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MCP-1 In Colorectal Cancer: Benefits of Exercise

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MCP-1 IN COLORECTAL CANCER: BENEFITS OF EXERCISE

by

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DEDICATION

I would like to dedicate this degree to my family who has been more supportive in this endeavor than I could have ever hoped. To my Mom who tells me so often that she loves me and how proud she is of me. To my sister and brother who take me on their vacations to get a break from reality when I need it the most. To my loving nieces; I love you two as my own and want to thank you for making me laugh and loving me so much. And in memory of my Dad; I know he would be so proud of me today. You are all such a huge support system for me. I love you all and thank you so much for everything you have done and continue to do for me.

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ABSTRACT

The etiology of colon cancer is a complex phenomenon that involves both genetic and environmental factors. However, only about 20% have a familial basis with the largest fraction being attributed to environmental causes that can lead to chronic inflammation. Tumors associated macrophages drive the pro-inflammatory response in the tumor micro-environment and are associated with poor prognosis in certain cancers. Monocyte chemoattractant protein 1 (MCP-1) is thought to be the most important chemokine for recruitment of macrophages to the tumor microenvironment. In chapter 1, we examined the timing and magnitude of the intestinal inflammatory cytokine response in relation to tumorigenesis in the $Apc^{Min/+}$ mouse. Then, in chapter 2, we examined the role of MCP-1 on tumor associated macrophages, inflammation, and intestinal tumorigenesis in $Apc^{Min/+}$ mice. In chapter 3, we examined the effects of exercise on markers associated with macrophages and select T cell populations in $Apc^{Min/+}$ mice and related this to polyp characteristics. The results in chapter 1 show that the increase in polyp burden with age is positively correlated with the increase in intestinal inflammatory cytokine expression (mRNA and protein) of MCP-1, IL-1β, IL-6 and TNF-α, with MCP-1 showing the highest association. Similarly, circulating MCP-1 was increased at 12 wks ($P<0.05$) and then again at 20 wks ($P<0.05$). MCP-1 deficiency decreased overall polyp number by 20% and specifically large polyp number by 45% ($P<0.05$). In chapter 2, we show that MCP-1 deficiency decreased F4/80 positive cells in both the polyp tissue and surrounding intestinal tissue ($P<0.05$) as well as expression of

markers associated with M1 (IL-12 & IL-23) and M2 macrophages (IL-13, CD206, TGFβ & CCL17) ($P<0.05$). MCP-1 knockout was also associated with increased CTLs and decreased Tregs ($P<0.05$). In chapter 3, we show that while there was no significant difference in overall polyp number between the groups (Sed: 23.3 ± 4.3 and Ex: 16.5 ± 4.3), Ex did have a reduction in the number of large polyps (Sed:6.1 ± 1.1 and Ex: 3.0 ± 0.6) ($P<0.05$). Similarly, Ex reduced mRNA expression of overall macrophages (F4/80) as well as markers associated with both M1 (IL-12) and M2 (CD206, CCL22, & Arg-1) subtypes ($P<0.05$). CD8 expression was increased while Foxp3 expression was decreased with Ex ($P<0.05$). We demonstrate that MCP-1 is an important mediator of tumor growth and immune regulation that may serve as an important biomarker and/or therapeutic target in colon cancer. This data also provides important new information on immune regulation as a possible mechanism for the documented benefits of exercise training on reducing colon cancer progression.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Colon cancer is the third most commonly diagnosed cancer among men and women in the United States (cancer.org). It is estimated that over 98,000 new cases will be diagnosed in 2014, causing over 50,000 deaths. The risk of developing colon cancer is 5% for men and women combined, and many of the risk factors are environmental and thus preventable. These preventable risk factors include physical inactivity, obesity, smoking, and heavy alcohol use (cancer.org). These environmental factors cause chronic inflammation driving the promotion, invasion, and metastasis of colon cancer. Inflammation involves the interaction between multiple factors to promote colon cancer progression. Cytokines, chemokines, and immune cells will be discussed, as well as a possible mechanism for the beneficial effects of physical activity on colon cancer progression.

The link between inflammation and cancer has been well established. Inflammation has been linked to every step involved in the development and progression of colon cancer, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (1-5). Hanahan and Weinberg have named inflammation as the 7th hallmark of cancer (6). The inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), largely produced by macrophages, have all been associated with poor outcome in colon cancer (1-4). In fact, we have previously shown that IL-6 overexpression using electron gene transfer

techniques can increase polyp burden in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis (7). Similarly monocyte chemoattractant protein 1 (MCP-1), a major chemokine for macrophage recruitment, has been associated with increased grade of the tumor in certain cancers (8, 9). While the link between inflammatory cytokines and colon cancer is well recognized, the timing and magnitude of this response in relation to tumorigenesis has not been described.

Previously it was thought that infiltration of immune cells into the tumor microenvironment would have antitumor effects but in recent years, it has been shown that these tumor-associated immune cells actually enhance the neoplastic capabilities of cells within the tumor microenvironment (6, 10-13). Multiple immune cell types are found within tumors which may be tumor-inhibiting or tumor-promoting. For example, cytotoxic T lymphocytes (CTLs) are tumor-inhibiting and patients with a higher percentage of these cells in the tumor have a better prognosis (6). On the other hand, patients with a higher percentage of macrophages in general have a worse prognosis (14-18). Therefore, identifying strategies to alter the responses of tumor-associated immune cells may have important implications for the progression of colon cancer.

MCP-1, also known as chemokine (C-C motif) ligand 2 (CCL2), has been identified as an essential chemokine for monocyte trafficking (19-21). Using targeted gene disruption Lu et. al (1998) created an MCP-1 deficient mouse and documented that MCP-1 is uniquely essential for monocyte recruitment in several inflammatory models (20). More recently in cancer models, MCP-1 has been suggested to be the most important chemokine for macrophage recruitment to the tumor microenvironment (22); both rodent and clinical studies have associated MCP-1 levels with tumor-associated

macrophage (TAM) abundance and subsequent cancer progression (23-25). For example, we have shown that MCP-1 is correlated with abundance of large polyps in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis. Similarly, Bailey et al. has reported that MCP-1 expression is associated with TAM number and stage of colorectal cancer in humans (24). Further, in a model of colitis-associated colon cancer it has been reported that mice deficient for an MCP-1 specific receptor have less macrophage infiltration, reduced COX-2 expression, and decreased tumor size (25). However, the specific role of MCP-1 on macrophage number and phenotype, inflammatory processes, and tumor progression in a genetic mouse model of colon cancer has yet to be determined.

Physical inactivity has been reported to account for 10% of all colon cancer cases (26), whereas physical activity has been associated with reduced risk for incidence of colon cancer. This inverse relationship between physical activity and colon cancer risk is supported by epidemiological studies as well as controlled experimental studies using rodent models. For example, nine weeks of treadmill running has been reported to decrease the total number of intestinal polyps as well as the number of large polyps in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis (27). Similarly a recent study in a multiethnic colon cancer screening population reported that exercising for one hour per week was associated with a lower prevalence of polyps and adenomas when compared to those who exercised less or not at all (28).

The mechanisms responsible for a preventative effect of exercise on colon cancer risk are complex and multifaceted. An exercise-induced alteration in immune system function is one possible mechanism that has not been widely explored. Exercise has been reported to influence macrophage behavior in various disease models. For instance, it

has been shown that exercise can reduce macrophage-mediated inflammation in an obese mouse model (29). Similarly, macrophage infiltration associated with the reversal of arterial inflammation was decreased by exercise training in aged mice (30). However, while it is well established that macrophages play a significant role in the pathogenesis of colon cancer and that exercise can influence the macrophage response, there is very little evidence on the benefits of exercise on macrophage behavior in the presence of colon cancer.

T cells have been shown to be altered by exercise (31, 32). It is well recognized that cytotoxic T lymphocytes (CTLs) constitute one of the most important effector mechanisms of anti-tumor immunity (33, 34). For example, adoptive transfer of CD8+ cells controls the growth of B16 melanoma in mice (35). On the other hand, regulatory T cells (Tregs) have been associated with accelerated tumor growth and immune evasion through their inhibitory actions on CTLs and helper T cells (36, 37). The T cell response to exercise has been reported to be highly variable and is likely dependent on the mode, intensity and duration of exercise. However, to date there is no evidence on the effects of exercise on T cells in a mouse model of colon cancer.

The $Apc^{Min/+}$ mouse model has been the most widely used genetically engineered mouse model for cancer studies that involve the gastro-intestinal tract (38, 39). It was the first mouse model to be generated by mutation of the ademonatous polyposis coli (Apc) gene through random chemical carcinogenesis (40). This gene is similarly mutated in patients with familial adenomatous polyposis (41). It has been shown to be responsive to treatment with anti-inflammatory agents, including both anti-inflammatory dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) (38, 39).

The purpose of the first study was to examine the temporal sequence and magnitude of the inflammatory cytokine response in relation to tumorigenesis in the *Apc*$^{Min/+}$ mouse to lead to the development of inflammatory mediator biomarkers to assess progression of this disease, and further, allow for the determination of appropriate timing of effective treatments to target inflammatory processes in this mouse model. The results from this study showed that gene expression of MCP-1 was highly correlated with large polyp number. In the second study, our aim was to examine the role of MCP-1 in colorectal cancer in the *Apc*$^{Min/+}$ mouse. The MCP-1 knockout mouse was crossed with the *Apc*$^{Min/+}$ mouse to generate a mouse model of intestinal tumorigenesis deficient for MCP-1. With this mouse model we were able to determine the role of MCP-1 on macrophage recruitment and phenotype in the tumor microenvironment. We show that MCP-1 deficiency in our model of intestinal tumorigenesis leads to a decrease in the total number of polyps as well as the number of large polyps, confirming the association between MCP-1 and large polyp number seen previously. The purpose of the third study was to determine if the effects of exercise on the immune response could be a mechanism for the beneficial effects of exercise on tumorigenesis in this model. We show that overall, certain markers associated with both the M1 and the M2 macrophage phenotype were reduced in *Apc*$^{Min/+}$ mice following exercise. Additionally, exercise resulted in an increased expression of CD8 and decreased expression of Foxp3, markers for CTLs and Tregs, respectively. These alterations in immune cell parameters following exercise training were accompanied by a decrease in the percentage of large polyps.

CHAPTER 1

TIMING OF INTESTINAL INFLAMMATION IN RELATION TO TUMORIGENESIS IN THE $APC^{MIN/+}$ MOUSE[1]

[1] McClellan, J.L.,Davis, J.M., Steiner, J.L., Day, S.D., Steck, S.E., Carmichael, M.D., Murphy, E.A. *Intestinal inflammatory cytokine response in relation to tumorigenesis in the Apc(Min/+) mouse.* Cytokine. **57**(1): p. 113-9. Reprinted here with permission from publisher.

ABSTRACT

The etiology of colon cancer is a complex phenomenon that involves both genetic and environmental factors. However, only about 20% have a familial basis with the largest fraction being attributed to environmental causes that can lead to chronic inflammation. While the link between inflammation and colon cancer is well established, the temporal sequence of the inflammatory response in relation to tumorigenesis has not been characterized. We examined the timing and magnitude of the intestinal inflammatory cytokine response in relation to tumorigenesis in the $Apc^{Min/+}$ mouse. $Apc^{Min/+}$ mice and wildtype mice were sacrificed at one of 4 time-points: 8, 12, 16, and 20 wks of age. Intestinal tissue was analyzed for polyp burden (sections 1, 4 and 5) and mRNA expression and protein concentration of MCP-1, IL-1β, IL-6 and TNF-α (sections 2 and 3). The results show that polyp burden was increased at 12, 16 and 20 wks compared to 8 wks ($P<0.05$). Gene expression (mRNA) of MCP-1, IL-1β, IL-6 and TNF-α was increased in sections 2 and 3 starting at wk 12 ($P<0.05$), with further increases in MCP-1, IL-1β and IL-6 at 16 wks ($P<0.05$). Protein concentration for these cytokines followed a similar pattern in section 3. Similarly, circulating MCP-1 was increased at 12 wks ($P<0.05$) and then again at 20 wks ($P<0.05$). In general, overall polyp number and abundance of large polyps were significantly correlated with the inflammatory cytokine response providing further support for a relationship between polyp progression and these markers. These data confirm the association between intestinal cytokines and tumorigenesis in the $Apc^{Min/+}$ mouse and provide new information on the timing and magnitude of this response in relation to polyp development. These findings may lead to the development of inflammatory mediators as

important biomarkers for colon cancer progression. Further, these data may be relevant in the design of future investigations of therapeutic interventions to effectively target inflammatory processes in rodent models.

INTRODUCTION

Colon cancer is a significant global health concern; despite advances in detection, surgery and chemopreventive treatment it remains the third most common malignancy and the fourth most common cause of cancer mortality worldwide (42-44). The etiology of colon cancer is a complex phenomenon that involves contribution from both genetic and environmental factors. However, only about 20% of colon cancer cases can be attributed to genetic factors (45) with the vast majority of cases being ascribed to environmental causes that can lead to chronic inflammation. For example, inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's colitis, all of which display chronic inflammation of the gastrointestinal mucosa, are associated with increased risk for the development of colon cancer (46, 47).

The link between inflammation and colon cancer is well established; inflammation has been linked to every step involved in the development and progression of colon cancer, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (1-5). Furthermore, chronic inflammation has been associated with "sickness behaviors" including circadian disruptions, anorexia, cachexia, fatigue, and decreased physical activity all of which can lead to a decreased quality of life, as well as poorer prognosis and survival in cancer patients (48-50). The inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), largely produced by macrophages, have all been associated with poor

outcome in colon cancer (1-4). In fact, we have previously shown that IL-6 overexpression using electron gene transfer techniques can increase polyp burden in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis (7). Similarly monocyte chemoattractant protein 1 (MCP-1), a major chemokine for macrophage recruitment, has been associated with increased grade of the tumor in certain cancers (8, 9). While the link between inflammatory cytokines and colon cancer is well recognized, the timing and magnitude of this response in relation to tumorigenesis has not been described.

The $Apc^{Min/+}$ mouse model has been the most widely used genetically engineered mouse model for cancer studies that involve the gastro-intestinal tract (38, 39). It was the first mouse model to be generated by mutation of the ademonatous polyposis coli (Apc) gene through random chemical carcinogenesis (40). This gene is similarly mutated in patients with familial adenomatous polyposis (41). It has been shown to be responsive to treatment with anti-inflammatory agents, including both anti-inflammatory dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) (38, 39). Despite this, intestinal inflammation has not been characterized in this model; to our knowledge there are no studies that have examined the relationship between inflammatory cytokines and tumor burden in the $Apc^{Min/+}$ mouse. The purpose of this study was to examine the temporal sequence and magnitude of the inflammatory cytokine response in relation to tumorigenesis in the $Apc^{Min/+}$ mouse. This may lead to the development of inflammatory mediators as important biomarkers to assess progression of this disease, and further, allow for the determination of appropriate timing of effective treatments to target inflammatory processes in mouse models.

