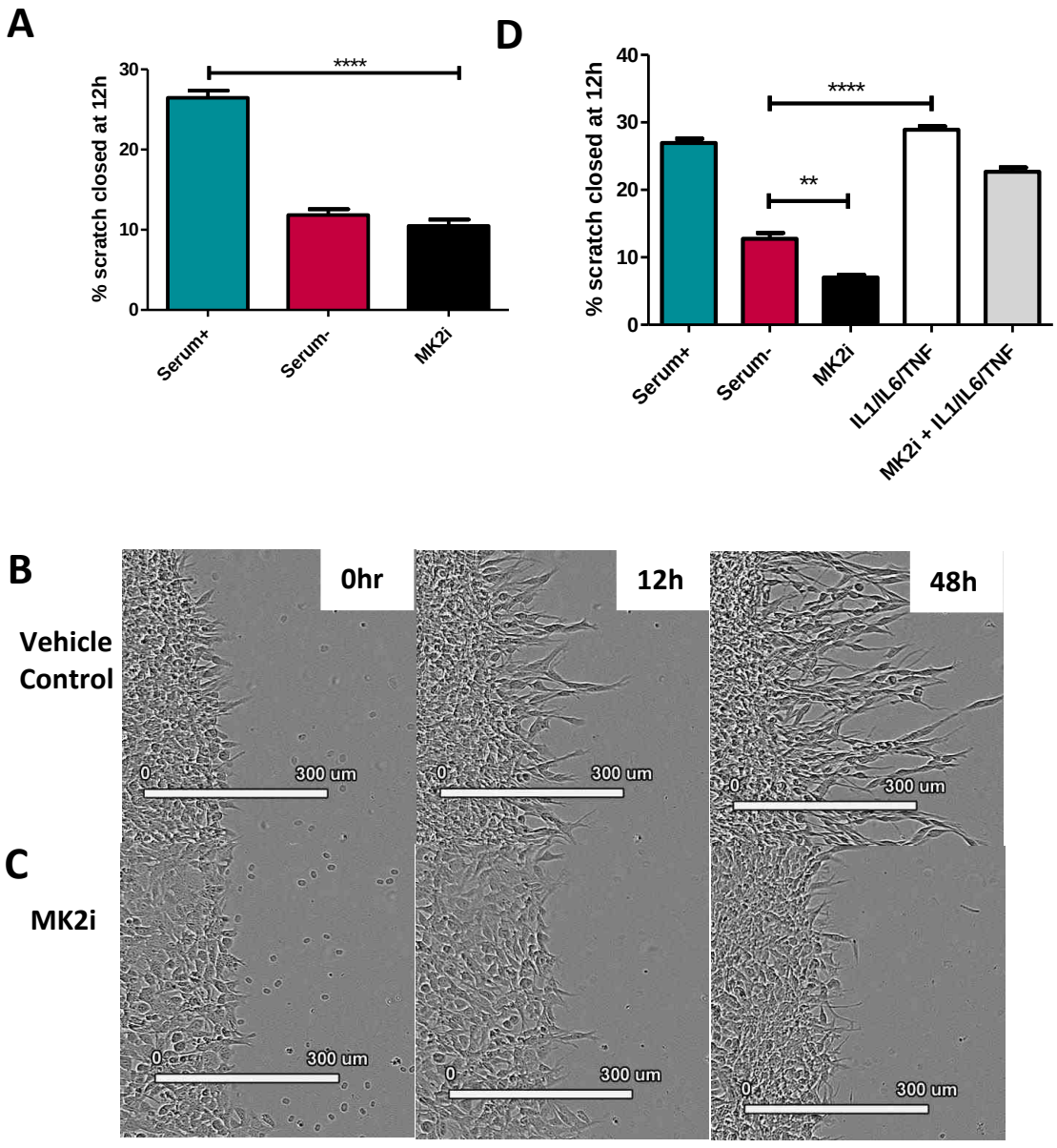

Figure 17. MK2 and MK2-induced cytokines increase invasion. In a matrigel scratch-wound assay, A) MK2 inhibition decreased scratch closure of tumor cells isolated from CT26 tumors. Control cells B) grew protrusions that decreased with C) MK2 inhibitor treatment. D) MK2 inhibition decreased and MK2-cytokines increased scratch closure of serum-starved tumor cells. N=9, in triplicate.

Figure 13) was administered intratumorally to CT26 tumors. Cytokine treatment began at day 2 and continued three times/week. Cytokine treatment increased the size of tumors from MK2-inhibited CT26 (Figures 18A-C). Tumors from uninhibited CT26 treated with cytokines also increased in size compared to PBS controls. These data suggest that the MK2 inhibition reduced cytokine levels, leading to smaller tumors, and that MK2-induced cytokines were responsible for the increase in tumor when injected intratumorally.

To further investigate the mechanism by which IL-1, IL-6, and TNF-α promote chronic inflammation and tumor growth, the effects of these cytokines on MK2 phosphorylation were examined. CT26 cells were serum starved to decrease signaling activity. Cells were treated with cytokines and MK2 phosphorylation was assayed. Addition of IL-1β, IL-6, or TNF-α activated pMK2, indicating that MK2 activity can be positively regulated by cytokines that MK2 induces (Figure 19A). This supports the idea that a positive feedback loop exists between MK2 and the cytokines produced in response to MK2 signaling.

MK2-inhibited tumors with no cytokine treatment produce much less of the MK2-induced cytokines than tumors grown from cells treated with MK2i. Cytokine-treated tumors from MK2i-treated cells secreted increased MK2-cytokines in organ culture (Figure 20A-C). Tumors from control CT26 that received additional IL-1β, IL-6, and TNF-α treatment showed increased IL-6. There was a trend towards increased TNF-α (mean of 21.4 pg/ml vs. 14.6 pg/ml) and IL-1β (mean of 14.1 pg/ml vs. 10.2 pg/ml), but the increase did not reach statistical

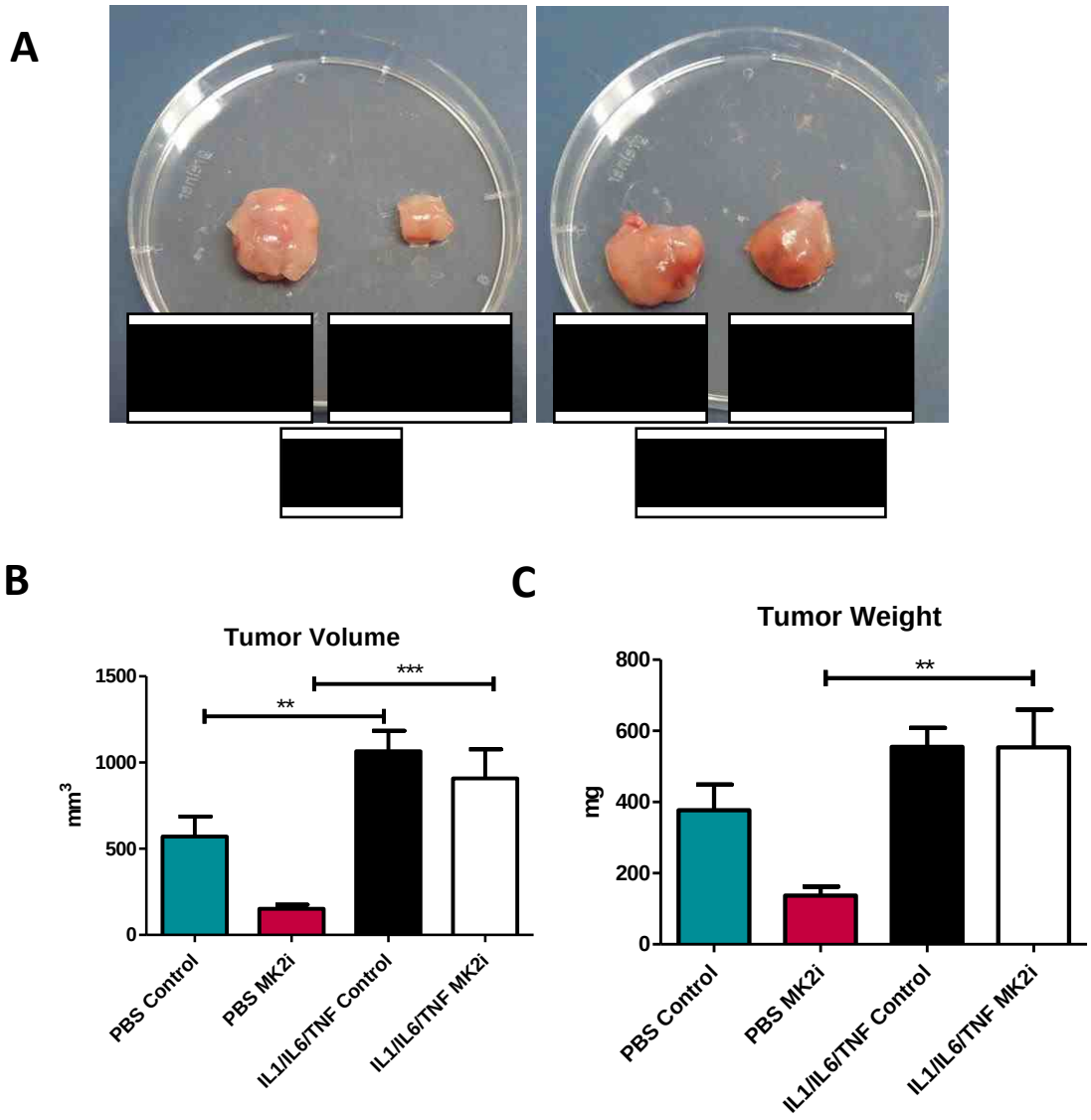

Figure 18. MK2-induced cytokines rescue tumor burden in MK2-inhibited tumors. Intratumoral injection of MK2-induced cytokines increased tumor size of tumors grown from MK2-inhibited CT26, as seen in A) a representative picture and B) volume and C) weight comparisons. Control tumors given MK2-induced cytokines were significantly larger. N=9 for PBS and 10 for cytokine-tx groups. ** = $p \leq 0.01$, *** = $p \leq 0.001$.