MATERIALS AND METHODS

2.1 Animals. *Apc*$^{Min/+}$ male mice on a C57BL/6 background (Jackson Laboratories) were purchased and bred with female C57BL/6 mice in the University of South Carolina's Center for Colon Cancer Research (CCCR). Offspring were genotyped as heterozygotes by RT-PCR for the *Apc* gene by taking tail snips at weaning. The primer sequences were sense: 5'-TGAGAAAGACAGAAGTTA-3'; and antisense: 5'-TTCCACTTTGGCATAAGGC-3'. Female *Apc*$^{Min/+}$ offspring were randomly assigned to one of four different timepoints: 8, 12, 16 or 20 wks of age (n=8-12/group). Wildtype C57BL/6 mice were used as age matched controls (n=6-11/group). Mice were maintained on a 12:12 h light-dark cycle in a low-stress environment (22^{o}C, 50% humidity and low noise) and provided food and water ad libitum. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2 Tissue collection. Mice were sacrificed at their respective group age (8, 12, 16 or 20 wks) for tissue collection using isoflurane overdose. All mice were sacrificed in the mornings between 9:00 and 11:00am. The small intestine was carefully dissected distally to the stomach and proximal to the cecum. The large intestine (section 5) was removed from the distal end of the cecum to the anus. Mesentery tissue was removed with tweezers, and the small intestine was cut into four equal sections (sections 1-4). All intestinal sections were flushed with PBS, opened longitudinally, and flattened with a cotton swab. Sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) for 24 h. Sections 2 and 3 were divided into 2 equal parts and mucosal scrapings were performed in iscoves

medium (Invitrogen, Carlsbad, CA) (containing 5% fetal bovine serum and a cocktail enzyme inhibitor (10 mM EDTA, 5 mM benzamidine HCl, and 0.2 mM phenylmethyl sulfonyl fluoride)) and TRIzol reagent (Invitrogen, Carlsbad, CA) for protein and gene expression analysis, respectively. Samples were stored at –80°C until analysis of inflammatory mediators. Blood was collected from the inferior vena cava using a heparinized syringe and spun in a microcentrifuge at 4,000rpm for 15 min. Plasma was then stored at –80^{o}C until assayed for MCP-1.

2.3 Polyp counts. Formalin-fixed intestinal sections from all animals were rinsed in deionized water, briefly stained in 0.1% methylene blue, and counted by the same investigator who was blinded to the treatments. Polyps were counted under a dissecting microscope, using tweezers to pick through the intestinal villi and identify polyps. Polyps were categorized by size (>2 mm, 1–2 mm, and <1 mm).

2.4 Expression of inflammatory markers. Procedures for RNA isolation from mucosal scrapings were performed as previously described (51, 52)**.** Briefly, mucosal tissue was homogenized under liquid nitrogen with a polytron, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The extracted RNA (2.5 µL of sample) was dissolved in DEPC-treated water and quantified spectrophotometrically at 260-nm wavelength. RNA quality was assessed on an Agylent 2100 BioAnalyzer. RNA was reverse transcribed into cDNA in a 50 µL reaction volume containing 19.25 µL RNA (1.5 µg) in Rnase-free water, 5 µL 10X RT Buffer, 11 µL 25 mM $MgCl_2$, 10 µL deoxyNTPs mixture, 2.5 µL random hexamers, 1 µL Rnase inhibitor, and 1.25 µL multiscribe reverse transcriptase (50U/µL). Reverse transcription was performed at 25^{o}C for 10 min, 37^{o}C for 60 min, and 95^{o}C for 5 min, followed by quick chilling on ice and storage at -20^{o}C

until subsequent amplification. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis was done per manufacturer's instructions (Applied Biosystems, Foster City, CA) using TaqMan® Gene Expression Assays (IL-1β, IL-6, TNF-α and MCP-1). DNA amplification was carried out in 12.5 µL Taqman Universal PCR Master Mix (AmpliTaq Gold DNA Polymerase, Passive Reference 1, Buffer, dNTPs, AmpErase UNG), 1 µL cDNA, 9 µL Rnase-free water, and 1.25 µL 18S primer (VIC) and 1.25 µL primer (FAM) (for endogenous reference and target gene) in a final volume of 25 µL/well. Samples were loaded in a MicroAmp 96-well reaction plate. Plates were run using Applied Biosystems Sequence Detection System. After 2 min at 50^{o}C and 10 min at 95^{o}C, samples were coamplified by 50 repeated cycles of which one cycle consisted of 15 s denaturing step at 95^{o}C and 1 min annealing/extending step at 60^{o}C. Data were analyzed using Applied Biosystems software using the CT, cycle threshold, which is the value calculated and based on the time (measured by PCR cycle number) at which the reporter fluorescent emission increases beyond a threshold level (based on the background fluorescence of the system), and it reflects the cycle number at which the cDNA amplification is first detected. All samples were run in duplicate. Quantification of cytokine gene expression for IL-1β, IL-6, TNF-α and MCP-1 were calculated using the delta CT method as described by Livak and Schmittgen (2001) (53). Briefly, delta CT (CT(FAM) – CT(VIC)) is calculated for each sample and control. Delta delta CT (delta CT(control) – delta CT(sample)) is then calculated for each sample and relative quantification is calculated as 2 delta delta CT. Initial exclusion criteria consist of FAM CT ≥40 and VIC CT ≥23. Multiple plates were used for each marker, however an equal number of samples from each time-point were run on the same plate and all plates

were run under the same conditions. The intra assay variability for all gene expression assays was < 1%.

2.5 Concentration of inflammatory markers. Mucosal tissue was homogenized using a polytron and samples were centrifuged at 10,000 rpm at 4^{o}C for 10min, and the supernatants removed and stored at 4^{o}C prior to the assay of IL-1β, IL-6, TNF-α and MCP-1 via ELISA (R&D Systems, Minneapolis, MN). The assay was performed according to the manufacturer's instructions. Total soluble protein was also determined using supernatant of homogenized samples via bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL.). Cytokine levels are expressed as a ρg per 100 μg total protein. Plasma levels of MCP-1 were also measured using ELISA techniques (R&D Systems, Minneapolis MN). As described above, multiple plates were used for each inflammatory marker with an equal number of samples from each time-point on the same plate. Intra assay and inter assay variability were < 3% and <5%, respectively for all proteins via ELISA.

2.6 Statistical Analysis. Polyp data was analyzed using a one-way ANOVA (Time) with Student Newman-Keuls post-hoc analysis. All inflammatory mediator data were analyzed using a two-way ANOVA (Time X Strain) with Student Newman-Keuls post-hoc analysis where appropriate. The correlation coefficients between polyp number and inflammatory mediator levels were determined by Spearman correlation analyses. Analysis was done using commercial software (SigmaStat, SPSS, Chicago, IL). Statistical significance was set with an alpha value of $p<0.05$. Data are presented as mean (±SEM).

RESULTS

3.1 Polyp incidence. Polyps were counted on formalin-fixed, methylene blue-stained intestinal sections. Overall polyp number was increased at 12 (60.3 ±6.3), 16 (63.4 ±7.6) and 20 wks (47 ± 4.4) versus 8 wks (26.4 ± 3.2) ($P<0.05$) (Figure 1.1A) and colon polyps at 20 wks (1.4 ± 0.4) were greater than 8 wks (0.28 ± 0.18), but this did not reach statistical significance ($P<0.1$) (Figure 1.1B). There were also differences in the polyp size distribution over time (Figure 1.1C) that were consistent with disease development; 8 and 12 wk old mice showed the greatest number of small polyps ($P<0.05$), 16 wk old mice had the greatest distribution of medium polyps ($P<0.05$), and large polyps were most abundant at 16 and 20 wks ($P<0.05$).

3.2 Tissue expression of inflammatory markers. Gene expression of inflammatory markers (MCP-1, IL-1β, IL-6 & TNF-α) were examined in regions 2 and 3 of the intestines of $Apc^{Min/+}$ over time (Figure 1.2). Regions 2 and 3 were chosen so as to represent a section of the intestine with low and high polyp incidence, respectively. All data was normalized to fold-increase from age-matched wildtype mice.

MCP-1: MCP-1 was elevated at 16 and 20 wks in both sections 2 and 3 (~ 20-35 fold) ($P<0.05$) and there was further elevation at 20 wks in section 2 only ($P<0.05$) (Figure 1.2A).

IL-1β: In both sections 2 and 3, there was an increase in IL-1β at 12, 16 and 20 wks (~ 4-10 fold) ($P<0.05$) and an additional increase was observed at 16 and 20 wks ($P<0.05$) (Figure 1.2B).

IL-6: IL-6 was significantly elevated at 16 and 20 wks in section 2 (~ 15-30 fold) ($P<0.05$), and at 12, 16 and 20 wks in section 3 (~ 10-30 fold) ($P<0.05$). The greatest magnitude of IL-6 expression was observed at 16 wks in both sections ($P<0.05$) (Figure 1.2C).

TNF-α: In sections 2 and 3, TNF-α expression was increased at 12, 16 and 20 wks (~ 4-6 fold) ($P<0.05$) (Figure 1.2D).

3.3 Tissue concentration of inflammatory markers. The concentration of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and MCP-1were examined in regions 2 and 3 of the intestines of $Apc^{Min/+}$ at 8, 12, 16 and 20 wks of age (Figure 1.3). The data was normalized to fold-increase from age-matched wildtype mice.

MCP-1: In section 2, MCP-1 was increased at 16 and 20 wks (~ 3-4 fold) ($P<0.05$) and in section 3 at 12, 16 and 20 wks (~ 5-7 fold) ($P<0.05$) and there was further elevation at 20 wks ($P<0.05$) in both sections (Figure 1.3A).

IL-1β: In section 2, IL-1β was increased at 20 wks (~ 1.5 fold) ($P<0.05$), and in section 3 at 12, 16 and 20 wks (~ 2 fold) ($P<0.05$) (Figure 1.3B).

IL-6: There were no differences in protein concentration of IL-6 across age in section 2. However, in section 3, IL-6 concentration was increased at 12, 16 and 20 wks (~ 2-3 fold) ($P<0.05$) (Figure 1.3C).

TNF-α: Similar to IL-6 there were no differences in protein concentration of TNF-α across age in section 2. In section 3, there was an increase in TNF-α only at 12 wks (~ 4 fold) ($P<0.05$) (Figure 1.3D).

3.4 Plasma MCP-1. Plasma concentration of MCP-1, a measure of systemic inflammation, was also measured in *Apc*$^{Min/+}$ mice and wildtype mice over time (Figure 1.4). MCP-1 was significantly increased in *Apc*$^{Min/+}$ at 12, 16 and 20 wks ($P<0.05$) and there was an additional increase at 20 wks ($P<0.05$).

3.5 Correlations. In order to further address the relationship between polyp progression (overall polyp number & abundance of large polyps) and inflammatory mediators we performed correlation analyses. Mice from all time-points were included in these analyses. Figure 1.5 shows the relationship between large polyp number and mRNA expression of MCP-1, IL-1β, IL-6 and TNF-α in section 3 of the intestines. All four inflammatory mediators were positively associated with the number of large polyps ($P<0.01$); MCP-1 showed the strongest correlation ($R^2=0.721$; Figure 1.5A) followed by IL-1β ($R^2=0.659$; Figure 1.5B), IL-6 ($R^2=0.566$; Figure 1.5C) and TNF-α ($R^2=0.398$; Figure 1.5D). This was generally consistent with the relationship observed between these mediators and the abundance of large polyps in section 2 ($P<0.001$) and overall polyp number in sections 2 and 3 ($P<0.001$) (data not shown). Similarly, there were positive correlations for protein concentration of pro-inflammatory mediators and polyp progression ($P<0.05$) (data not shown) although this relationship was not as strong as that observed for gene expression.

DISCUSSION

Epidemiological evidence links colon cancer to chronic intestinal inflammation (54). Further support for a role of inflammation in colon cancer comes from studies using mouse models that have been shown to be responsive to treatment with anti-

inflammatory agents, including both anti-inflammatory dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) (39). Despite this, the timing and magnitude of the inflammatory cytokine response in relation to tumorigenesis has not been characterized. This study used an established mouse model of intestinal tumorigenesis to examine this response in the $Apc^{Min/+}$ mouse. Our findings indicate that an elevation in the intestinal inflammatory cytokine (MCP-1, IL-1β, IL-6 and TNF-α) response occurs at12 wks of age in association with the rapid increase in polyp number. Further elevations in these mediators with age were associated with increased polyp size. In fact, all inflammatory mediators were positively correlated with the abundance of large polyps as well as overall polyp number (data not shown). These data also generally show a greater increase in the cytokine response in intestinal section 3, a region of high polyp incidence, compared to section 2 that is considered a region of relatively low polyp incidence (55). These findings contribute to the growing evidence on the association between inflammatory cytokines and colon cancer and provide important new data on the magnitude and timing of this response in relation to tumorigenesis in the $Apc^{Min/+}$ mouse.

The $Apc^{Min/+}$ mouse model has been the most widely used genetically engineered model for cancer studies that involve the gastro-intestinal tract. In fact over 600 articles have been published using this model. It has been shown to be responsive to treatment with anti-inflammatory agents, including both anti-inflammatory dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) (39). For example, Greenspan et al. reported that the NSAID Sulindac can decrease intestinal polyp number in the $Apc^{Min/+}$ mouse that is associated with a decrease in proliferative and inflammatory prostaglandins in the small intestine and colon (56). Similarly, another study reported

that Celecoxib reduced the formation of intestinal polyps that was associated with lower levels of COX-2 activity (57). And we have shown that the anti-inflammatory dietary component curcumin can offset intestinal inflammatory cytokines and tumorigenesis in this mouse model (52); a decrease in mRNA expression of IL-1β, IL-6, TNF-α and MCP-1 as well as protein concentration of IL-1β and MCP-1 was observed in the intestines following 14 wks of curcumin treatment. Another study reported that the dietary component Silibinin can decrease intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse in association with a decreased expression of TNF-α, IL-1β but not MCP-1. However, the timing and magnitude of the intestinal pro-inflammatory response in relation to tumor progression in this model has not been characterized. This is surprising given the preponderance of studies that have used this model to test anti-inflammatory agents (39). Most studies have focused on the effects of various anti-inflammatory treatments over the life-span of these mice without consideration for the temporal sequence of inflammation in relation to cancer. To our knowledge there is only one study that has examined any inflammatory mediators in the *Apc*$^{Min/+}$ mouse over time. Kettunen et al., (2003) examined the intestinal immune response in *Apc*$^{Min/+}$ mice at 5, 8 and 15 wks of age and reported an increase in prostaglandin E_2 (PGE_2) at 15 wks but not TNF-α or IL-12 (58). However, MCP-1, IL-6 and IL-1β were not measured in this study and there were no attempts made to measure gene expression of any inflammatory mediators.