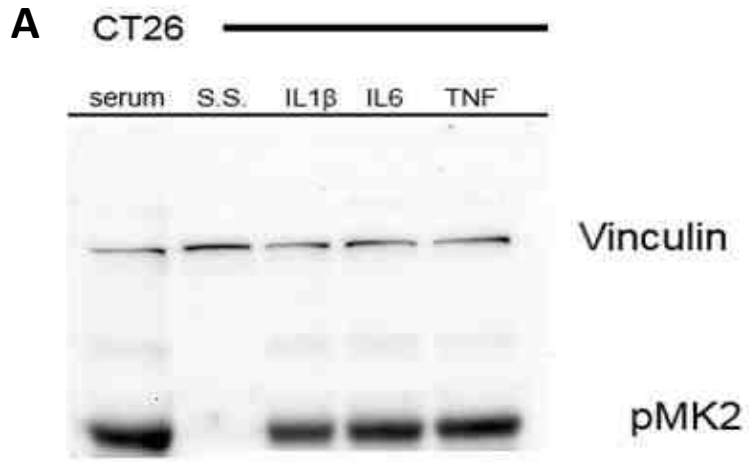

Figure 19. MK2-induced cytokines activate MK2. A) Addition of IL-1β, IL-6, or TNF-α for 30m restores pMK2 levels in serum-starved (S.S.) CT26 culture. Vinculin is used here as a loading control.

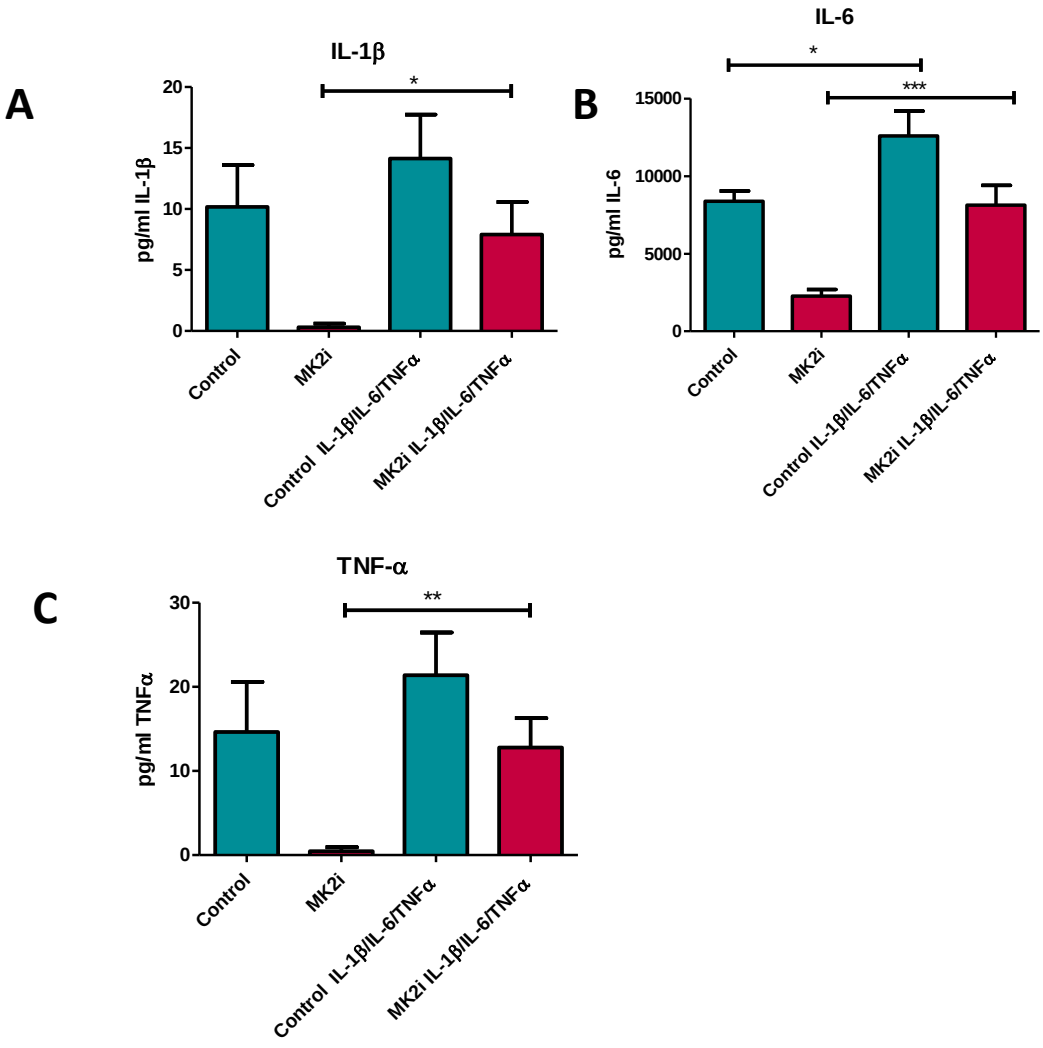

Figure 20. Organ cultures from cytokine-treated tumors have increased MK2-induced cytokines. Treatment with MK2-induced cytokines restores production of A) IL-1β, B) IL-6, and C) TNF-α in organ cultures of tumors from MK2-inhibited CT26 cells. N=9-10. * = $p \leq 0.05$, ** = $p \leq 0.01$.

significance.

Measurement of cytokine mRNA confirms that cytokine treatment increased mRNA levels of MK2-induced cytokines. IL-1β, IL-6, and TNF-α levels in cytokine-treated pre-MK2i tumors are elevated above the control tumors (Figure 21A-C). The increase in cytokine levels is supportive evidence that changes to cytokine production within the tumor occur as a result of cytokine treatment, indicating positive feedback. Combined with the ability of IL-1β, IL-6, or TNF-α to activate MK2 behavior, and evidence that MK2 is the primary regulator of IL-1, IL-6, and TNF-α, these data support a feedback loop that is interrupted by MK2 inhibition and restored by IL-1β, IL-6, and TNF-α treatment. The cytokine mRNA levels of tumors grown from MK2-inhibited CT26 is statistically lower than controls for IL-1β; however, it is not significantly different for IL-6 or TNF-α. The comparable mRNA levels suggest that MK2 regulates the activity of cytokines through additional mechanisms besides mRNA stability.

For tumors from MK2-inhibited CT26, then, the decrease of tumor burden and cytokine production was contingent on maintaining low levels of MK2-induced cytokines. Treatment with cytokines restored tumor burden and inflammation, resulting in a tumor phenotype that was similar in size and cytokine profile to tumors grown from noninhibited CT26. These data support the critical role of MK2-induced cytokines in CRC growth, mediated through MK2.

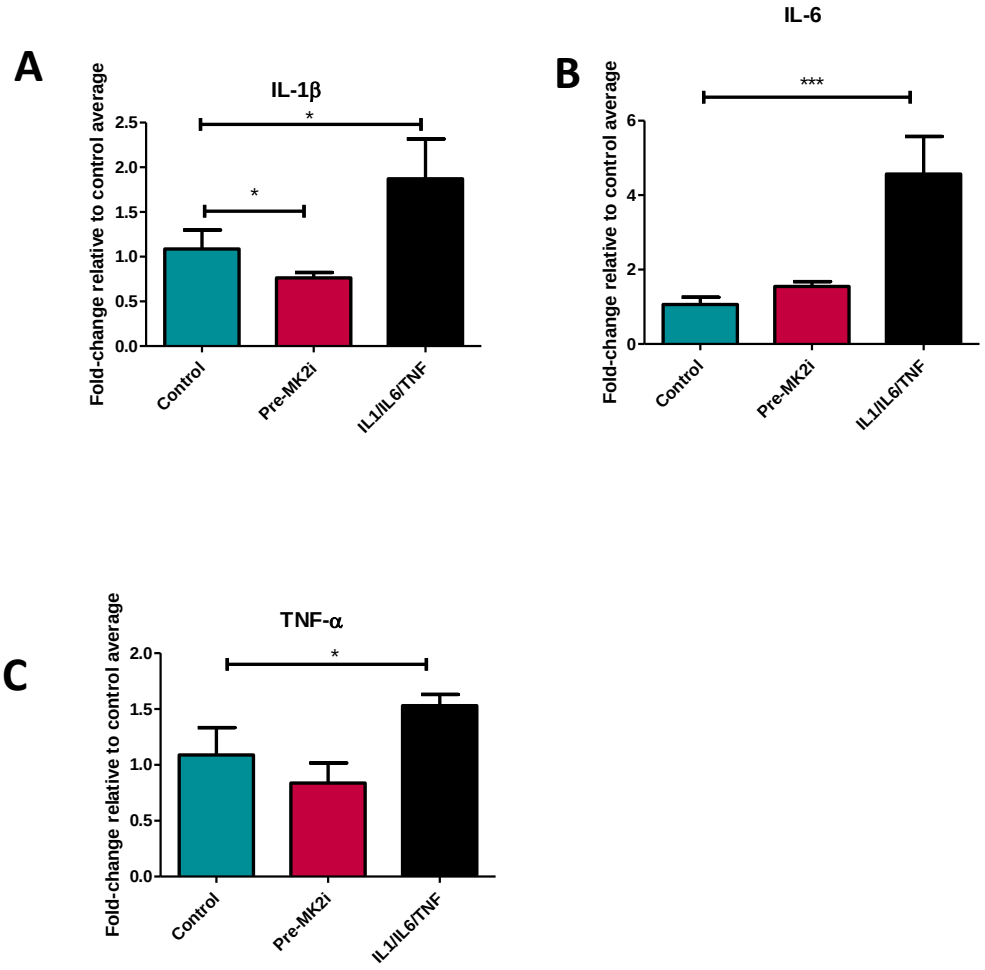

Figure 21. MK2-induced cytokines increase cytokine mRNA levels in pre-MK2i tumors. Pre-MK2i CT26 tumors treated with MK2 cytokines (IL1/IL6/TNF) make more A) IL-1β, B) IL-6, and C) TNF-α mRNA. N=5. *=p≤0.05, ***=p≤0.001.

IL-1β alone promotes production of MK2-induced cytokines and tumor growth

IL-1β, TNF-α, and IL-6 cross-regulate one another, and each is capable of leading to p-MK2 on its own (Figure 19A). Addition of a mixture of MK2-induced cytokines restores the presence of cytokine mRNA, supporting the idea that MK2-induced cytokines promote MK2 activation. If addition of MK2-induced cytokines does create MK2 activation, then addition of a single cytokine might be sufficient to activate MK2 activity and cytokine production within the tumor. IL-1β was chosen because it was produced in relatively small amounts within the tumor, and not at all by cultured tumor cells, suggesting that it is produced by infiltrating cells or tumor-associated stroma. As quantities of cytokines were decided on the basis of what was observed from control tumor, addition of IL-1β models the potential for inflammatory non-tumor cells to activate inflammation within the tumor microenvironment.

IL-1β treatment restored the size of pre-MK2i tumors (Figure 22A). Unlike the combination cytokine treatment shown in Figure 18, the tumors that received IL-1β were not significantly larger than the PBS control, although the mean size was higher (834 mm^3 to 570 mm^3). TNF-α and IL-6 levels from tumor supernatants were increased compared to PBS pre-MK2i. As all three MK2-induced cytokines showed a similar increase in proliferation speed in the proliferation tests (Figure 16B), the increased tumor size is likely due to the combination of cytokines, rather than the presence of IL-1β alone.

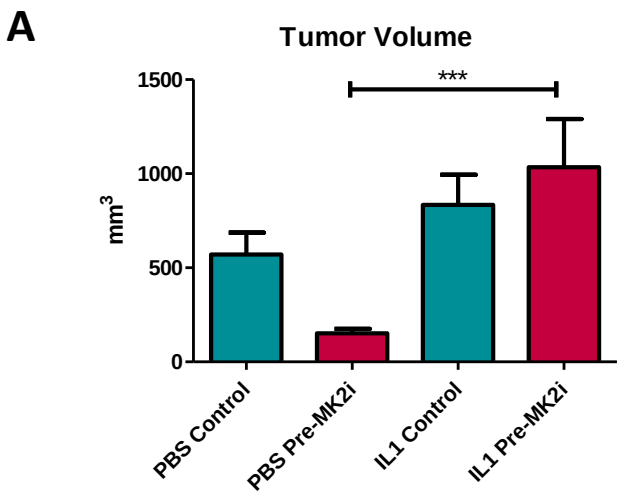

Figure 22. IL-1β restores MK2-induced cytokines and tumor size to pre-MK2i tumors. Intratumoral IL-1β A) restores tumor burden in tumors grown from MK2-inhibited CT26. N=5, *** = $p \leq 0.001$.

MK2 regulates MIP-1α, MIP-2, and MCP-1, pro-invasive macrophage-attracting factors

In MK2$^{-/-}$ mice, macrophages are decreased in the colon both in PBS and AOM/DSS-treated mice (Chapter 3). Macrophages are an important source of IL-1β, IL-6, and TNF-α in that model, and addition of bone marrow macrophages increased the neoplasm size in wild-type mice. Macrophage chemoattractant protein MCP-1 was regulated by MK2 in colon culture in that model. To investigate a potential role of MK2 for regulating macrophages in CT26 tumors, supernatants from whole tumors and cultured tumor cells were tested for cytokines that affect macrophage infiltration. CT26 tumors produce quantities of MCP-1, but also MIP-1α and MIP-2 (Figure 23A-C). Tumors grown from MK2-inhibited CT26 had decreased levels of these MCP-1, MIP-1α, and MIP-2; and the cultured tumor cells produced barely detectable levels of MCP-1, and drastically reduced MIP-1α and MIP-2 (Figure 23 D-F). These three cytokines are important in monocyte and macrophage migration, but have also been shown to promote invasion directly in tumor cells (239-241). To examine the effects on invasion, a cocktail of MIP-1α, MIP-2, and MCP-1 were added to a matrigel scratch-wound invasion assay. Like IL-1β, IL-6, and TNF-α, MIP-1α, MIP-2, and MCP-1 activated MK2 in CT26 cells (Figure 24A). Although MIP-1α, MIP-2, and MCP-1 are primarily considered to promote monocyte and macrophage migration, they all showed the ability to activate MK2 signaling in epithelial cells. In cultured cells from CT26 tumors, addition of MIP-1α, MIP-2, and MCP-1 increased the speed of scratch closure, indicating pro-migratory characteristics (Figure 24B). These cytokines rescued the anti-invasive effects of MK2 inhibitor treatment, a