We show here for the first time the timing and magnitude of the inflammatory cytokine response in intestinal tissue across time in the *Apc*$^{Min/+}$ mouse. In addition to the inflammatory cytokines that have well documented effects on tumor progression (1-5), MCP-1 was examined as it has been identified as a major chemokine for macrophage

recruitment in several human tumors including the colon (59, 60) and has been associated with increased inflammation and tumor grade in various cancers (8, 9). Our data show an increased mRNA expression of MCP-1, IL-1β, IL-6 and TNF-α that is evident at 12 wks of age and is consistent with the increase in polyp number that occurs at this time; total polyp number (sections 1, 4 and 5) increases from 26 ± 3 at 8 wks to 60 ± 6 at 12 wks. Any further increases in the inflammatory response appear to be associated with a change in polyp size; 12 wk old $Apc^{Min/+}$ mice had the largest number of small polyps (<1mm) whereas 20 wk old mice had the greatest number of large polyps (>2mm). In fact, a positive correlation was observed between the abundance of large polyps and all inflammatory mediators. Similarly, protein concentration of these cytokines generally follows the same trend. It is worth noting that section 3, a section of high polyp incidence shows a greater magnitude of inflammation than section 2, a section of low polyp incidence (55) that provides further evidence for the association between inflammation and tumor burden in this model. Whether the changes in polyp number and size that occurs in this model is a result of the elevated inflammatory response or vice versa cannot be determined from this investigation. Future studies are necessary to identify whether inflammation is a cause and/or effect of the changes in polyp number and size across time in this mouse model.

These findings may have important implications for colon cancer. Firstly, these results may contribute to the future development of biomarkers to assess colon cancer progression. The current literature supports a positive relationship between systemic IL-6 and increasing tumor stages and tumor size, metastasis and decreased survival (61) in colon cancer. Similarly, MCP-1 has been correlated with increased grade of the tumor

(9) advanced tumor stage, lymph node involvement (62) and poor prognosis in breast cancer (8, 60), but no studies of this nature have been carried out with colon cancer. However, our data shows a strong relationship between polyp progression and MCP-1 in a mouse model of colon cancer. Future studies are necessary to fully evaluate the potential of these inflammatory cytokines as prognostic indicators for colon cancer. Secondly, these data provide important new information that can be used for the determination of appropriate timing of effective treatments that can be used in the design of future investigations that target inflammatory processes in mouse models of colon cancer. Based on these data it is likely that anti-inflammatory treatments would be more effective as a preventive approach (i.e administered before 12 wks) to reduce polyp number and an intervention approach (i.e administered after 12 wks) to reduce polyp size in this model.

In summary, the *Apc*$^{Min/+}$ mouse is the most widely used model in studies that examine the effect of anti-inflammatory treatments including bioactive food components and NSAIDs on intestinal tumorigenesis. Despite this there is very little information on the temporal sequence of inflammation in relation to tumorigenesis in this model. This is the first systematic investigation of the timing and magnitude of the inflammatory cytokine (IL-1β, IL-6 and TNF-α) and MCP-1 response in the *Apc*$^{Min/+}$ mouse. These data contribute to the growing evidence on the association between inflammation and colon cancer and provide important new data that could be used in the development of biomarkers as well as in the design of future investigations of anti-inflammatory treatments.

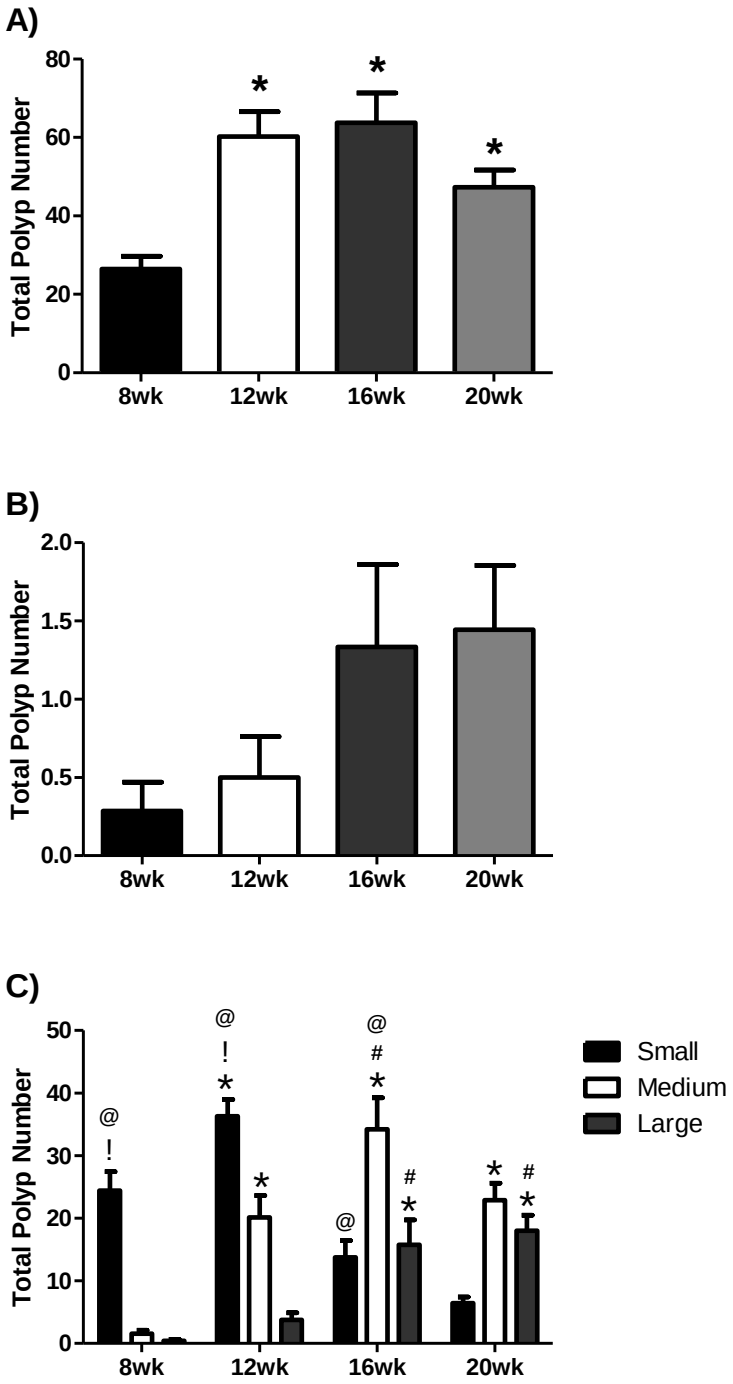

Figure 1.1. Polyp number in the *Apc*$^{Min/+}$ mouse at 8, 12, 16 and 20wks of age. Differences in (A) total polyp number (B) colon polyp number and (C) polyp size distribution were observed over time (n=6-12/group). P<0.05 * greater than 8 wks, # greater than 12 wks, ! greater than 16wks and @ greater than 20 wks.

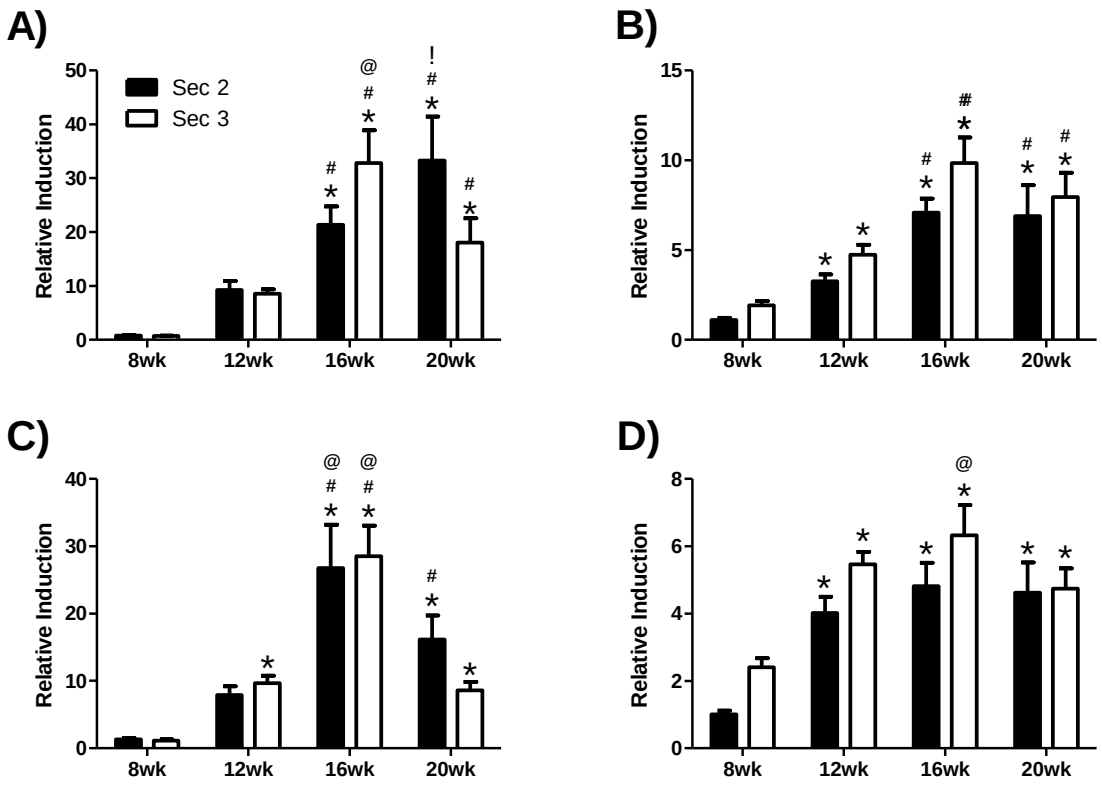

Figure 1.2. mRNA gene expression of inflammatory mediators in the *Apc*$^{Min/+}$ mouse at 8, 12, 16 and 20wks of age. Differences in mRNA gene expression of (A) MCP-1, (B) IL-1β, (C) IL-6, and (D) TNF-α were observed in sections 2 and 3 of the intestines over time (n=6-12/group). $P<0.05$ * greater than 8 wks, # greater than 12 wks, ! greater than 16wks and @ greater than 20 wks.

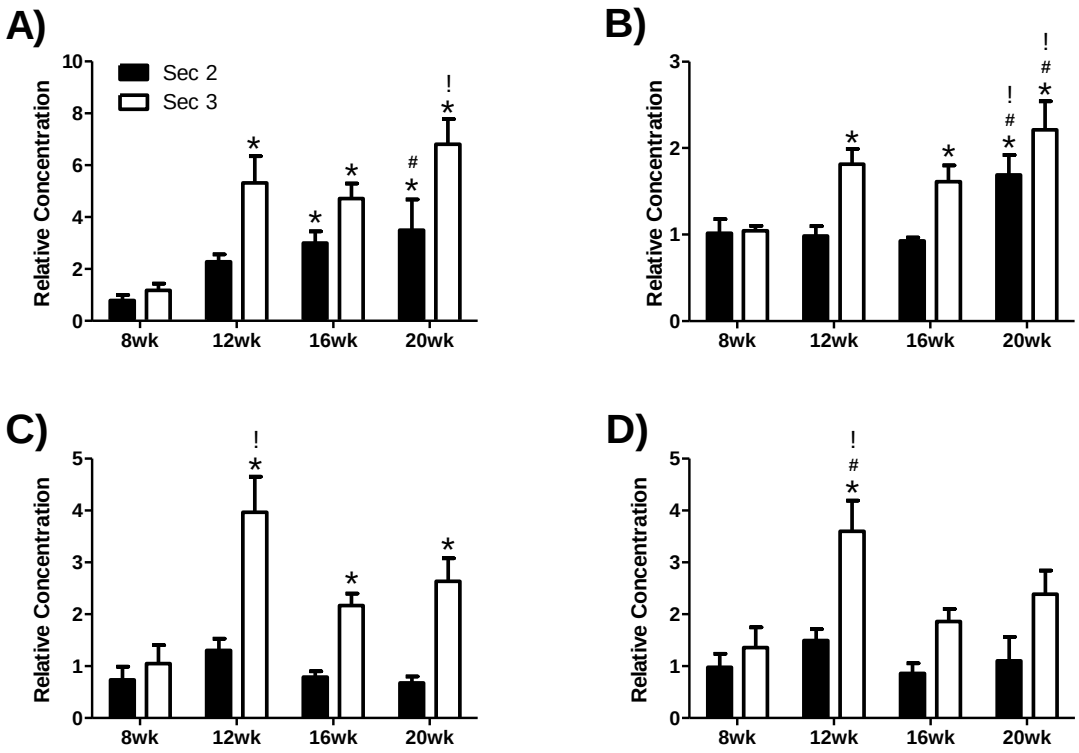

Figure 1.3. Protein concentration of inflammatory mediators in the *Apc*$^{Min/+}$ mouse at 8, 12, 16 and 20wks of age. Differences in protein concentration of (A) MCP-1, (B) IL-1β, (C) IL-6, and (D) TNF-α were observed in sections 2 and 3 of the intestines over time (n=6-12/group). P<0.05 * greater than 8 wks, # greater than 12 wks, ! greater than 16wks and @ greater than 20 wks.

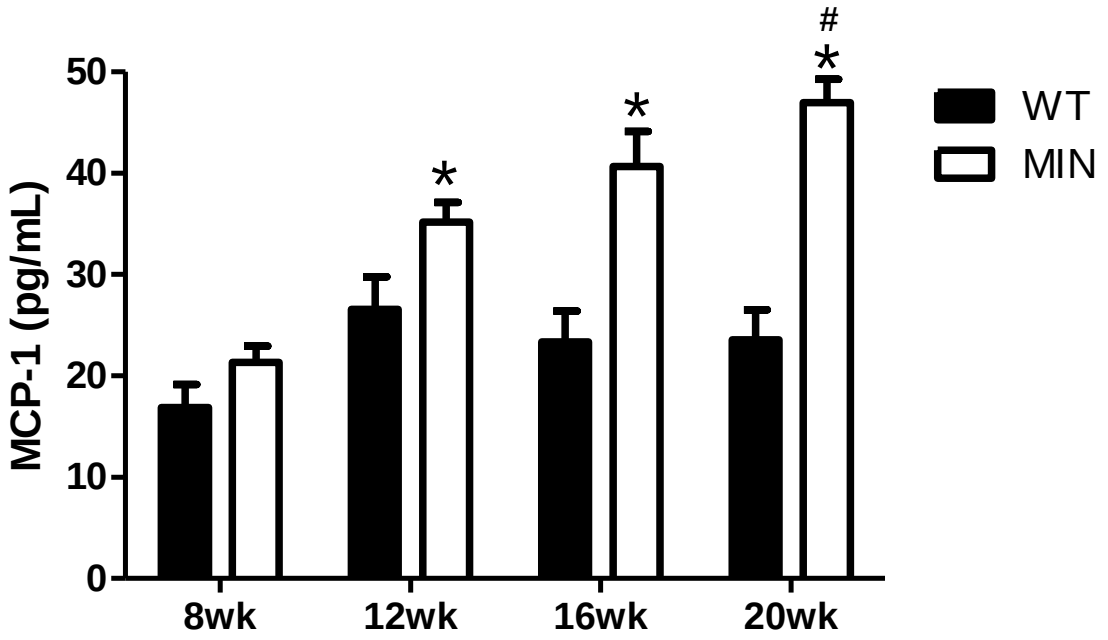

Figure 1.4. Plasma MCP-1 is increased in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis. Wildtype and Apc$^{Min/+}$ mice were sacrificed at various time-points (8, 12, 16 or 20 wks) and plasma was analyzed for MCP-1 concentration using ELISA. * MCP-1 was increased at 12, 16 and 20 wk versus WT ($P<0.05$). $P<0.05$ * greater than 8 wks and # greater than 12 wks.