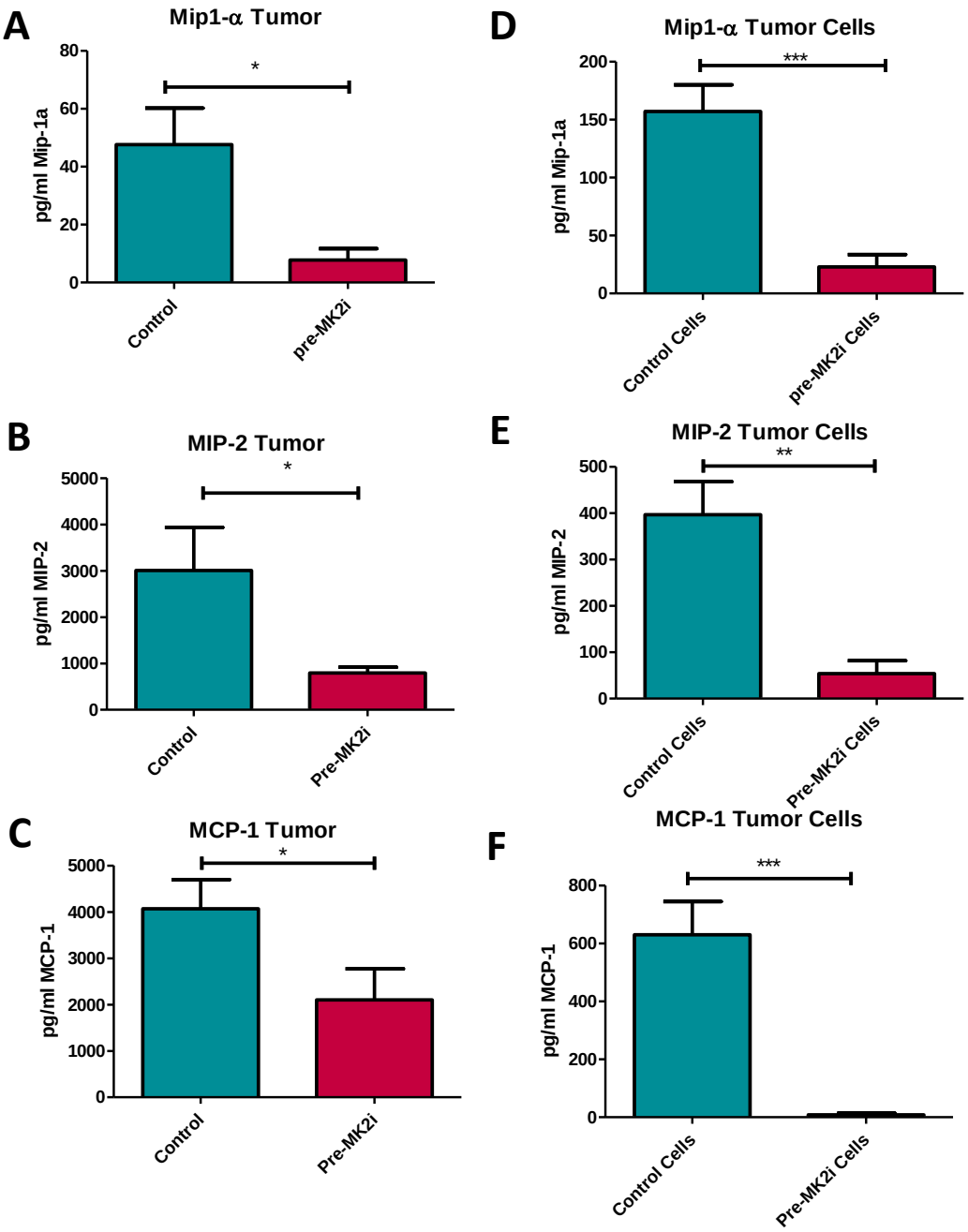

Figure 23. MK2 regulates macrophage migration cytokines. Tumors from MK2-inhibited CT26 produce less A) MIP-1α, B) MIP-2, and C) MCP-1. Cultured tumor cells produce little detectable D) MIP-1α, E) MIP-2, or F) MCP-1. N=7-8 for tumors and 6 for cultured tumor cells. * p = ≤ 0.05, ** p = ≤ 0.01, *** p = ≤ 0.001.

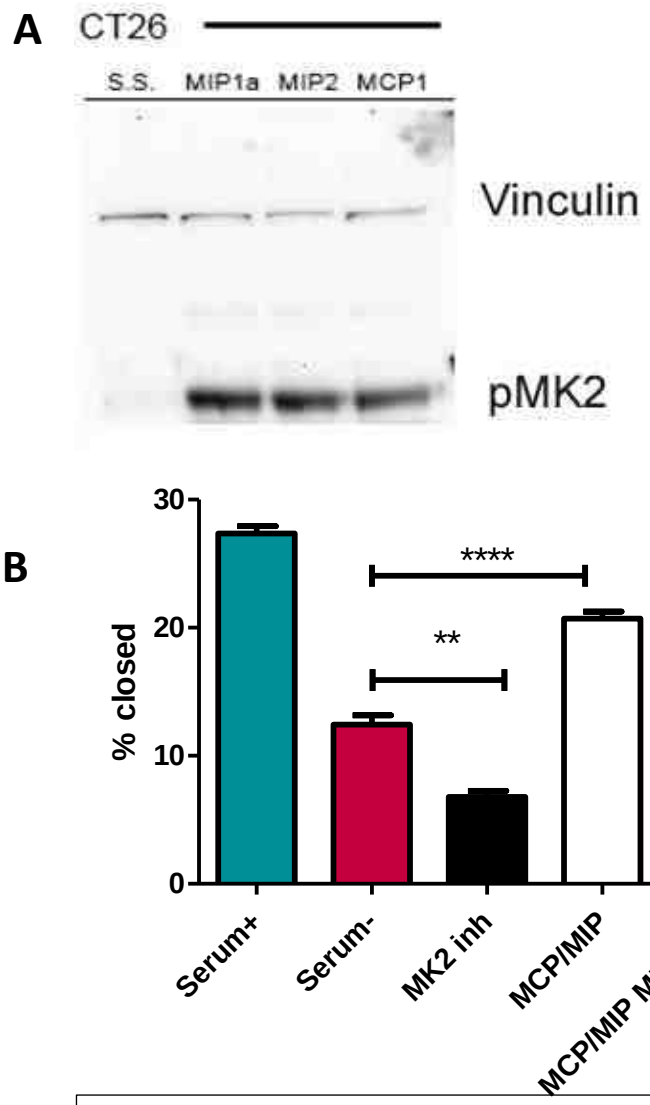

Figure 24. MCP-1, MIP-1α, and MIP-2 promote MK2 activation and invasion. MCP-1, MIP-1α, and MIP-2 cause MK2 phosphorylation of serum-starved cells as seen in A) a Western blot of pMK2. In a matrigel scratch-wound assay, B) tumor cells treated with 10 ug/ml of MIP-1α, MIP-2, and MCP-1 invade matrigel more quickly. MK2 inhibitor reduces invasion but is rescued by MIP/MCP treatment. N=9, in triplicate. ** = $p \leq 0.01$, **** = $p \leq 0.0001$.

similar pattern to that observed in MK2-induced cytokines. The observed increase in invasion suggests that MIP-1α, MIP-2, and MCP-1 may be important not only in attracting and activating monocytes, but also by directly supporting tumor progression.

Restoration of macrophage-attracting cytokines or macrophages induces tumor growth and MK2-induced cytokine production

Infiltrating macrophages have been shown to respond to CT26- produced cytokines by migrating to the invasive edge of the tumor promote CT26 growth and invasion (242). In Chapter 3, we found that macrophages are important providers of MK2-induced cytokines. Addition of macrophages in the colitis-associated CRC model increased cytokine production and tumor burden. In the CT26 model, MIP-1α, MIP-2, and MCP-1 were added to tumors as a cytokine cocktail, similar to the MK2-induced cocktail. Intratumoral MIP and MCP-1 rescued the phenotype of tumors from MK2-inhibited CT26 (Figure 25A). These cytokines also increased CT26 invasion. To separate the macrophage-attracting effects from any direct effects on CT26 cells, bone marrow macrophages were injected intratumorally. The macrophages also induced tumor growth to sizes comparable to the control.

Cytokine production from the MIP/MCP-treated group and the macrophage tumors was also increased. In the MIP/MCP group, IL-6 and TNF-α production were increased sharply, and IL-1β levels went from nearly undetectable to approximately half the amount of the control tumors (Figure 25 B-D). Some of this increase may be due to directly activating MK2, as these cytokines are able to cause phosphorylation of MK2 (Figure 24), but another potential source of MK2-induced cytokines is inflammatory

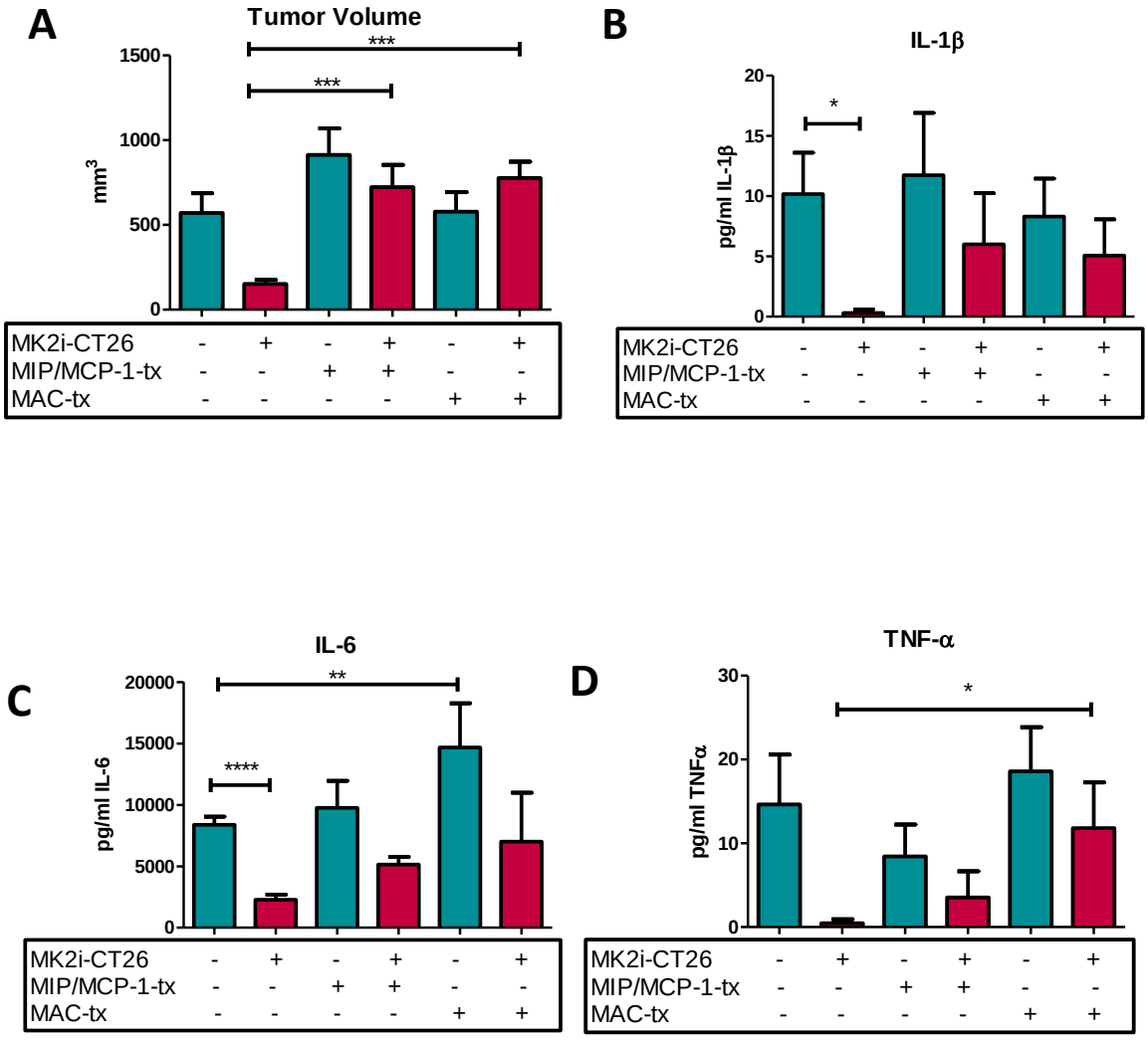

Figure 25. Macrophages and macrophage-attracting cytokines restore pre-MK2i tumor burden. Intratumoral MCP-1, MIP-1α, and MIP-2 or bone marrow macrophages increases pre-MK2i A) tumor burden and whole tumor production of B) IL-1β, C) IL-6, and D) TNF-α. N=4-5 for macrophage and 10 for other groups.

macrophages, which can be attracted and activated by these cytokines. There was no significant difference in IL-6, TNF-α, or IL-1β levels between the control tumors and the macrophage-treated tumors from MK2-inhibited cells; the macrophage treatment fully restored MK2-induced cytokine production as well as tumor burden (Figure 25 B-D). These data support the importance of the cell make-up in the tumor microenvironment, particularly of macrophages, which are regulated by MK2 activity within the tumor.

Discussion

MK2 is a target of growing interest in multiple cancers. In glioblastoma cells, MK2 activity supports pro-tumorigenic protein Khsrp and promotes secretion of IL-6, which promotes invasion and growth in glioblastoma just as it can in CRC (138;139). MK2 knockout significantly prevented tumor development in a model of skin cancer (140). Targeting MK2 in leukemia cells enhances apoptosis (243). The production of IL-1β, IL-6, and TNF-α are critical elements in aggressive tumors in the colon as well as other tissues. Identification of MK2 as the primary control point opens possibilities for intervention. As aggressive tumors have increased levels of inflammation, inhibition of MK2 may be a viable solution to reduce the growth and invasion (18;203). As TNF-α, IL-1β, and IL-6 are each important in their own right, their simultaneous control under MK2 offers a way to inhibit multiple pathways of CRC growth and invasion at once. MK2 also participates in cell cycle pathways, which may support its importance in non-inflammatory survival pathways; this could be of potential interest in dual therapies with chemotherapeutic drugs (244). Inflammation is a primary mediator of tumor

growth, as observed in both colitis-associated and spontaneous models. The total restoration of tumor burden in tumors from MK2-inhibited CT26 cells is very suggestive of the importance of MK2-induced cytokines. Another indication of the importance of these cytokines and MK2 activity is the absence of neoplasms in MK2-inhibited mice in the inflammation-driven AOM/DSS model. Clearly, the MK2-cytokine loop is an important mechanism for tumor growth in CRC. Models of invasive spontaneous CRC and of colitis-associated CRC were used to further investigation of MK2 activity. We found that inhibition of MK2 reduced or eliminated tumor burden. The decrease in tumor burden to 25% of the control group in CT26 tumors, and the absence of tumor burden in 78.6% of the AOM/DSS mice, suggests that MK2 activity is a critical component in CRC, and is a promising therapeutic target (Fig. 1-2). MK2-induced cytokines are a potential mechanism to explain these differences.

MK2 activity increases production of MK2-induced cytokines, and MK2-induced cytokines, in turn, activate MK2 (Figure 19). Thus, initiation of MK2 signaling can create an inflammatory positive feedback loop. As cytokines are produced, they can signal in a paracrine manner, activating more MK2 and more cytokine production. The chronic inflammation is part of the tumor microenvironment. Inflammation changes infiltrating cell types and behavior. Because pro-inflammatory cytokines alter migration and behavior in nearly every cell type, the composition of the tumor microenvironment is dependent on the cytokine milieu. In addition to IL-1, TNF-α, and IL-6, MK2 regulates important chemoattractants for macrophage populations. The addition of these cytokines, without any further inflammatory addition, is enough to overcome reductions

in tumor burden in MK2-inhibited CT26 tumors, and increases the MK2-induced cytokines produced by tumors. These data support the role of MIP-1α, MIP-2, and MCP-1 as important regulators of tumor inflammation. Interrupting the MK2 to cytokine to MK2 feedback causes a cascade of alterations by reducing cytokine amounts. In our colitis-associated model, no gross signs of inflammation were observed in the colons of MK2 inhibited mice, and in CT26 tumors, inhibition of the MK2 pathway resulted in persistent reduction in cytokine production both from isolated tumor cells and from whole tumor supernatants. These observations support the idea that there are lasting effects of interruption of MK2 signaling.

MK2 inhibitor treatment lead to a greater reduction in tumor burden than other studies have reported with single-cytokine targeted therapies. IL-1β blockade in an AOM/DSS model reduced tumor number by approximately half, but all mice developed tumors (217). IL-6 produced by both tumor and non-tumor cells contributes the tumor burden, and inhibition of IL-6 reduced tumor weight by, again, approximately 50% (245). Anti-TNF-α therapy reduced the number of tumors in obese mice by approximately half, and volume by approximately two thirds (229). In the two MK2 inhibitor groups in the AOM/DSS model, neoplasms were completely absent in 11/14 mice. The same trend was observed in a model of skin cancer, where TNF-$\alpha^{-/-}$ mice developed fewer tumors than wild-type mice, but more than MK2$^{-/-}$. These differences may be due to MK2's control of multiple cytokines, and ability of IL-1, IL-6, and TNF-α to overlap and reinforce proliferative pathways in CRC.

One reason a reduced tumor burden is seen in MK2-inhibited tumors compared to any single cytokine pathway blockade is the way that inflammatory pathways promote production of pro-inflammatory cytokines. Inflammasome activation is essential for IL-1β production. The inflammasome can be activated by microbial changes during colitis, but also by TNF-α in a sterile manner (246;247). In turn, IL-1β can drive IL-6 production. Thus, blocking TNF-α can reduce IL-1β and IL-6 levels, but if the inflammasome is activated by invasion of microbiota through the damaged epithelium, there is a backup pathway that will result in production and secretion of IL-1β and IL-6. Cross-activation was observed in mice treated with IL-1β, which restored tumor burden and MK2-induced cytokine in tumor supernatants. Inhibition of MK2 adds additional feedback checkpoints, where both IL-1β and IL-6 would be inhibited.

Interruption of the MK2-cytokine feedback loop can have long-term effects. Although MK2 inhibition was not continued after initiation of CT26 tumors, tumors and tumor cells from MK2-inhibited CT26 had reduced cytokine production. Properly timed inhibition can have continuing anti-inflammatory effects weeks after the inhibitor is no longer applied to cells. Adding MK2-induced cytokines to tumor cells from MK2-inhibited CT26-initiated tumors resulted in restored proliferation, cytokine production, and tumor growth, so the positive feedback loop can be restored by inflammatory signaling. However, the effects linger as long as no further sources of inflammation are introduced, as with the intratumoral injection of recombinant cytokines. Interestingly, Intratumoral restoration of physiological levels of MK2-regulated MIP and MCP proteins also resulted in rescued tumor burden, suggesting that macrophages have the potential

to drive the necessary inflammatory signaling that results in tumor growth. These cytokines also promote invasion in cultured tumor cells, so their effects on tumor behavior are not limited to attracting macrophages. Direct administration of bone marrow macrophages also restored tumor burden and cytokine production, indicating that macrophages may be an important source of cytokines, or can initiate the same inflammatory response as administration of recombinant MK2-induced cytokines does. The lasting effects of MK2 inhibition bode well for therapeutic applications of this pathway, as doses may be able to be spaced farther apart. With proper timing, MK2 inhibition need not be constant to provide a significant reduction in tumor size.