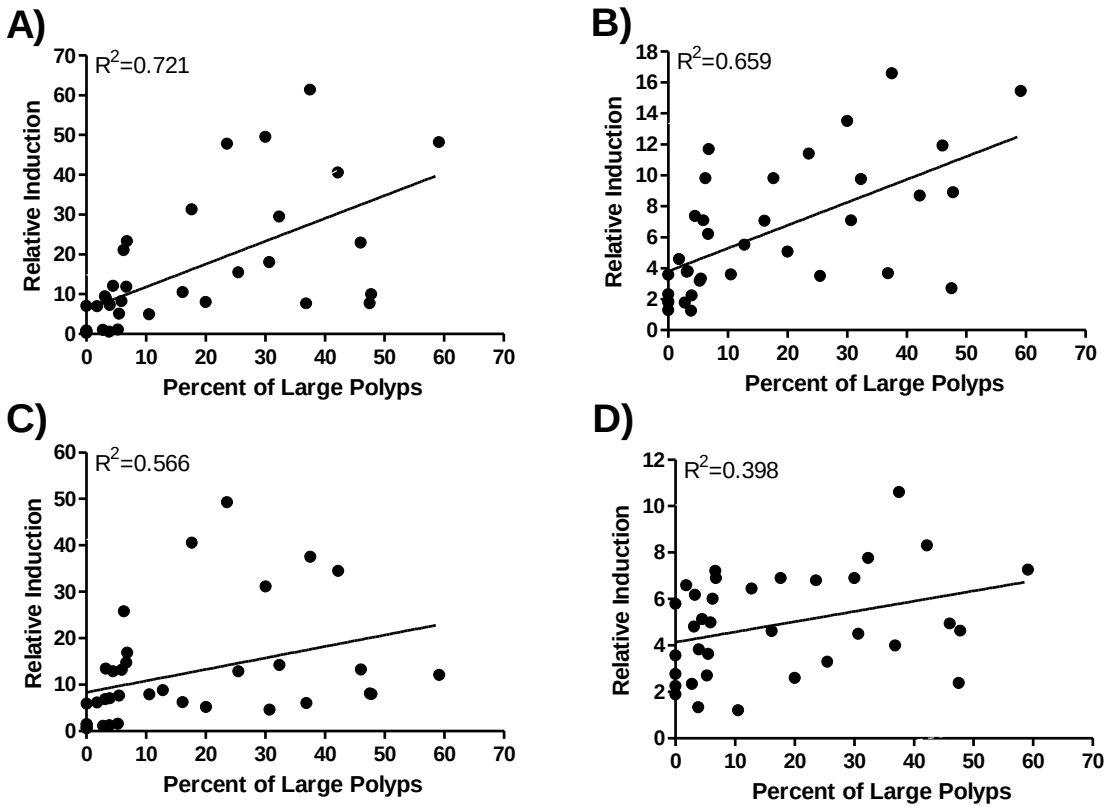

Figure 1.5. Inflammatory mediators are positively correlated with polyp progression in the *Apc*$^{Min/+}$ mouse. Correlations were performed between abundance of large polyps and mRNA expression of MCP-1 (A), IL-1β (B), IL-6 (C) and TNF-α (D) in section 3 of the intestines. All inflammatory mediators showed a positive relationship with abundance of large polyps ($P<0.01$).

CHAPTER 2

LINKING TUMOR ASSOCIATED MACROPHAGES, INFLAMMATION, AND INTESTINAL TUMORIGENESIS: ROLE OF MCP-1[2]

[2] McClellan, J.L.,Davis, J.M., Steiner, J.L., Enos, R.T., Jung, S.H., Carson, J.A., Pena, M.M., Carnevale, K.A., Berger, F.G., Murphy, E.A. *Linking tumor-associated macrophages, inflammation, and intestinal tumorigenesis: role of MCP-1.* Am J Physiol Gastrointest Liver Physiol. **303**(10): p. G1087-95. Reprinted here with permission from publisher.

ABSTRACT

Tumor associated macrophages are associated with poor prognosis in certain cancers. Monocyte chemoattractant protein 1 (MCP-1) is thought to be the most important chemokine for recruitment of macrophages to the tumor microenvironment. However, its role on tumorigenesis in a genetic mouse model of colon cancer has not been explored. We examined the role of MCP-1 on tumor associated macrophages, inflammation, and intestinal tumorigenesis. Male $Apc^{Min/+}$, $Apc^{Min/+}$/MCP-$1^{-/-}$ or wildtype mice were sacrificed at 18 wks of age and intestines were analyzed for polyp burden, apoptosis, proliferation, β-catenin, macrophage number & phenotype, markers for cytotoxic T lymphocytes (CTLs) and regulatory T cells (Tregs), and inflammatory mediators. MCP-1 deficiency decreased overall polyp number by 20% and specifically large polyp number by 45% ($P<0.05$). This was consistent with an increase in apoptotic cells ($P<0.05$), but there was no change detected in proliferation or β-catenin. MCP-1 deficiency decreased F4/80 positive cells in both the polyp tissue and surrounding intestinal tissue ($P<0.05$) as well as expression of markers associated with M1 (IL-12 & IL-23) and M2 macrophages (IL-13, CD206, TGFβ & CCL17) ($P<0.05$). MCP-1 knockout was also associated with increased CTLs and decreased Tregs ($P<0.05$). In addition, MCP-$1^{-/-}$ offset the increased mRNA expression of IL-1β & IL-6 in intestinal tissue and IL-1β & TNF-α in polyp tissue ($P<0.05$), and prevented the decrease in SOCS1 expression ($P<0.05$). We demonstrate that MCP-1 is an important mediator of tumorgrowth and immune regulation that may serve as an important biomarker and/or therapeutic target in colon cancer.

INTRODUCTION

There is a well-established association between inflammation and cancer risk. In fact, inflammation has been suggested to represent the 7th hallmark of cancer (63). It has been linked to every step involved in the development and progression of colon cancer, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (1, 4). Macrophages have recently emerged as major players in the connection between inflammation and cancer (14, 15); they represent up to 50% of the tumor mass and produce a wide array of inflammatory mediators with pro-tumoral functions (14, 15). Further, they have been associated with alterations in adaptive immune responses within the tumor microenvironment that may lead to reduced immune surveillance (14, 64). In general, high levels of tumor associated macrophages (TAMs) are associated with poor prognosis in colon cancer (14-18). Therefore, identifying strategies to alter the responses of TAMs may have important implications for the progression of colon cancer.

Monocyte chemoattractant protein 1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), has been identified as an essential chemokine for monocyte trafficking (19-21). Using targeted gene disruption Lu et. Al (1998) created an MCP-1 deficient mouse and documented that MCP-1 is uniquely essential for monocyte recruitment in several inflammatory models (20). More recently in cancer models, MCP-1 has been suggested to be the most important chemokine for macrophage recruitment to the tumor microenvironment (22); both rodent and clinical studies have associated MCP-1 levels with TAM abundance and subsequent cancer progression (23-25). For example, we have shown that MCP-1 is correlated with abundance of large polyps in the *Apc*$^{Min/+}$

mouse model of intestinal tumorigenesis (65). Similarly, Bailey et al. has reported that MCP-1 expression is associated with TAM number and stage of colorectal cancer in humans (24). Further, in a model of colitis-associated colon cancer it has been reported that mice deficient for an MCP-1 specific receptor have less macrophage infiltration, reduced COX-2 expression, and decreased tumor size (25). However, the specific role of MCP-1 on macrophage number and phenotype, inflammatory processes, and tumor progression in a genetic mouse model of colon cancer has yet to be determined.

The purpose of this study was to examine the role of MCP-1 on macrophage number and phenotype (M1 & M2), markers for certain T cell subsets including cytotoxic T lymphocytes (CTLs) and regulatory T cells (Tregs), and selected inflammatory mediators (IL-1β, IL-6 TNF-α & SOCS1) in the intestines and the tumor microenvironment in a genetic mouse model of intestinal tumorigenesis, and further, to relate these to tumor progression (polyp number and size). This was done by crossing the $Apc^{Min/+}$ mouse, the most widely used genetic mouse model for cancer studies that involve the gastro-intestinal tract (38, 39), with an MCP-1 knockout mouse to develop an MCP-1 deficient model of intestinal tumorigenesis. $Apc^{Min/+}$ mice are heterozygotes for a mutation in the adenomatous polyposis coli (*Apc*) gene and spontaneously develop intestinal and colon adenomas. Since the *Apc* gene is mutated in a large percentage of human colon cancer cases, this is a common model for studying factors that may influence progression of colon cancer. We hypothesized that MCP-1 deficiency would reduce macrophage number in the tumor environment and that this would be associated with a decrease in inflammation, and a reduction in polyp burden.

In this report, we demonstrate that MCP-1 plays a necessary role in macrophage recruitment, immune regulation, inflammation, and polyp burden in colon cancer. MCP-1 deficiency reduced macrophage number, altered markers of CTLs and Tregs, and prevented the increased mRNA expression of inflammatory cytokines in the tumor microenvironment that was associated with a reduction in overall polyp number and an increase in apoptosis.

MATERIALS AND METHODS

2.1 Animals. *Apc*$^{Min/+}$ and MCP-1$^{-/-}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME). All experimental mice (*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/MCP-1$^{-/-}$) were bred in the University of South Carolina's Center for Colon Cancer Research (CCCR) Mouse Core Facility. Specifically, *Apc*$^{Min/+}$ male mice were bred with female C57BL/6 mice to generate *Apc*$^{Min/+}$ mice. Offspring were genotyped as heterozygotes by RT-PCR for the *Apc* gene by taking tail snips at weaning. The primer sequences were sense: 5'-TGAGAAAGACAGAAGTTA-3'; and antisense: 5'-TTCCACTTTGGCATAAGGC-3'. The *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mouse was generated by introducing MCP-1$^{-/-}$ into the *Apc*$^{Min/+}$ model. This colony was maintained by breeding male *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mice with female MCP-1$^{-/-}$ mice. Offspring were genotyped for the mutant *Apc* allele as described above and for MCP-1. The primer sequences for MCP-1 were as follows: mutant GCCAGAGGCCACTTGTGTAG, wild type forward TGACAGTCCCCAGAGTCACA and common TCATTGGGATCATCTTGCTG. Male *Apc*$^{Min/+}$, *Apc*$^{Min/+}$/MCP-1$^{-/-}$ and wild type (n=10/group) mice were maintained on a 12:12h light-dark cycle in a low-stress environment (22^{o}C, 50% humidity and low noise)

and provided food and water ad libitum. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2 Tissue Collection. Body weight was measured prior to sacrifice (18 wks of age). Mice were sacrificed for tissue collection as previously described (65). Sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) for 24 h. For intestinal section 3, polyps were excised and mucosal scrapings were performed in TRIzol reagent (Invitrogen, Carlsbad, CA). Samples were stored at –80°C until analysis for macrophage phenotype, T cell markers, and inflammatory mediators. Previously reported findings from our laboratory has shown that intestinal section 3, a region of high polyp incidence, has a larger inflammatory response than section 2, an area of low polyp occurrence (65), and therefore we included only section 3 for these analyses. Blood was collected from the inferior vena cava using a heparinized syringe. Blood parameters were examined immediately using a Vetscan blood analyzer (Abaxis, Union City, CA). The remaining blood was spun in a microcentrifuge at 4,000rpm for 15 min. Plasma was then stored at –80^{o}C until assayed for IL-6 (R&D Systems, Minneapolis MN).

2.3 Polyp Counts. Formalin-fixed intestinal sections from all animals were rinsed in deionized water, briefly stained in 0.1% methylene blue, and counted by the same investigator who was blinded to the treatments. Polyps were counted under a dissecting microscope and were categorized according to size (>2 mm, 1–2 mm, and <1 mm).

2.4 Immunohistochemistry. Intestinal sections were processed to identify apoptotic positive cells, proliferating cells, β-catenin and macrophage number. Formalin-fixed, paraffin-embedded intestinal sections were Swiss-rolled and cut on a microtome in 4-µm

sections. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay as per the manufacturer's instructions (Apo-Tag kit, Millipore, Billerica, MA). For proliferation, slides were stained using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Antigens were unmasked using proteinase K (Millipore, Billerica, MA) and peroxidase activity was inhibited using BLOXALL (Vector Laboratories, Burlingame, CA) for 10 min. Sections were incubated with rabbit polyclonal PCNA antibody (1:200; Abcam, UK) for 1 h at RT. For β-catenin and macrophage staining the HRP-DAB Cell and Tissue staining kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Sections were incubated with rat monoclonal F4/80 (1:50; Serotec, Raleigh, NC) and rabbit polyclonal β-catenin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 2 h at RT. Color detection was visualized by exposing sections to 3,3´-diaminobenzidine (DAB). TUNEL positive cells, PCNA positive cells and β-catenin were visualized using the DAKO Chromavision Systems ACIS 3 system (66). The threshold for positive staining was chosen by the user for automated scoring. Polyps were outlined for qualitative analysis and the percentage of each polyp that met the positive staining threshold was averaged for each sample. Data was then normalized to fold change from $Apc^{Min/+}$ mice. To quantify macrophages, F4/80 positive cells within each polyp (positive cells/μm^2) and villi (10 villi/mouse) were counted (40X magnification) and averaged for each animal (67-69).

2.5 mRNA Gene Expression. Quantification of gene expression for macrophage phenotype (IL-12 & IL-23 (M1 macrophage phenotype), IL-13, CD206, TGF-β & CCL17 (M2 macrophage phenotype)), T cell makers (CD8 & Foxp3), and inflammatory

mediators (IL-1β, IL-6, TNF-α, SOCS1 (suppressor of cytokine signaling 1)) were performed as previously described (65, 70). Briefly, mucosal tissue and polyps were homogenized under liquid nitrogen with a polytron, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA). RNA was reverse transcribed into cDNA and quantitative RT-PCR analysis was done per manufacturer's instructions (Applied Biosystems, Foster City CA) using TaqMan® Gene Expression Assays. Quantification of cytokine gene expression was calculated using the ΔΔCT method.

2.6 Statistical Analysis. Analysis was done using commercially available software (SigmaStat, SPSS, Chicago, IL). For comparisons between *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/MCP-1$^{-/-}$ (polyp burden, apoptosis, proliferation, β-catenin, macrophage number, and gene expression in the polyps) a t-test was performed. When the wild type group was included in the analysis (body weight, gene expression in the mucosal tissue, and blood parameters) a one-way ANOVA with Student Newman-Keuls post-hoc analysis was performed. Statistical significance was set with an alpha value of $P<0.05$. Data are presented as mean (±SEM).