MK2 inhibitors are a promising target for combination therapies, and published data are promising. Inhibiting both MK2 and the cell cycle protein Chk1 results in increased apoptosis across multiple therapy-resistant cell lines (248). In an autochthonous model of non-small-cell lung cancer, MK2 inhibition sensitized p53-deficient tumors to cisplatin treatment (249). Many chemotherapeutics, including those commonly used in CRC, promote inflammation (250). Inflammation as a result of therapy is a concern not just because of the pro-tumorigenic results of inflammation, but because chemoresistance has been directly linked with IL-6 and IL-1 (251-253). Decreasing chemoresistant tumor cells could increase clearance of tumor cells and reduce recurrence rates. It is possible that the anti-inflammatory effects of MK2 inhibition could help prevent chemoresistant subpopulations developing in CRC. MK2 promotes not only pro-inflammatory pathways, but other pathways that control cell cycle. $MK2^{-/-}$ mice have shown resistance to skin cancer, where they grew fewer tumors than wild-type mice. Part of this reduction is

likely due to reduced TNF-α production, as TNF-$\alpha^{-/-}$ animals also showed reduced tumor size, but also linked to changes in cell cycle and DNA repair signaling (254). In addition to inflammatory mediators, MK2 substrates include cell cycle proteins, DNA damage repair, and proteins associated with epithelial-to-mesenchymal transition (255-257). In addition to the observed changes in pro-inflammatory cytokines, MK2 may have a significant effect on one or more of these areas within CRC, and further investigation is warranted.

CRC is one of the most common malignancies in the United States, and incidence among young adults is increasing (258). Identification of targets is ongoing due to the complicated, multi-faceted nature of CRC, but will be essential to improve outcomes, particularly in tumors identified in advanced stages. MK2, with its connections to inflammation and positioning as an intermediary within forward feedback loops, presents a potential weak spot for attack. We show here that inhibition of MK2 results in decreases in pro-tumorigenic cytokines and reduction or reversal of tumor growth.

Chapter 5: Conclusions

Introduction

Advanced CRC is difficult to eliminate, with extremely poor prognosis, but interrupting inflammatory pathways may provide an important opportunity for future treatments (1;121;259). These cancers are genetically diverse, but aggressive and advanced cancers have much higher levels of inflammation, which predict increased metastasis, invasion, and mortality (18;121;199;259-262). Increases in pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in patient serum are correlated with tumor progression and predictors of tumor aggression and patient mortality (23;262). Each of these cytokines has, individually, been recognized as supporting cancer development and progression (18;29;233;263;264). Because of the widely recognized role that inflammation plays in CRC, pro-inflammatory molecules are under investigation for therapeutic targets.

The three major cytokines associated with CRC initiation and progression are controlled through the p38 pathway (265). p38, which is activated by cell stress and inflammatory signaling, has been investigated as a target in multiple diseases in which inflammation plays a significant role, including CRC (112;266). Unfortunately, p38 inhibitors display significant toxicity in animal models (111;113). Clinical trials have showed mixed results, but currently there are no inhibitors that have progressed beyond phase II clinical trials (18;29;111;111;233;263;264). Some investigators are moving upstream in signaling pathways in the search for anti-inflammatory interventions (113). However, moving

downstream is also a strategy that can reduce toxicity (267); it may offer the opportunity to more accurately target cytokine production without inhibiting pathways also initiated by p38 that can be helpful in cancer or cause toxicity (268).

MK2, a kinase directly phosphorylated by p38, provides a promising downstream target for control of CRC through inflammatory cytokines (269). Reports from $MK2^{-/-}$ animals show that IL-1β, IL-6, and TNF-α were reduced in many disease models, often to or below normal levels (186;191;209). Critically, MK2 inhibitors do not have the same adverse effects as observed with p38 inhibition; even mice that are $MK2^{-/-}$ are viable and healthy (186). The control of inflammatory cytokine production, paired with a reduction in toxicity, makes MK2 an excellent target for intervention in CRC inflammation. At the initiation of this project, investigation into MK2 in cancer was limited, but in a skin cancer model, $MK2^{-/-}$ mice showed reduced tumor incidence, and copy number had reported to vary in lung cancer patients (132;135;140). In the time since we began investigating MK2, MK2 has been increasingly implicated in tumor promotion in other cancer models and cell lines (24;134;136;137). Preliminary data produced by our lab showed that mRNA from human tumor samples had higher MK2 compared to normal controls (data not shown). These data and studies formed the basis for further investigation into the role of MK2 in CRC, with the hope for uncovering a potential target for therapeutic intervention. Based on the literature and preliminary findings, we crafted two aims to look at the role of MK2 in CRC.

Aim 1.

To determine the extent that MK2 drives neoplasm development and cytokine production in a colitis-associated model of colorectal cancer. For this aim, we hypothesized that $MK2^{-/-}$ mice would have decreased IL-1, IL-6, and TNF-α and reduced neoplasm development. We further hypothesized that one important source of the MK2-induced cytokines was macrophage populations.

Aim 2.

To examine MK2's role in CRC growth as mediated by MK2-induced cytokines, and to evaluate the use of MK2 inhibitors as therapeutic intervention in CRC models. We hypothesized that interruption of MK2 activity in a tumor would result in decreased IL-1β, IL-6, and TNF-α, and a lower tumor burden.

These two aims were designed to investigate the interplay between MK2, MK2-induced cytokines, and CRC across two different mouse models, and to gather evidence into the therapeutic value of MK2 inhibition. The results gathered from this investigation strongly support the prominence of MK2 in CRC development and progression, and provide evidence that MK2 inhibition is a promising strategy.

Results

MK2 is vital to CRC development. In Chapter 3, we found that $MK2^{-/-}$ mice are profoundly resistant to development of neoplasms in a colitis-associated CRC model. None of the $MK2^{-/-}$ mice had neoplasms at harvest, although the WT mice developed an average of 3.2 neoplasms. Histological examination of the colon revealed that the $MK2^{-/-}$

colons are grossly normal. Using colon supernatants to examine the production of cytokines revealed that the pro-tumorigenic MK2-induced cytokines IL-1, IL-6, and TNF-α were all profoundly decreased in comparison to WT AOM/DSS mice. MK2 thus appears to be the major pathway for the production of these cytokines in this model. The lack of compensatory pathways makes MK2 a better candidate for intervention, and underscores its essential role; MK2 is indispensable to colitis-associated CRC development.

Macrophages are an important source of inflammatory cytokines in colitis-associated CRC, so we investigated the cell-specific role of MK2 in macrophages. $MK2^{-/-}$ mice had decreased macrophages both before and after AOM/DSS treatment. This decrease indicates that MK2 is important in the attraction or maintenance of macrophages in the gut both during homeostasis and CRC development. Macrophage-attracting factors GM-CSF and MIP-1 were reduced in organ supernatants, suggesting one mechanism by which the decrease in macrophages is occurring. Fewer of the macrophages isolated from $MK2^{-/-}$ mice produce MK2-induced cytokines. This is true for both PBS and AOM/DSS-treated mice. We hypothesized that the lack of pro-inflammatory macrophages could also contribute to the lack of neoplasm development observed. To test this hypothesis, we injected WT macrophages intraperitoneally into WT or $MK2^{-/-}$ mice. In WT mice, the additional macrophages resulted in an increase in TNF-α and IL-6, as well as GM-CSF and MIP-1. The average number of neoplasms doubled in these mice, supporting the importance of macrophages and MK2-associated signaling in tumor burden. In $MK2^{-/-}$ mice, there was an increase in the MK2-induced cytokines. Levels of

IL-6, GM-CSF, and MCP-1 were the same as WT AOM/DSS mice that had not received supplementary macrophages. However, this increase was not enough to cause neoplasm development; all $MK2^{-/-}$ mice remained neoplasm-free. Thus, while $MK2^{+/+}$ macrophages can provide high levels of MK2-induced cytokines, their presence alone does not alleviate the profound tumor suppression in MK2-deficient mice.

Tumor cells themselves can promote inflammation through the secretion of cytokines, including IL-1, IL-6, and TNF-α, as well as by the production of other proteins that attract immune cells and activate inflammatory phenotypes in surrounding tissue. In Chapter 4, we explored MK2 inhibition in established or establishing tumors, and found that MK2 inhibition reduces tumor burden and inflammation. Using the colitis-associated CRC model utilized in Chapter 3, we treated mice with one of two MK2 inhibitors at the time that neoplasm formation is observable in 85% of WT mice. After three weeks, we ceased treatment. Both of the MK2 inhibitor-treated groups had regression of tumors: 5/7 and 6/7 of the mice in the two inhibitor-treated groups were neoplasm-free. MK2-induced cytokines were the same as matched normal tissue from the PBS group; IL-6 was significantly decreased compared to normal. Only two of the neoplasm-bearing mice from the inhibitor groups had tumors large enough to sample for cytokine production. Both of these tumors produced high levels of IL-1β, and low levels of IL-6 compared to control neoplasms. In tumors grown from CT26 cell line, we found that pre-treating CT26 cells to inhibit MK2 activity before injection resulted in tumors less than half the size of the controls. These pre-MK2i tumors produced decreased MK2-induced cytokines. Isolated and cultured tumor cells grown from these tumors also showed

decreased production of MK2-induced cytokines. In vitro, we found that MK2-induced cytokines increased pMK2 levels in CT26 cells, suggesting that MK2 and MK2-induced cytokines participated in a forward feedback loop. The existence of the feedback loop was supported by an injection of IL-1β into pre-MK2i tumors. Like the combination of MK2 cytokines, IL-1β alone restored tumor size, and tumor supernatants contained comparable levels of the other MK2-induced cytokines. We injected MK2-induced cytokines into pre-MK2i and control tumors. Pre-MK2i tumors grew to the size of control tumors, suggesting that the size difference was cytokine-dependent. Both the tumor and cultured tumor cell supernatants from pre-MK2i tumors also produced less MCP-1, MIP-1α, and MIP-2. These are chemokines that promote infiltration of macrophages. Restoration of MCP-1, MIP-1α, and MIP-2 also increased pre-MK2i tumor volume to the size of the vehicle control. We injected macrophages intratumorally and observed an increase of tumor size. Tumors grown from MK2-inhibited CT26 can thus be "rescued" from their decreased growth by administering inflammatory cytokines, attracting pro-inflammatory macrophage populations, or delivering macrophages directing to the site.