RESULTS

3.1 Body Weight. Previous published data from our group has shown that *Apc*$^{Min/+}$ mice develop cachexia that is positively correlated with polyp burden (7). As expected *Apc*$^{Min/+}$ mice show a decrease in body weight compared to wild type mice (22.2 ± 0.4 versus 26.7 ± 0.5) ($P<0.05$) and MCP-1 deficiency offsets this effect; *Apc*$^{Min/+}$/MCP-1$^{-/-}$ had an average body weight of 25.6 ± 0.6 that was not different from wild type mice.

3.2 Polyp incidence. At 18 wks of age, mice (*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/MCP-1$^{-/-}$) were sacrificed, intestinal tissue was harvested and polyps were counted on formalin-fixed,

methylene blue-stained sections. Overall polyp number (sections 1, 4 and 5) was decreased by 20% in *Apc*$^{Min/+}$/MCP-1$^{-/-}$ (42.8 ± 3.0 versus 54.2 ± 2.5) (P<0.05) (Figure 2.1A). To examine polyp size (Figure 2.1B), we classified polyps as being large (> 1mm in diameter), medium (<2 > 1 mm in diameter) or small (< 1 mm in diameter). Interestingly, we found that the difference in overall polyps between the groups can be largely attributed to a reduction in the number of large polyps; *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mice had 45% fewer large polyps than *Apc*$^{Min/+}$mice (16.4 ± 5.0 versus 29.9 ± 1.4) (P<0.05) but there were no significant differences in the number of small or medium polyps. We also examined changes across location (Figure 2.1C) and found that polyp number was decreased in *Apc*$^{Min/+}$/MCP-1$^{-/-}$ by ~40% in section 1 (3.3 ± 0.5 versus 5.6 ± 0.5) (P<0.05), ~15% in section 4 (37.7 ± 2.7 versus 45.1 ± 2.6), and ~45% in section 5 (colon) (1.8 ± 0.4 versus 3.4 ± 0.6) (P<0.05).

3.3 Apoptosis. Apoptosis, or programmed cell death, is typically evaded by tumors and leads to uncontrolled tumor growth (71). TUNEL staining was used to localize apoptotic cells within polyps (Figure 2.2A). This analysis was done only in section 4, a region of high polyp incidence, in order to examine the largest number of polyps possible. MCP-1 deficiency increased the number of TUNEL-positive cells (P<0.05); after normalizing the *Apc*$^{Min/+}$ group to 1.0 we found a 3.3 fold increase in the percentage of the polyp area staining positive for TUNEL in *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mice (P<0.05).

3.4 Proliferation. Sustaining proliferative signaling is one of the hallmarks of cancer. PCNA staining was done in section 4 to quantify proliferating cells (Figure 2.2B). There were no differences detected in the percentage of the polyp area staining positive for PCNA (1.0 ± 0.2 versus 1.2 ± 0.1 for *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mice, respectively).

3.5 β-catenin. Immunohistochemical staining for β-catenin, a central molecule of the Wnt-signaling pathway that is involved in cellular replication and which has been correlated with poor prognosis in colon cancer was also performed (67-69). This analysis was also done in section 4 to allow for examination of the largest number of polyps possible. We did not detect a difference in the percentage of polyp area staining positive for β-catenin (1.0 ± 0.1 versus 1.0 ± 0.1 for $Apc^{Min/+}$ and $Apc^{Min/+}$/MCP-$1^{-/-}$ mice, respectively) (Figure 2.2C).

3.6 Macrophage Number & Phenotypic Markers. Immunohistochemistry using F4/80 antibody was used to identify macrophages in section 4 of the intestine. F4/80-positive cells were counted within the villus (Figure 2.2D) (10 villi were counted per mouse) as well as the polyps (Figure 2.2E) (positive cells/μm^2) and averaged per animal. We found a significant decrease (~25%) in the average number of intestinal villi macrophages in $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (23.7 ± 2.6 versus 31 ± 2.5) ($P<0.05$). Similarly, F4/80 positive cells were decreased (~40%) in the polyps of $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (53.6 ± 8.1 versus 92.2 ± 7.4) ($P<0.05$). Further, gene expression of macrophage phenotypic markers (IL-12 & IL-23 (M1 macrophage phenotype), IL-13, CD206, TGF-β and CCL17 (M2 macrophage phenotype)) were examined in the mucosal scrapings (Figure 2.3C) of intestinal tissue and in the polyps (Figure 2.3D). For mucosal scrapings, data was normalized to fold-change from age-matched wild type mice and for polyps, to fold-change from $Apc^{Min/+}$ mice. In the mucosal tissue, there was an increase in mRNA expression of IL-23, IL-13, CD206 and CCL17 in the $Apc^{Min/+}$ mouse ($P<0.05$) and MCP-1 deficiency blunted this response ($P<0.05$). Similarly, in the polyps, we detected a

significant decrease in mRNA expression of IL-12, IL-23, IL-13, CD206, TGF-β and CCL17 in $Apc^{Min/+}$/MCP-$1^{-/-}$ versus $Apc^{Min/+}$ mice (P<0.05).

3.7 T cell Markers. We also performed gene expression analysis of markers associated with CTLs (CD8) and Tregs (Foxp3) (Figure 2.4). In the mucosal tissue (Figure 2.4A), there was no difference in mRNA gene expression of CD8 across the groups. There was however, a significant increase in Foxp3 in the $Apc^{Min/+}$ mouse and this effect was blunted in the $Apc^{Min/+}$/MCP-$1^{-/-}$ group. For the polyps (Figure 2.4B), we found a significant increase in mRNA expression of CD8 in the $Apc^{Min/+}$/MCP-$1^{-/-}$ group (P<0.05). And similar to the mucosal data, there was a decrease in Foxp3 expression in the polyps of $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (P<0.05).

3.8 Inflammatory Modifiers. Gene expression of inflammatory cytokines (IL-1β, IL-6 & TNF-α) and SOCS1 was examined in the mucosal scrapings of intestinal tissue as well as the polyps (Figure 2.5). In the mucosal tissue (Figure 2.5A), IL-1β and IL-6 were increased in $Apc^{Min/+}$ mice (P<0.05) and this effect was blunted for IL-6 and completely blocked for IL-1β when MCP-1 was depleted (P<0.05). There were no differences in TNF-α across the groups. Consistent with the inflammatory cytokine data, SOCS1 mRNA expression was decreased in $Apc^{Min/+}$ mice (P<0.05) but there was no difference in SOCS1 expression between wild type mice and $Apc^{Min/+}$/MCP-$1^{-/-}$ mice. In the polyps (Figure 2.5B), there was a decrease in mRNA expression of IL-1β and TNF-α in $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (P<0.05) but there were no significant differences detected for IL-6. Similar to the mucosal tissue data, SOCS1 was decreased in polyps of $Apc^{Min/+}$ mice compared to $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (P<0.05).

3.9 Complete Blood Count & Plasma IL-6. A complete blood count was performed as both white blood cell (WBC) and red blood cell (RBC) counts have been shown to be altered during progression of intestinal tumorigenesis in this mouse model (Table 2.1) (72). We found that overall WBC counts were increased in $Apc^{Min/+}$ mice (13.6m/mm^3 ± 1.8) compared to wild type (5.7m/mm^3 ± 0.6) ($P<0.05$) and MCP-1 deficiency blunted this response (7.1m/mm^3 ±0.9) ($P<0.05$). In general, these findings were consistent across all cell types (lymphocytes, monocytes & granulocytes). For RBCs, $Apc^{Min/+}$ mice showed an overall large decrease (2.6m/mm^3 ± 0.2) versus wild type mice (7.9m/mm^3 ± 0.2) ($P<0.05$) and MCP-1 deficiency offset this effect (3.9m/mm^3 ± 0.2) ($P<0.05$). Similar results were seen for hematocrit and hemoglobin. We also examined circulating levels of IL-6 in plasma as a marker of systemic inflammation. Consistent with previous findings from our laboratory (7), we confirm an increase in plasma IL-6 in $Apc^{Min/+}$ mice (40.0 ± 6.3 ρg/mL) compared to wild type mice (0.8 ± 0.8 ρg/mL) ($P<0.05$), and report that this effect was blunted in the MCP-1 deficient $Apc^{Min/+}$ mice (19.3 ± 1.9 ρg/mL) ($P<0.05$).

DISCUSSION

Macrophages play a key role in tumorigenesis; they are a major player in the inflammatory response that contributes to cellular transformation, promotion, apoptosis, proliferation, invasion, angiogenesis, and metastasis (1, 4, 14, 15, 73, 74). MCP-1 has been identified as the most important chemokine for recruitment of macrophages to the tumor microenvironment (22). However, the link between MCP-1, macrophages, inflammation, and tumorigenesis in colon cancer has not yet been established. Through the use of the $Apc^{Min/+}$/MCP-1$^{-/-}$ mouse bred in our laboratory, we report the novel finding

that MCP-1 is a link between macrophages, inflammation, and tumorigenesis in colon cancer. MCP-1 deficiency decreased overall polyp number and abundance of large polyps that was consistent with an increase in apoptosis. Overall macrophage number and markers associated with both the M1 and the M2 phenotype were reduced in *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mice. Further, MCP-1$^{-/-}$ mice had increased expression of CD8 and decreased expression of Foxp3 in the tumor microenvironment, markers for CTLs and Tregs, respectively. MCP-1 deficiency also reduced the expression of inflammatory cytokines in the intestinal tissue and polyps as well as circulating levels of IL-6.

Both rodent and clinical studies have associated MCP-1 levels with tumorigenesis (23-25). However, there have been relatively few studies that have specifically linked MCP-1 to colon cancer progression. Here we show that *Apc*$^{Min/+}$ mice deficient for MCP-1 have a reduction in overall intestinal polyp number (~20%) as well as the number of large polyps (~45%). We interpret this to mean that MCP-1 can affect both development and growth of polyps. However, it appears as if it plays a larger role in progression of growth as opposed to initiation of development. While the current investigation was limited to one sacrifice time-point (18 wks), given that *Apc*$^{Min/+}$ mice develop most of their polyps by 12 wks of age, it is likely that an even larger disparity in polyp growth, but not number, would be observed if mice had been sacrificed at a later time-point. These findings are consistent with a recent report from our laboratory showing that MCP-1 is correlated with the number of large polyps in this same mouse model (65). And similarly, with a recent rodent study that shows a decrease in the size of tumors in an MCP-1 receptor deficient mouse model of colitis-associated colon cancer (25). Further, it has been reported that ablation of D6, a promiscuous decoy receptor that

scavenges inflammatory chemokines, increases colitis and results in more severe malignancies in colitis-associated cancer (75). Our findings are also confirmed by the only available clinical study reporting an association between MCP-1 expression and stage of colorectal cancer (24).

In addition to decreased polyp number, MCP-1 deficiency increased apoptosis suggesting that MCP-1 knockout may lead to prevention of the dysregulation of apoptosis in colon cancer. While there have been no other reported studies that have examined the role of MCP-1 on apoptosis in colon cancer, our findings are supported by recent *in vitro* data in other cancer models. For example, knock down of MCP-1 has been shown to enhance tumor cell apoptosis in mammary carcinoma cells (76) and MCP-1 inhibited apoptosis in PC-3M prostate colon cancer cells (77). However, we did not detect an effect of MCP-$1^{-/-}$ on the number of proliferating cells. This is inconsistent with a recent report showing a decrease in proliferation in both a breast cancer and prostate cancer model following pharmacological inhibition of MCP-1(78). It is possible that the effects of MCP-1 on proliferation may be dependent on the specific cancer model used; to our knowledge there are no reports of an effect of MCP-1 on proliferation in colon cancer. Similarly, there was no change in β-catenin with MCP-1 knockout. In contrast, a recent study reported less nuclear β-catenin accumulation in an MCP-1 receptor deficient mouse model of chronic colitis (25). However, a different mouse model was used in that investigation, which may explain, at least in part, the variability in findings across studies.

In addition to the polyp characteristics, we also examined the role of MCP-1 on macrophage number in the tumor environment and surrounding intestinal tissue. Recent

data has shown that MCP-1 has been associated with infiltration of macrophages to the tumor microenvironment (22, 23). These macrophages can represent up to 50% of the tumor mass producing a wide array of inflammatory mediators with pro-tumoral functions (14, 15). Further, abundance of TAMs has been associated with poor prognosis in colon cancer (14-18, 24). For example, one study reported a reduction in macrophage infiltration that was consistent with a decrease in the size of colon polyps in an MCP-1 receptor deficient mouse model of colitis-associated colon cancer (25). Further, in a clinical study it was documented that macrophage accumulation within the tumor was correlated with stage of progression (24). Consistent with these reports, our MCP-1 deficient $Apc^{Min/+}$ mice have decreased macrophage number in both the polyps and surrounding intestinal tissue. Because it is now well accepted that macrophages constitute an extremely heterogeneous population that is divided into two main classes (M1 and M2) (14), we also examined the role of MCP-1 on expression of M1 and M2 phenotypic markers. In general, it is thought that M1 macrophages are cytotoxic against neoplastic cells, whereas M2 macrophages exert pro-tumoral functions (14). We report the novel finding that MCP-1 deficiency reduces the expression of both M1 (IL-12 and IL-23) and M2 (IL-13, CD206, TGF-β and CCL17) macrophage phenotypic markers in the tumor environment as well as surrounding intestinal tissue in the $Apc^{Min/+}$ mouse model of colon cancer. While our data suggests that a reduction in both M1 and M2 macrophages is associated with better prognosis in colon cancer, a greater understanding of the roles of these macrophage subsets within the tumor microenvironment is necessary.

It is well recognized that CTLs constitute one of the most important effector mechanisms of anti-tumor immunity (33, 34). For example, adoptive transfer of CD8+ cells has been show to control the growth of B16 melanoma in mice (35). On the other hand, Tregs have been associated with accelerated tumor growth and immune evasion through their inhibitory actions on CTLs and helper T cells (36, 37). To date, there is very little evidence on the relationship between MCP-1, macrophages and T cell responses in colon cancer. We examined the role of MCP-1 on markers associated with CTLs (CD8) and Tregs (Foxp3). Our findings illustrate a regulatory role of MCP-1 on expression of CD8 and Foxp3 in colon cancer; MCP-1 deficiency increased expression of CD8 in the polyps and decreased expression of Foxp3 in the mucosal tissue and polyps. Our results are consistent with previously reported data showing that blockade of MCP-1 can result in activation of CTLs in a mouse model of lung cancer and a reduction in Tregs in TC1 tumor-bearing mice (79, 80). These findings should be substantiated using fluorescence activated cell sorting methodology to firmly establish a role of MCP-1 on immune regulation in the tumor microenvironment.

The inflammatory cytokines IL-1β, IL-6, and TNF-α have been associated with poor outcome in colon cancer (1, 4). It has been proposed that the mutual interaction of macrophages with cancer cells enhances production of inflammatory cytokines that transform the tumor microenvironment so that it favors the survival, growth and motility of cancer cells (3, 21, 81). Our study in $Apc^{Min/+}$/MCP-$1^{-/-}$ mice is the first to report that a reduction in macrophage number corresponds to a reduction in the inflammatory cytokine response in the tumor environment as well as circulating IL-6 in a mouse model of colon cancer. We also examined expression of SOCS1, an important negative

physiological regulator of cytokine responses. A recent study reported spontaneous colorectal carcinoma development and nuclear β-catenin accumulation in SOCS1 deficient mice suggesting its role as an anti-oncogene that prevents inflammation-mediated carcinogenesis (82). Our data show a reduction in SOCS1 expression in the polyps and surrounding intestinal tissue in *Apc*$^{Min/+}$ mice that is eliminated when MCP-1 is knocked down. This finding suggests that MCP-1 plays a role in dysregulation of cytokine signaling through silencing of SOCS1. It is important to point out that we did not distinguish between macrophage-mediated inflammation and inflammation facilitated by the tumor cells themselves; it is likely that MCP-1 regulates inflammation in both cell types (83). It is also important to note that only mRNA gene expression of these inflammatory markers was measured in this study. However, a recent study from our laboratory shows that mRNA expression of several of these same inflammatory mediators is mirrored by elevations in protein concentrations (65).

Changes in both WBC and RBC counts are characteristic in colon cancer and have been show to occur in the *Apc*$^{Min/+}$ mouse (72). We therefore also performed a complete blood count to determine the role of MCP-1 on these parameters in the *Apc*$^{Min/+}$ mouse. In general, our data show that MCP-1 deficiency prevents the overall increase in WBCs as well as the increase in specific subsets including lymphocytes, monocytes and granulocytes. Similarly, the decrease in RBCs, hematocrit and hemoglobin that occurs in the *Apc*$^{Min/+}$ mouse is offset when MCP-1$^{-/-}$ is knocked out.

In summary, we report that MCP-1 is an important regulator of macrophages, T cells, and inflammatory responses in the tumor microenvironment that can lead to increased polyp burden in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis. Using

an *Apc*$^{Min/+}$/MCP-1$^{-/-}$ mouse that we developed in our laboratory, we show a decrease in overall polyp number and large polyp abundance that was consistent with a reduction in macrophage number, an alteration in T cell markers that are associated with improved immune surveillance, and a decrease in inflammatory processes in the polyp tissue and surrounding intestinal tissue as compared to MCP-1 sufficient *Apc*$^{Min/+}$ mice. Given the important regulatory role of MCP-1 in these facets of cancer, development of effective pharmacological or antibody approaches to inhibit MCP-1 may have important implications for the prevention and/or treatment of colon cancer.

Table 2.1. Circulating levels of white blood cells, red blood cells, and IL-6 are altered in *Apc*$^{Min/+}$ mice deficient for MCP-1. Values are means ± SEM. Data are representative of three experimental blocks. * significantly different from wild type (P<0.05), # significantly different from *Apc*$^{Min/+}$ (P<0.05), ** trend to be different from wild type (P<0.1), and ## trend to be different from *Apc*$^{Min/+}$ (P<0.1).

	WT	MIN	MIN/MCP
WBC (m/mm3)	5.7 ± 0.58	13.6 ± 1.88 *	7.1 ± 0.89 #
Lym (m/mm3)	4.4 ± 0.43	11.3 ± 1.61 *	5.9 ± 0.75 #
Mon (m/mm3)	0.20 ± 0.01	0.25 ± 0.02	0.16 ± 0.02 #
Gra (m/mm3)	1.2 ± 0.16	1.7 ± 0.24 **	0.94 ± 0.10 #
RBC (m/mm3)	7.9 ± 0.16	2.6 ± 0.22 *	3.9 ± 0.18 * #
Hct (%)	34.9 ± 1.17	14.6 ± 0.84 *	20.0 ± 1.2 * #
Hb (g/dL)	12.2 ± 0.25	3.9 ± 0.33 *	6.4 ± 0.33 * #
Plasma IL-6 (pg/mL)	0.78 ± 0.8	40.01 ± 6.3 *	19.25 ± 1.9 * #

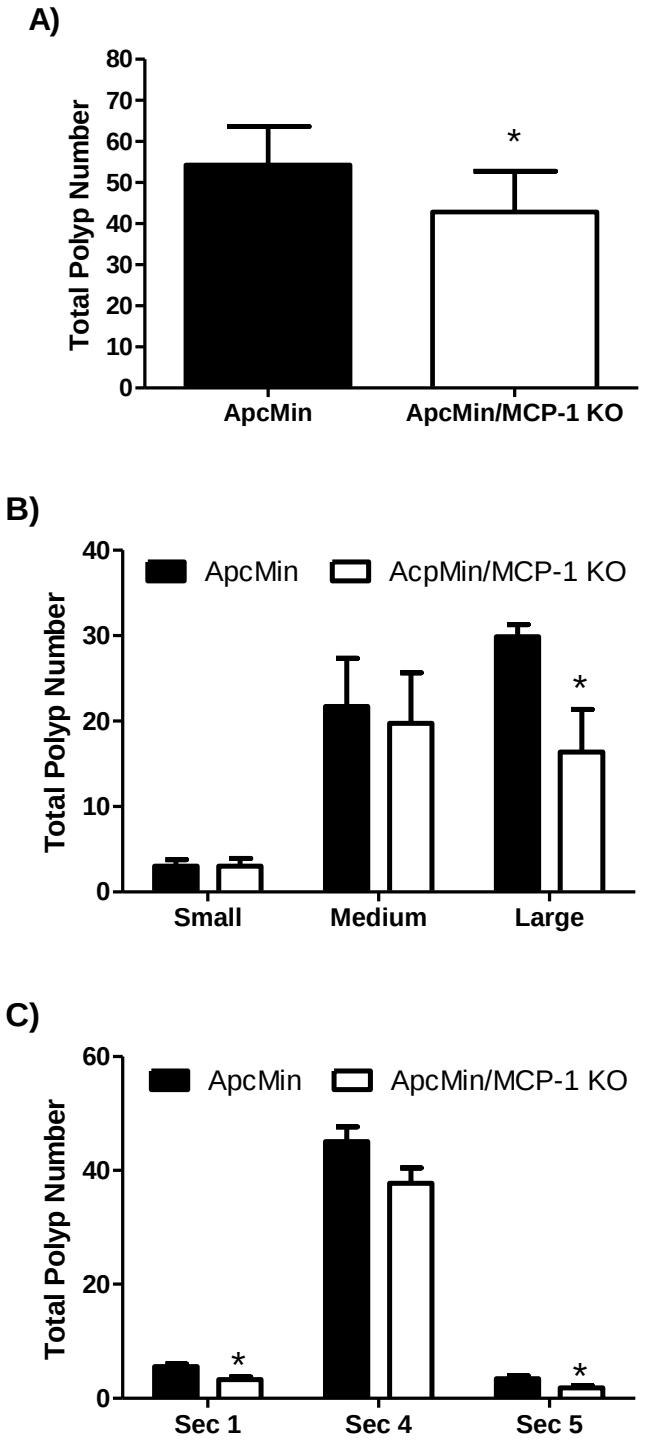

Figure 2.1. MCP-1 deficiency reduces tumorigenesis in the *Apc*$^{Min/+}$ mouse. Differences in total polyp number (A), polyp size (B), and polyp distribution (C) were examined in *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$/MCP-1$^{-/-}$ (n=10/group) mice at 18 wks of age. Values are means ± SEM. Data are representative of three experimental blocks. * significantly different (P<0.05).

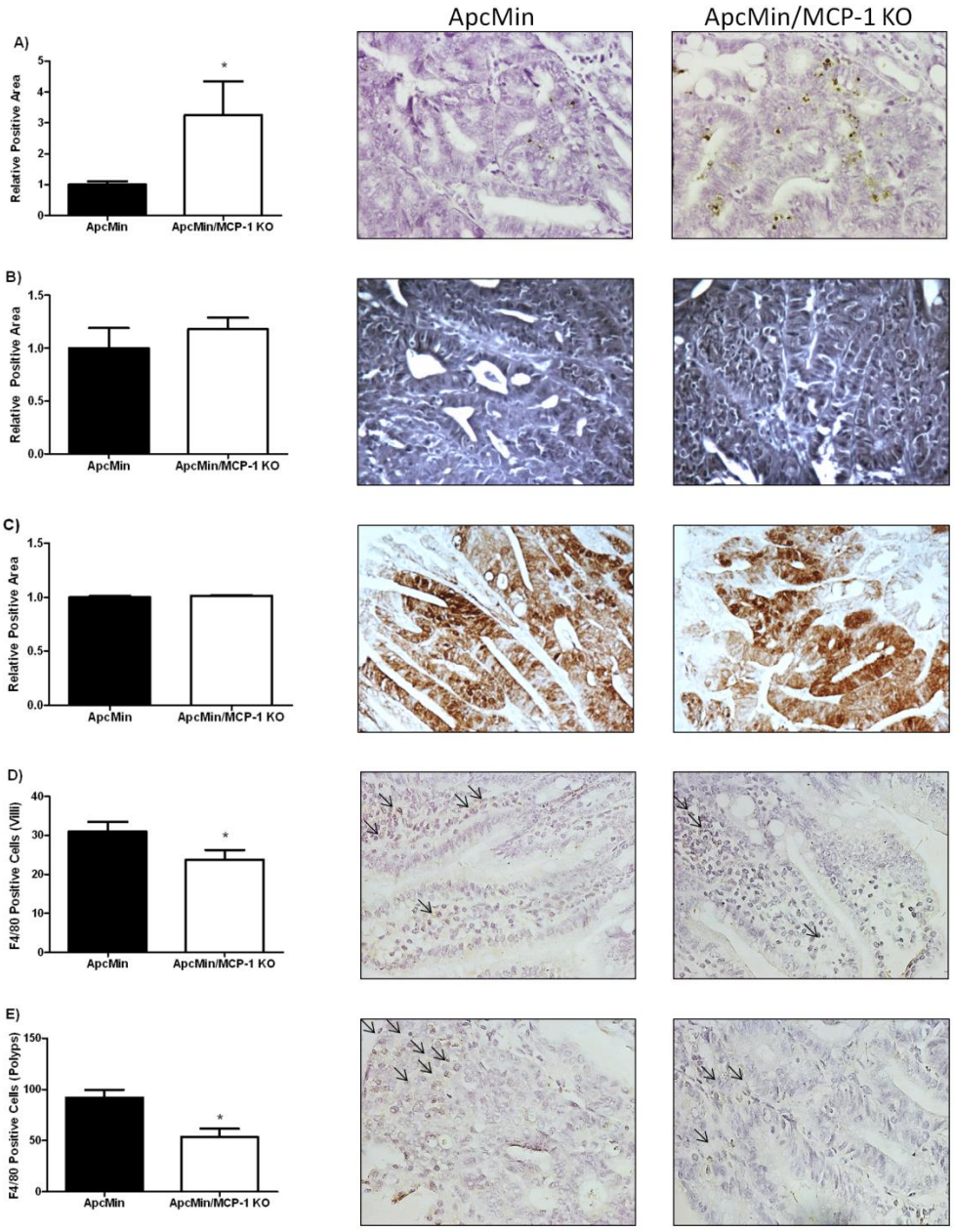

Figure 2.2. MCP-1 deficiency increases the number of apoptotic cells and decreases macrophage number but does not affect proliferation or β-catenin. TUNEL-positive cells were assessed in polyps located in intestinal section 4 of $Apc^{Min/+}$ and $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (n=10/group) at 18 wks of age (A). Polyps were stained for PCNA-positive cells in $Apc^{Min/+}$ and $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (n=10/group) (B). β-catenin was examined in section 4 of the intestines in $Apc^{Min/+}$ and $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (n=10/group) (C). The number of F4/80 positive cells was counted in the villi (D) and polyps I from intestinal section 4 of $Apc^{Min/+}$and $Apc^{Min/+}$/MCP-$1^{-/-}$ mice (n=10/group). Values are means ± SEM. Data are representative of three experimental blocks. * significantly different ($P<0.05$).

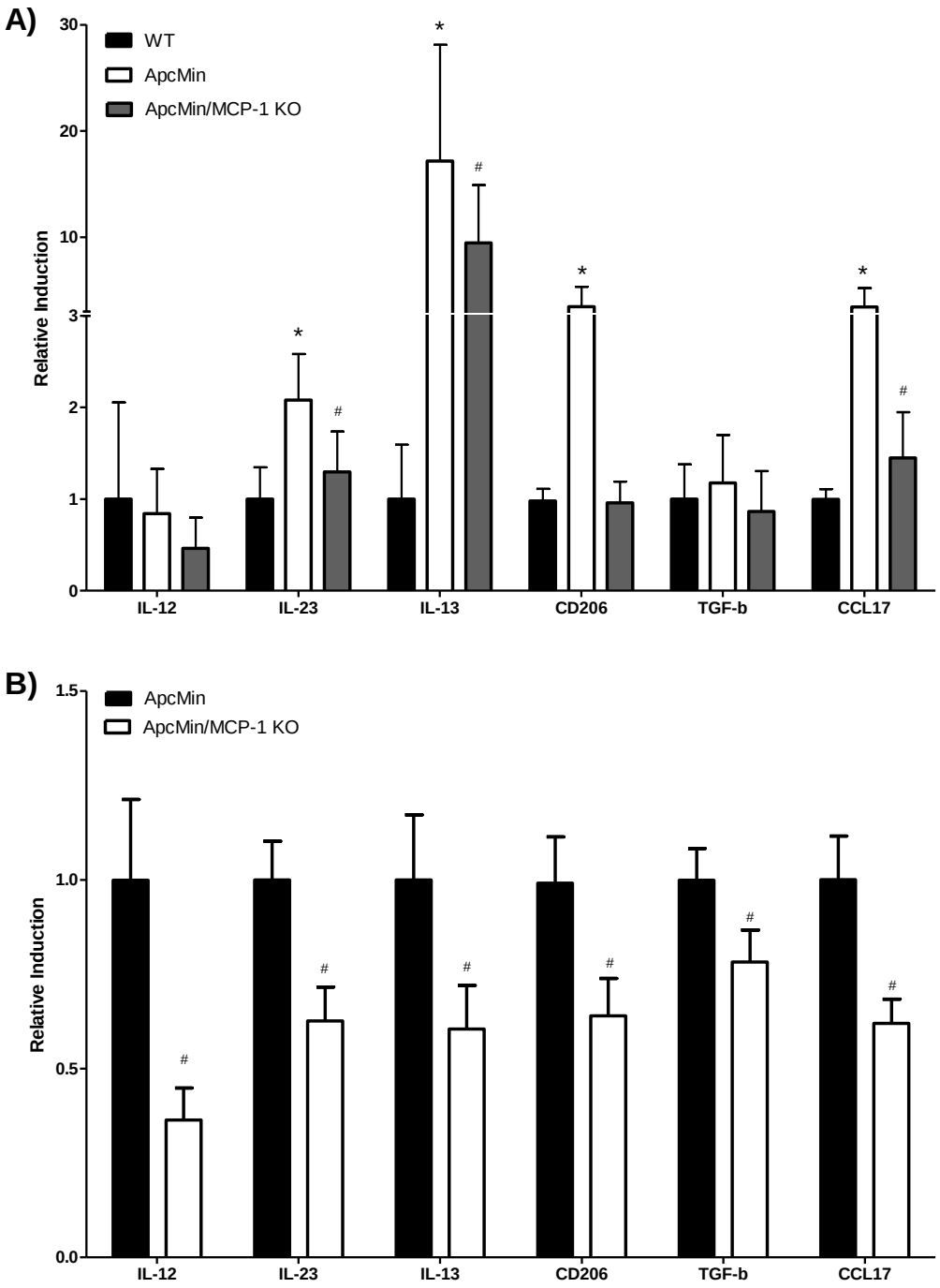

Figure 2.3. Macrophage number and expression of M1 and M2 phenotypic markers are reduced in *Apc*$^{Min/+}$ mice deficient for MCP-1. RT-PCR was performed to determine mRNA gene expression of macrophage phenotypic markers in the mucosal scrapings (A) and polyps (B) from intestinal section 3 of *Apc*$^{Min/+}$, *Apc*$^{Min/+}$/MCP-1$^{-/-}$ and wild type mice (mucosal scrapings only) (n=10/group) at 18 wks of age. Values are means ± SEM. Data are representative of three experimental blocks. * significantly different from wild type (P<0.05), # significantly different from *Apc*$^{Min/+}$ (P<0.05)