The addition of IL-1β, IL-6, and TNF-α to the tumor increased the levels of each of these cytokines in the supernatant of tumors, The increase could partially be attributed to remaining recombinant cytokines in the tissue, but mRNA for these cytokines was also significantly increased for cytokine-treated tumors. In addition, CT26 tumors grown from MK2-inhibited cells also showed restored cytokine production when treated only with IL-1β, suggesting that the cytokines are a result of activation of pro-inflammatory

cytokine production, and not solely due to recombinant cytokines. Administration of MIP-1α, MIP-2, and MCP-1 increased IL-1β, IL-6, and TNF-α in tumors from MK2-inhibited CT26. Intratumoral delivery of macrophages also increased MK2-induced cytokines; IL-6 and TNF-α reached the level of control tumors. In macrophage-treated tumors grown from control CT26, IL-6 was significantly increased. MCP and MIP proteins and infiltrating macrophages are thus implicated in the control of MK2-induced cytokine production.

Discussion and Future Directions

This work demonstrates the critical role that MK2 has in CRC development and progression, and identifies MK2 as a potential therapeutic target in CRC. These studies provide a foundation for further development of translational research that may improve outcomes for CRC patients. It also examines the cell-specific roles of MK2 in tumor cells and macrophages. MK2 blockade was completely protective against neoplasm development in the clinically relevant AOM/DSS model (169). Production of MK2-induced cytokines associated with development of CRC was decreased; in the case of IL-1α and TNF-α, these cytokines were at normal levels. Inhibition of IL-1α has shown promise in a Phase III clinical trial (270), so the reduction seen here backs up the relevance of the lack of neoplasm development. Introduction of $MK2^{+/+}$ macrophages increased MK2-induced cytokines, suggesting that the macrophage population promotes tumorigenic inflammation and is an important source for these cytokines. The increase in neoplasms observed when macrophages were injected into WT, but not $MK2^{-/-}$, mice suggests that the increase in inflammation introduced by macrophages

promotes tumor growth but is inadequate to initiate neoplasms independently. MK2 inhibitors, administered after neoplasms began to develop, reversed neoplasm growth and left the majority of mice neoplasm free. MK2 signaling continues to be critical after CRC initiation, and is a good target in this model. CT26 cells that were MK2 inhibited when they were injected into mice produced smaller tumors that made less pro-tumorigenic cytokines. Interestingly, differences in the production of MK2-induced cytokines were observed long after the cells were no longer actively inhibited. Intratumoral injection of a cocktail of MK2-induced cytokines restored growth of inhibited tumors. This data suggests that the difference in size was not due to increased death, but to a lack of inflammation in the tumor cells that originated the tumor.

Administration of IL-1β rescued the phenotype of tumors from MK2-inhibited CT26 cells. IL-1β increased proliferation of CT26 cells in culture, but to an equal extent as either TNF-α or IL-6 alone. A combination of cytokines increased proliferation *in vitro. In vivo,* IL-1β was nearly as effective as the combination of the three MK2-induced cytokines. IL-1β treated tumors produced TNF-α and IL-6 on a similar level to control tumors, suggesting that IL-1 was able to initiate the MK2 forward feedback loop, activating MK2 activity and driving cytokine production. The phenotype seen in tumors from MK2-inhibited cells is thus sensitive to inflammatory signaling.

One potential source of inflammatory signaling is cytokines from macrophages. In the AOM/DSS model, macrophages produced high levels of MK2-induced cytokines. As MK2 also regulates production of MIP-1α, MIP-2, and MCP-1, this could adversely affect macrophage infiltration and activation. Addition of macrophages to wild-type mice in

the AOM/DSS model increase tumor burden, and addition of macrophages or tumor-produced levels of MIPs and MCP-1 increased tumor size of pre-MK2i CT26 tumors. MK2 is an important regulator of inflammation not only through alteration of IL-1, IL-6, and TNF-α, but also by controlling infiltration of macrophage populations. In turn, macrophages drawn to tumors can promote tumor growth and invasion by creating an inflammatory environment and activating MK2 in the tumor microenvironment.

These experiments allow for a hypothesis as to the order of events in MK2-supported tumors. In the CT26 model, tumor-specific inhibition of MK2 reduced tumor burden, which suggests that the IL-6 and TNF-α made by the tumor cells was necessary for the inflammatory response seen in CT26 tumors. These cells did not produce IL-1β, so that cytokine was likely produced by stroma and infiltrating immune cells after initiation of inflammation. Both IL-6 and TNF-α activate MK2 activity in cell lines, so autocrine and paracrine signaling from these cytokines drives continuing MK2 signaling in the tumor cells, and is likely to activate MK2 in the surrounding tissue. As MK2-induced cytokines are made, they attract infiltrating immune cells. IL-1β and IL-6 are directly associated with macrophage migration (271-274). TNF-α indirectly induces endothelial activation that increases infiltration of macrophages (275). In Chapter 4, it was demonstrated that MIP-1α, MIP-2, and MCP-1 are also regulated by MK-2; these are important for recruiting macrophages to the colon (150;152;153). Inhibition of MK2 reduces multiple important sources of macrophage attraction. Macrophages are important sources of MK2-induced cytokines, which increase CRC tumor size. Addition of macrophages to neoplasms in AOM/DSS increases tumor size and cytokine production; in CT26 tumors, it

restored the growth in pre-MK2i tumors and increased cytokine production. While MK2 activity is ongoing, this creates a powerful forward feedback loop, where additional IL-1β, IL-6, and TNF-α are produced by inflammatory macrophages, promoting further MK2 signaling. These cytokines drive tumor growth as they promote further cytokine production and attraction of inflammatory macrophages. Inhibition of tumor cell-specific MK2 prevents these steps, and maintains a tumor microenvironment with much lower levels of inflammation. MK2 activity and cytokine production remains suppressed as tumors grow, resulting in a lower tumor burden. See Figure 26 for a graphical representation of this proposed pathway.

Future Direction: Mechanism of IL-1 and IL-6 Induction by MK2

MK2 has a number of substrates, but none have been confirmed to directly affect IL-1 and IL-6 production. The substrate may affect mRNA quantities, as with TNF-α, which is regulated through mRNA stabilization (121;276). Not all inflammatory cytokine production in the p38 pathway is due to MK2; in a model of parasite infection, p38 inhibition decreased levels of IL-6, but not of IL-1β or TNF-α (277). Although so far all three cytokines have been regulated through MK2 in the models of CRC used, that may not be the same for all cancers or conditions. An understanding of how MK2 regulates cytokine production would shed light on cases where cytokine regulation appears to differ, such as when MK2 inhibition increases TNF-α levels in an acute myeloid leukemia model (278). One potential avenue for investigation is MK2's control of p38 localization. Because MK2 has a nuclear export signal that is exposed when MK2 is phosphorylated, MK2 activity can control the location of the p38-MK2 complex (279). Cytosolic

localization of p38 is thus regulated by MK2 phosphorylation. MK2 can also act as a platform for complex formation, modulating binding availability for p38 substrates. Changes in localization of p38 would affect its ability to activate other substrates that could lead to cytokine production, for example Jun and NFκB. These both result in increased pro-inflammatory cytokine production (92).