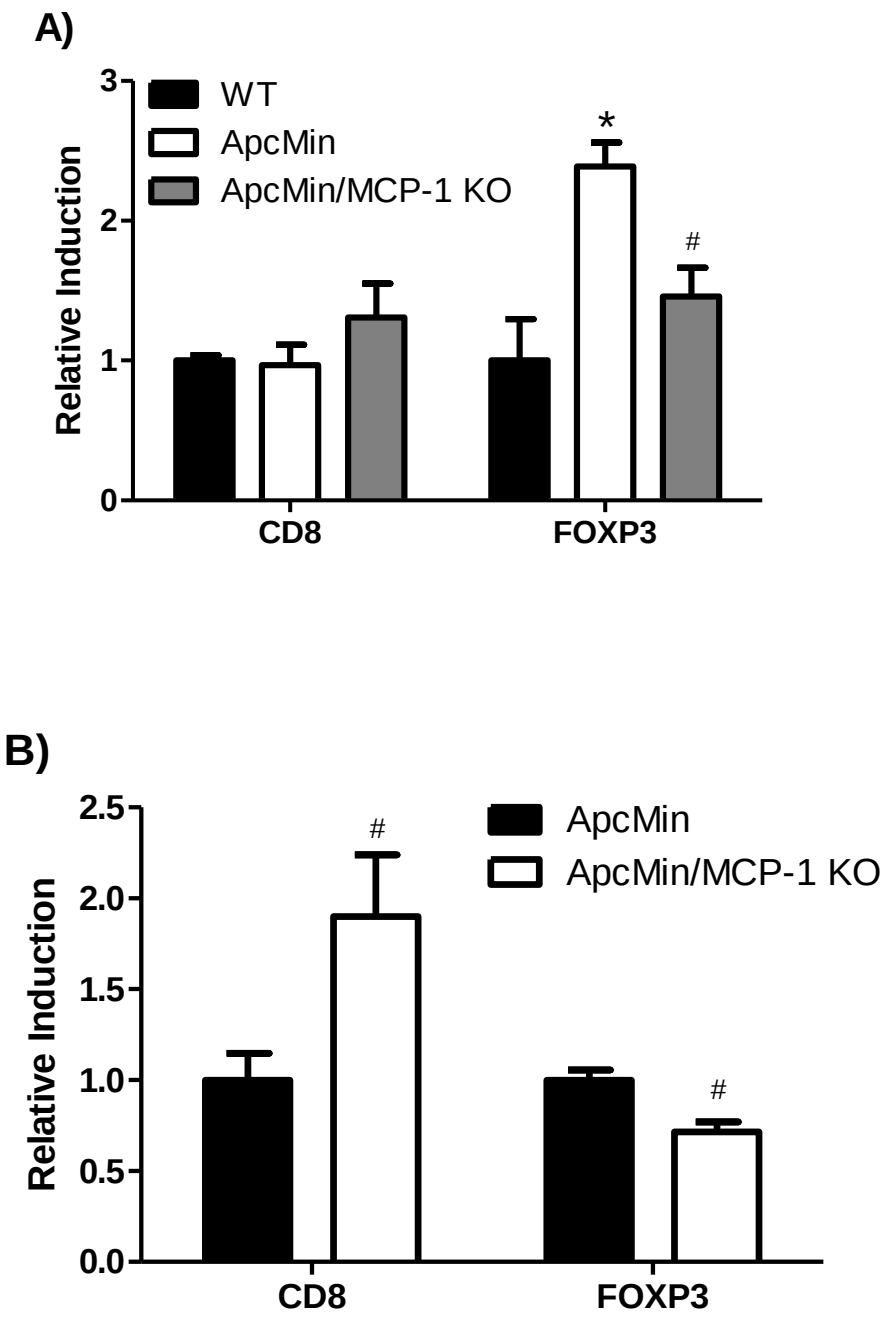

Figure 2.4. MCP-1 deficiency alters markers associated with CTLs and Tregs in *Apc*$^{Min/+}$ mice. RT-PCR to determine mRNA gene expression of CD8 and Foxp3 was performed in mucosal scrapings (A) and polyps (B) from intestinal section 3 of *Apc*$^{Min/+}$, *Apc*$^{Min/+}$/MCP-1$^{-/-}$ and wild type mice (mucosal scrapings only) (n=10/group) at 18 wks of age. Values are means ± SEM. Data are representative of three experimental blocks. * significantly different from wild type ($P<0.05$), # significantly different from *Apc*$^{Min/+}$ ($P<0.05$).

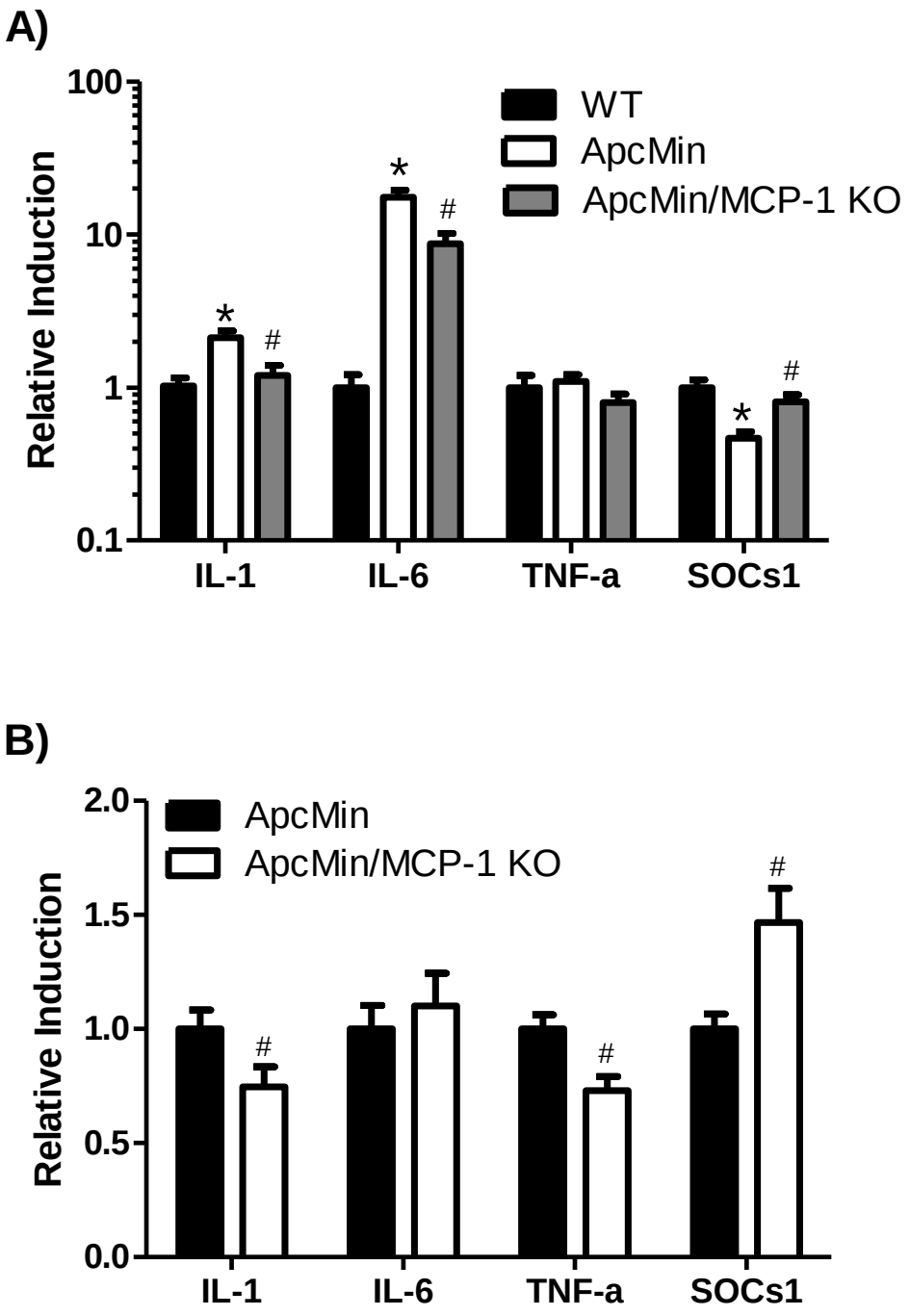

Figure 2.5. MCP-1 knockout alters the inflammatory response in *Apc*$^{Min/+}$ mice. RT-PCR to determine mRNA gene expression of IL-1β, IL-6, TNF-α, and SOCS1 was performed in mucosal scrapings (A) and polyps (B) from intestinal section 3 of *Apc*$^{Min/+}$, *Apc*$^{Min/+}$/MCP-1$^{-/-}$ and wild type mice (mucosal scrapings only) (n=10/group) at 18 wks of age. Values are means ± SEM. Data are representative of three experimental blocks. * significantly different from wild type ($P<0.05$), # significantly different from *Apc*$^{Min/+}$ ($P<0.05$).

CHAPTER 3

EXERCISE EFFECTS ON POLYP BURDEN AND IMMUNE MARKERS IN THE APC$^{MIN/+}$ MOUSE MODEL OF INTESTINAL TUMORIGENESIS[3]

[3] McClellan, J.L.,Steiner, J.L., Day, S.D., Enos, R.T., Davis, J.M., Singh, U.P., Murphy, E.A. *Exercise effects on polyp burden and immune markers in the ApcMin/+ mouse model of intestinal tumorigenesis.* Int J Oncol. **45**(2): p. 861-8. Reprinted here with permission from publisher.

ABSTRACT

Many observational epidemiologic studies suggest an association between exercise and colon cancer risk. The mechanisms contributing to a preventative effect of exercise on colon cancer are complex and multifaceted. Altered immune system function is one possible mechanism that has been largely unexplored. Therefore, the purpose of this study was to examine the effects of exercise on markers associated with macrophages and select T cell populations in a mouse model of intestinal tumorigenesis and to relate this to polyp characteristics. Male $Apc^{Min/+}$ mice were randomly assigned to either sedentary (Sed) or exercise (Ex) treatment (n=6-9/group). The exercise treatment consisted of treadmill running for 1hr/day and 6 days a wk at 15m/min from 4wks of age until 16 wks of age. Intestinal polyps were counted and categorized by size. Mucosal tissue was analyzed for mRNA expression of overall macrophages (F4/80), for genes associated with M1 (IL-12, IL-23, & Nos2) and M2 (CD206, IL-10, IL-4, CCL17, CCL22, & Arg-1) macrophages and the macrophage chemoattractants MCP-1, Fetuin A, & CXCL14. Markers for cytotoxic T cells (CTLs) and regulatory T cells were also examined by measuring mRNA expression of CD8 and Foxp3, respectively. While there was no significant difference in overall polyp number between the groups (Sed: 23.3 ± 4.3 and Ex: 16.5 ± 4.3), Ex did have a reduction in the number of large polyps (Sed:6.1 ± 1.1 and Ex: 3.0 ± 0.6) ($P<0.05$). This was consistent with a decrease in spleen weight ($P<0.05$). Similarly, Ex reduced mRNA expression of overall macrophages (F4/80) as well as markers associated with both M1 (IL-12) and M2 (CD206, CCL22, & Arg-1) subtypes ($P<0.05$) but there was no significant decrease in macrophage chemoattractants. CD8 expression was increased while Foxp3 expression was decreased with Ex ($P<0.05$).

Overall this data provides important new information on immune regulation as a possible mechanism for the documented benefits of exercise training on reducing colon cancer progression.

INTRODUCTION

Colon cancer remains a significant global health concern; it is the third most common malignancy and the fourth most common cause of cancer mortality(42, 43, 84, 85). Annually, it accounts for approximately 600,000 deaths worldwide. However, the vast majority (~80%) of these cases can be ascribed to environmental causes and are therefore potentially preventable (45). For instance, physical inactivity has been reported to account for 10% of all colon cancer cases (26), whereas physical activity has been associated with reduced risk for incidence of colon cancer. This inverse relationship between physical activity and colon cancer risk is supported by epidemiological studies as well as controlled experimental studies using rodent models. For example, nine weeks of treadmill running has been reported to decrease the total number of intestinal polyps as well as the number of large polyps in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis (27). Similarly a recent study in a multiethnic colon cancer screening population reported that exercising for one hour per week was associated with a lower prevalence of polyps and adenomas when compared to those who exercised less or not at all (28).

The mechanisms responsible for a preventative effect of exercise on colon cancer risk are complex and multifaceted. An exercise-induced alteration in immune system function is one possible mechanism that has not been widely explored. Macrophages, cells of the innate immune system, have recently emerged as major components in the

development of colon cancer given their ability to produce a wide array of inflammatory mediators with pro-tumoral functions (14, 15). In general, these cells have been associated with poor prognosis in colon cancer (14-18, 24). For example, one study reported a reduction in macrophage infiltration that was consistent with a decrease in the size of colon polyps in an MCP-1 receptor deficient mouse model of colitis-associated colon cancer (25). Further, in a clinical study it was documented that macrophage accumulation within the tumor was positively correlated with stage of progression (24). Consistent with these reports, we have documented that MCP-1 deficient $Apc^{Min/+}$ mice have decreased macrophage number in both the polyps and surrounding intestinal tissue and this was associated with a reduction in total polyp and large polyp number. Exercise has been reported to influence macrophage behavior in various disease models. For instance, it has been shown that exercise can reduce macrophage-mediated inflammation in an obese mouse model (29). Similarly, macrophage infiltration associated with the reversal of arterial inflammation was decreased by exercise training in aged mice (30). However, while it is well established that macrophages play a significant role in the pathogenesis of colon cancer and that exercise can influence the macrophage response, there is very little evidence on the benefits of exercise on macrophage behavior in the presence of colon cancer.