The two most likely paths for direct MK2 signaling paths for IL-6 and IL-1 production are through stabilization of the mRNA or activation of activating transcription factor 1 (ATF-1). mRNA stabilization can be affected direct through MK2 phosphorylation of proteins that bind AU-rich elements (ARE) in mRNA, or through signal transducer and activator of transcription 3 (STAT3). For IL-6, the same mechanisms that regulate TNF-α mRNA stability can also affect IL-6 mRNA, but since TNF-α can also affect IL-6 mRNA, further investigation is necessary to show if this is the primary pathway for MK2 regulation (121;259). Another potential pathway worth investigating is the STAT3 pathway. In the nucleus, active MK2 phosphorylates TRIM28, a transcriptional repressor protein that binds to STAT3 (280). Once phosphorylated, TRIM28 dissociates from the STAT3 gene, allowing transcription (281). STAT3 can also affect mRNA stability of IL-6 mRNA, through the same mechanism as MK2 (282). However, it has not been confirmed whether this regulation is the most important for MK2-regulated IL-6. IL-1β can also be regulated by STAT3 (283). Another potential target is activating transcription factor 1 (ATF-1), a MK2 substrate that can promote IL-6. ATF-1 binds to the IL-6 transcription site, and has been

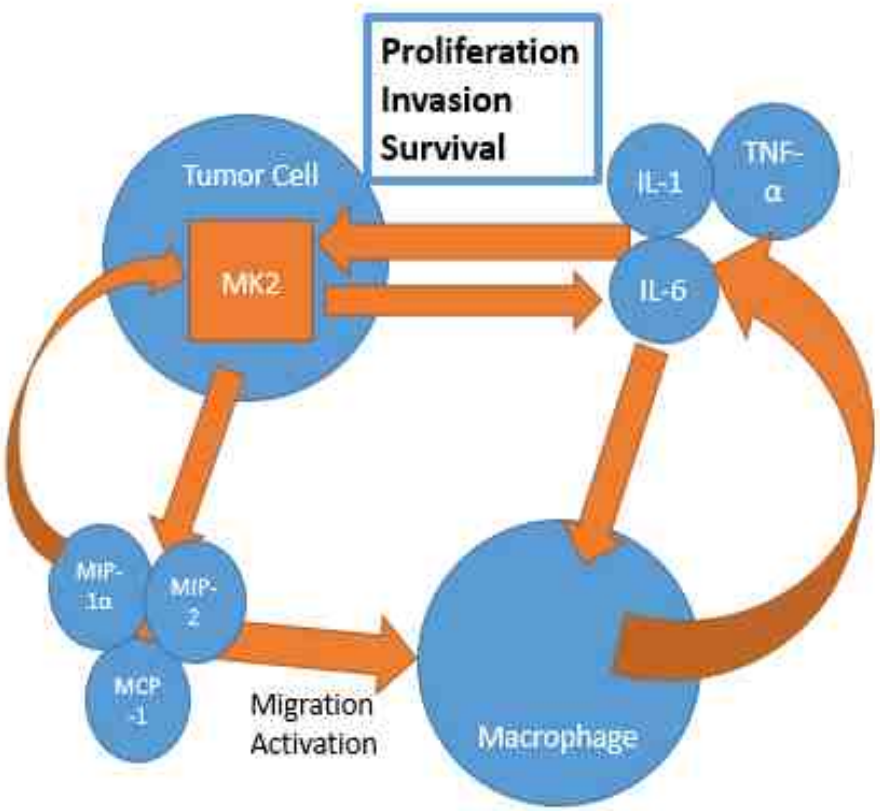

Figure 26. MK2 promotes colorectal cancer through cytokine production and activation and recruitment of immune cells. MK2 activity in tumor cells supports production of two critical groups of cytokines: a) IL-1, IL-6, and TNF-α, and b) MIP-1α, MIP-2, and MCP-1. Both of these clusters promote further MK2 signaling, creating positive feedback. Both support invasion and migration of tumor cells. MIPs and MCP-1 attract and activate macrophages, which contribute to the pool of IL-1, IL-6, and TNF-α. These cytokines drive tumor proliferation.

implicated in IL-6 production in macrophages (284;285). ATF-1 also binds to the promoter site of IL-1, but it is unknown the extent to which it influences mRNA production (286). Investigation into these pathways might reveal primary mechanisms for IL-1 and IL-6 production. Understanding how MK2 promotes cytokine production and whether the path is the same or differs for each might also reveal further downstream targets. For example, if all cytokines are primarily regulated through one or more ARE-binding proteins, that could provide a way of blocking MK2-induced cytokines while leaving the rest of MK2 activity intact. Investigating this mechanism could help clarify the extent of other MK2 signaling pathways in promoting and progressing cancers, and provide insight into the co-regulation of inflammatory cytokines within the immune system.

Therapeutic Pursuit: Dual Therapy and Chemoresistance

Most new therapies are used not as replacements for existing drug treatments, but as co-treatments to increase efficacy, decrease side effects, or otherwise improve outcomes of existing therapies. MK2 inhibition thus warrants further investigation to its effects alongside traditional treatments of CRC. Anti-tumor drugs commonly used in mouse models are applicable to human patients, which makes testing of dual therapies relatively straightforward. Examining the role of MK2 in chemoresistance and effects of adding MK2 inhibitors to standard therapies in mice would be a necessary step in investigating MK2 for potential therapeutic value. MK2 could contribute through chemoresistance directly, through intracellular signaling that supports survival and DNA

repair, or through increased production of MK2-induced cytokines, which can support development and promote survival of chemoresistant tumors.

Limitations of this Study

Every scientific study has limits on the variables examined and the conclusions made, and this work is no different. When the conclusions are promising, particularly in work that might be of clinical relevance, caution is especially warranted. Small and large limitations of this project are discussed throughout, intertwined with the results and discussions sections of Chapter 3 and 4. However, I would like to highlight two of the big limitations of these studies so far.

Crosstalk and overlap of MK2-induced cytokine signaling

The immune system is inextricably interwoven; signaling pathways are often overlapping and redundant. IL-1β, IL-6, and TNF-α are all downstream of MK2, but they can also activate cytokine receptors on cells. Cytokine signaling directly leads to p38 activation in the case of IL-1α, IL-1β and TNF-α (95;126). p38, in turn, activates MK2 and promotes more cytokine production. This positive feedback loop is part of why MK2 inhibition appears to be so powerful and long-lasting. However, because cytokines regulate each other, it is difficult to distinguish the effects of a single MK2-induced cytokine.

In addition, many of the cytokines influence the same tumor processes; they induce growth, survival, invasion, and more inflammation. Because there is overlap between MK2-induced cytokine activities, delineating a clear role for each is difficult. Separating

out the effects of IL-1, IL-6, and TNF-α is especially true because they can induce one another. There are other MK2-indepedent mechanisms by which cytokines are made. In our models, MK2 is the primary regulator of cytokine production; this may not be true in all tissues or for all genetic backgrounds. Because MK2 appears to be responsible for much of the cytokine production, it is easy to overlook these other cytokine production pathways. Alternate signaling paths could provide a potential escape point for tumors, particularly after prolonged MK2 inhibition. One potential pathway would be to allow mice treated with AOM/DSS and MK2 inhibitors a longer period after treatment ceased to investigate tumor recurrence in the long-term absence of treatment.

Alternate MK2 pathways to affect cancer

Inflammation is an important factor in many cancers, not just CRC, but this may not be the only mechanism by which MK2 influences tumor outcomes. MK2 substrates also include cell cycling proteins CDC25B and CDC 25C (306;307). CDC25B and CDC25C are cell cycle promotion proteins that are part of a complex that moves cells from the G2 phase to the M phase (308). In the absence of DNA damage, CDC25B may be phosphorylated by CHK1 as well as MK2 (309). Inhibition of both MK2 and CHK1 has shown to induce apoptosis in multiple tumor cell lines that are normally resistant to apoptotic death (244;309). The CDC25 family has been identified as potential therapeutic targets in cancer, so MK2's activation may be one angle to examine control of two members of the family (310). The phosphorylation sites on CDC25B and C inhibit activity, preventing progression into the cell cycle (311). Although cancer is normally associated with uncontrolled proliferation, checkpoints during DNA damage can assist in

tumor survival. Chemotherapeutics often cause DNA damage; rapidly dividing cells, such as tumors, are especially vulnerable to cell death due to this damage. The activation of DNA repair pathways requires suspension of replication. If these proliferation checkpoints are not available, tumor cell death greatly increases. In a dual-treatment system, MK2 inhibitors may sensitize cells to DNA damaging agents. Increased sensitivity has been observed in p53-deficient tumors, where p53 and MK2 deficient cells are far more sensitive to DNA damage (312). Investigating these other roles that MK2 plays may reveal more about the cell-specific ways MK2 supports cancer, and shed light into the mechanisms and potential effectiveness of MK2 inhibition. MK2's downstream signaling may play an important part in tumor survival and chemoresistance in a way that is not inflammation-mediated. Investigation into dual therapies, discussed above under Future Directions, would be one promising avenue to further pursue the different ways MK2 can affect survival and proliferation.

List of Abbreviations

AOM	Azoxymethane
ATF-1	Activating transcription factor 1
CFSE	Carboxyfluorescein succinimidyl ester
CRC	Colorectal cancer
DMSO	Dimethyl sulfoxide
DSS	Dextran sodium sulfate
HBSS	Hank's balanced salt solution
hnRNP A0	Heterogeneous nuclear ribonucleoprotein A0
HuR	Human antigen R
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
IP	Intraperitoneal
IT	Intratumoral
MIP-1α	Macrophage inflammatory protein 1-α (aka CCL3)
MIP-2	Macrophage inflammatory protein 2 (aka CXCL2)
MCP-1	Monocyte chemoattractant protein (aka CCL2)

MK2	Mitogen-activated protein kinase-activated protein kinase 2 (aka MAPKAPK2)
PBS	Phosphate-buffered saline
STAT3	Signal transducer and activator of transcription 3
TNF-α	Tumor necrosis factor alpha
TTP	Tristetraprolin

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