T cells also play a significant role in tumorigenesis and have been shown to be altered by exercise (31, 32). It is well recognized that cytotoxic T lymphocytes (CTLs) constitute one of the most important effector mechanisms of anti-tumor immunity (33, 34). For example, adoptive transfer of CD8+ cells controls the growth of B16 melanoma in mice (35). On the other hand, regulatory T cells (Tregs) have been associated with

accelerated tumor growth and immune evasion through their inhibitory actions on CTLs and helper T cells (36, 37). The T cell response to exercise has been reported to be highly variable and is likely dependent on the mode, intensity and duration of exercise. However, to date there is no evidence on the effects of exercise on T cells in a mouse model of colon cancer.

The purpose of this investigation was to examine the effects of exercise on expression of markers associated with macrophages and certain T cell subsets in a mouse model of intestinal tumorigenesis and to relate this to polyp characteristics. We used the $Apc^{min/+}$ mouse, the most widely used genetic mouse model for cancer studies that involve the gastro-intestinal tract (38). Since the *Apc* gene is mutated in a large percentage of human colon cancer cases, this is a common model for studying factors that may influence progression of colon cancer. The exercise protocol was designed to mimic a lifestyle that encompasses daily physical activity; mice were exercised for 1h/day, 6 days/week, for a 12 week period. We hypothesized that daily exercise would reduce the expression of markers associated with macrophages and alter the T cell expression profile in the tumor environment, and that this would be associated with a reduction in polyp burden.

MATERIALS AND METHODS

2.1 Animals. $Apc^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME). All experimental mice ($Apc^{Min/+}$) were bred in the University of South Carolina's Center for Colon Cancer Research (CCCR) Mouse Core Facility. Specifically, $Apc^{Min/+}$ male mice were bred with female C57BL/6 mice to generate $Apc^{Min/+}$ mice. Offspring were genotyped as heterozygotes by RT-PCR for the

Apc gene by taking tail snips at weaning. The primer sequences were sense: 5'-TGAGAAAGACAGAAGTTA-3'; and antisense: 5'-TTCCACTTTGGCATAAGGC-3'. Male $Apc^{Min/+}$ mice were used in this experiment and were maintained on a 12:12h light-dark cycle in a low-stress environment (22^{o}C, 50% humidity and low noise) and provided food (AIN-76A) and water *ad libitum*. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2 Exercise Protocol. Male $Apc^{Min/+}$ mice were randomly assigned to either the sedentary (Sed; n=9) or exercise (Ex; n=6) treatment. Mice in the Ex group ran on the treadmill at 15m/min and at a 5% incline for 1h per day (starting at 7pm), 6 days per week from 4 to 16 weeks of age. Mice in the Sed group remained in their cages in the treadmill room throughout the exercise bouts but were exposed to similar handling and noise in an attempt to control for extraneous stresses, and were deprived of food and water during the exercise sessions.

2.3 Tissue Collection. Body weight was measured weekly from 4 to 16 weeks of age. Mice were sacrificed for tissue collection as previously described (65, 86). Briefly, sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) for 24 h. For intestinal section 3, mucosal scrapings were performed in TRIzol reagent (Invitrogen, Carlsbad, CA). Samples were stored at –80°C until analysis for the expression of markers associated with macrophage phenotype and certain T cell subsets. Previously reported findings from our laboratory have shown that intestinal section 3 has a greater polyp incidence than section 2 (65), and therefore we included only section 3 for these analyses. Visceral fat pads (retroperitoneal, epididymal, and mesentery) were dissected and weighed for the

measurement of total visceral fat as it has been reported that *Apc*$^{Min/+}$ mice become cachectic with disease progression (87). Spleen was also weighed as splenomegaly has been shown to be associated with increased polyp number in this model (86). Blood was collected from the inferior vena cava using a heparinized syringe and blood parameters were examined immediately on fresh whole blood using a Vetscan blood analyzer (Abaxis, Union City, CA).

2.4 Polyp Counts. Formalin-fixed intestinal sections from all animals were rinsed in deionized water, briefly stained in 0.1% methylene blue, and counted by the same investigator who was blinded to the treatments. Polyps were counted under a dissecting microscope and were categorized according to size (>2 mm, 1–2 mm, and <1 mm).

2.5 mRNA Gene Expression. Quantification of gene expression for total macrophages (F4/80), markers associated with macrophage phenotype (IL-12, IL-23, & Nos2 (M1 macrophage phenotype), CD206, IL-10, IL-4, CCL17, CCL22, & Arg-1 (M2 macrophage phenotype)), macrophage chemoattractants (MCP-1, Fetuin A, & CXCL14), and T cell subsets (CD8 & Foxp3) were performed as previously described (65, 70). Briefly, mucosal tissue was homogenized under liquid nitrogen with a polytron, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA). The extracted RNA (2.5µL of sample) was dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified spectrophotometrically at 260nm wavelength. RNA was reverse transcribed into cDNA and quantitative RT-PCR analysis was done per manufacturer's instructions (Applied Biosystems, Foster City CA) using TaqMan® Gene Expression Assays. Quantification of cytokine gene expression was calculated using the ΔΔCT method (88).

2.6 Statistical Analysis. Analysis was done using commercially available software (SigmaStat, SPSS, Chicago, IL). A two-way repeated measures ANOVA was performed on body weight measurements. T-tests were performed on all other data. Statistical significance was set with an alpha value of $P<0.05$. Data are presented as mean (±SEM).

RESULTS

3.1 Body Weight and Visceral Fat Mass. It has been reported that *Apc*$^{Min/+}$ mice develop cachexia that is positively correlated with polyp burden (7). Therefore, we examined the potential benefits of exercise on body weight and visceral fat mass in the *Apc*$^{Min/+}$ mouse. At 15 and 16 weeks of age, there was an apparent difference (~1-2 g) between the groups; the Sed group weighed 26.1g ± 1.6 and 25.9g ± 1.4 at 15 and 16 weeks, respectively, whereas the Ex group weighed 27.3g ± 0.9 and 27.3g ± 0.9 ($P<0.1$) (Figure 3.1A). However, this did not quite reach statistical significance. Visceral fat tissue (retroperitoneal, epididymal, and mesentery) was collected at sacrifice and weighed to determine any influence of exercise on cachexia-related fat mass. There was no protective effect of exercise on total visceral fat pad weight (Sed: 1262.2mg ± 189.2, Ex: 1471.2mg ±197.7) (Figure 3.1B).

3.2 Spleen Weight. Increased spleen weight has been positively associated with polyp burden in this model (86). Therefore, spleens were harvested during sacrifice at 16 weeks of age, weighed, and expressed relative to body weight. Exercise significantly decreased spleen weight versus the Sed group (0.5mg/Kg ± 0.0 versus 0.68mg/Kg ±0.01, respectively) ($P=0.05$) (Figure 3.1C).

3.3 Complete Blood Count. A complete blood count was performed at sacrifice as both white blood cell (WBC) and red blood cell (RBC) counts have been shown to be altered

during progression of intestinal tumorigenesis in this mouse model (Figure 3.2) (86). WBC count at 16 weeks of age tended to be decreased by exercise (13.7 m/mm^3 ± 3.2 versus 7.4 m/mm^3 ± 0.6) (P=0.07). Similarly, there was a trend for an exercise-induced increase in hematocrit (Hct) compared to Sed (34.6 % ± 2.0 versus 27.1 % ± 3.8) (P=0.07). But there were no apparent differences among the groups for RBCs or hemoglobin (Hb).

3.4 Polyp incidence. At 16 weeks of age, mice (Sed and Ex) were sacrificed, intestinal tissue was harvested and polyps were counted on formalin-fixed, methylene blue-stained sections. Overall polyp number (sections 1, 4 and 5) was not significantly changed by exercise (23.3 ± 4.3 versus 19.2 ± 4.2 for Sed and Ex, respectively) (Figure 3.3A). To examine polyp size (Figure 3.3B), we counted and classified polyps as being large (> 1mm in diameter), medium (<2 > 1 mm in diameter) or small (< 1 mm in diameter). Interestingly, we found a significant reduction in the number of large polyps with exercise; the Ex group had 48% fewer large polyps than the Sed group (3.2 ± 0.7 versus 6.1 ± 1.1) (P<0.05) but there were no significant differences in the number of small or medium polyps.

3.5 Macrophage Number & Phenotypic Markers. Gene expression of macrophage phenotypic markers (IL-12, IL-23 & Nos2 (M1 macrophage phenotype), CD206, IL-10, IL-4, CCL17, CCL22 & Arg-1 (M2 macrophage phenotype), were examined in the mucosal scrapings (Figure 3.4A) of intestinal tissue. Data was normalized to fold-change from Sed mice. There was a significant decrease in mRNA expression of F4/80, a general macrophage marker (P<0.05). Similarly, there was a decrease in mRNA expression of the M2 associated macrophage markers, CD206, CCL22 and Arg in the Ex

mice (P<0.05), and a trend for a decrease in CCL17 (P<0.06). Even though not all markers associated with M2 macrophages were significantly reduced with exercise, all were consistently decreased. IL-12, a marker associated with the M1 macrophage phenotype was also decreased in the Ex mice (P<0.05) but there was no change in IL-23 or Nos2. Gene expression of macrophage-associated chemokines MCP-1, Fetuin A and CXCL14 were also examined in the mucosal scrapings. While mRNA expression of each of these markers was decreased with exercise (Figure 3.4B), statistical significance was not reached.

3.6 Changes in T cell expression. Given the role of T cell subsets in tumorigenesis, we also performed gene expression analysis of markers associated with CTLs (CD8) and Tregs (Foxp3) (Figure 3.5). CD8, a marker for CTLs that represents one of the most important effector mechanisms of anti-tumor immunity, was increased with exercise (P<0.05). Conversely, Foxp3, a marker for Tregs that are known to suppress immune function and that have been associated with increased tumorigenesis, was decreased with exercise (P<0.05).

DISCUSSION

There is an inverse relationship between physical activity and colon cancer risk (89). A multitude of mechanisms, including immune function dysregulation, have been implicated in this response. Macrophages and T cells play a significant role in the pathogenesis of colon cancer and exercise can influence the actions of these cells; however, there is very little evidence on the benefits of exercise on macrophage and T cell responses in the settings of colon cancer. We examined the effects of exercise on markers associated with macrophages and select T cell subsets in a mouse model of

intestinal tumorigenesis in relation to polyp characteristics. Overall, certain markers associated with both the M1 and the M2 macrophage phenotype were reduced in *Apc*$^{Min/+}$ mice following exercise. Additionally, exercise resulted in an increased expression of CD8 and decreased expression of Foxp3, markers for CTLs and Tregs, respectively. These alterations in immune cell parameters following exercise training were accompanied by a decrease in the percentage of large polyps.

Animal models provide a tool to examine the effects of exercise on colon cancer in an experimental environment in which the type and intensity of exercise can be controlled. They allow for detailed study of stage-specific responses to exercise, and help to identify the optimal mode, intensity and duration of exercise. The benefits of exercise on colon cancer risk have been well documented in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis (27, 67, 87, 90). For example, nine weeks of treadmill running has been reported to decrease the total number of intestinal polyps by 29% as well as the number of large polyps (38%) in male mice in this model (91). Similarly, exercise was reported to reduce total intestinal polyp number by 50% and the number of large polyps by 67% in this same model (67). Our findings are somewhat consistent with these investigations in that we report a 48% reduction in the number of large polyps. In contrast to the findings by Baltgalvis et al., we did not find a significant reduction in the number of total polyps; however, this is likely due to the smaller sample size in our study and/or to the slightly lower intensity of the exercise protocol (15m/min versus 18m/min). Nonetheless, the benefits of regular exercise training in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis are evident across studies and it is clear from our findings and

those of others that exercise plays a larger role in reducing the progression of growth as opposed to the initiation of development, at least in this model.

In addition to polyp characteristics, we measured body weight, fat mass, spleen weight, and markers of anemia. These outcomes have been associated with increased tumorigenesis and ultimately poorer prognosis in the *Apc*$^{Min/+}$ mouse (7, 86). Previous published data has shown that *Apc*$^{Min/+}$ mice develop cachexia that is positively correlated with polyp burden (7). In our study, the exercise mice tended to be heavier than the sedentary mice at 15 and 16 weeks of age, although this did not reach statistical significance. Further, there were no differences in fat mass between the groups. However, the lack of positive findings is likely due to the timing of sacrifice as mice were sacrificed prior to the onset of severe cachexia. This was done to eliminate any possible influence of cachexia or illness on the ability to perform the exercise protocol. Therefore, it is not surprising that we did not see a statistically significant effect of exercise at these time points. Spleen weight has been associated with increased polyp number and systemic inflammation (92). Our data indicate a reduction in spleen weight with exercise. This is consistent with previously reported literature; Baltgalvis et al. also reported a reduction in spleen weight in male Apc$^{Min/+}$ mice following exercise (92). We have recently reported an increase in markers of anemia in this mouse model(86). While exercise did prevent the characteristic decrease in Hct in these mice, the effect was not found to be statistically significant. Again, this is likely due to the timing of sacrifice as in our previous study mice were sacrificed at 18 weeks, a time in which the disease is much more severe. Thus, any benefits of exercise would likely be more evident had the mice been sacrificed at a later time point.

We next examined the effects of exercise on markers associated with macrophages in the mucosal tissue. Macrophages can represent up to 50% of the tumor mass producing a wide array of inflammatory mediators with pro-tumoral functions (14, 15). Further, abundance of tumor associated macrophages has been associated with poor prognosis in colon cancer (14-18, 24). Our data show a reduction in the expression of F4/80, an overall macrophage marker, with exercise. This is consistent with a previous study by Baltgalvis et al. that reported a reduction in macrophage number following exercise training in this model (27). Because it is now well accepted that macrophages constitute an extremely heterogeneous population that is divided into two main classes (M1 and M2) (14), we next examined the effects of exercise on expression of markers associated with both the M1 and M2 phenotype. In general, it is thought that M1 macrophages are cytotoxic against neoplastic cells, whereas M2 macrophages exert pro-tumoral functions (14). We report the novel finding that exercise reduces the expression of certain markers that are associated with the M1 (IL-12) and M2 (CD206, CCL17, CCL22 and Arg-1) phenotype in the mucosal tissue. It is important to note that given the limited available tissue, macrophage markers were not measured in the polyps themselves. However, previous data from our group shows a similar response for these outcomes when comparing the mucosal tissue and polyp tissue in this model (86). While our data suggests that a reduction in both M1 and M2 macrophages with exercise is associated with a reduction in polyp growth in this model, a greater understanding of the roles of each macrophage subset within the tumor microenvironment is necessary.

Given the reduction in macrophage markers with exercise, we next examined the effects of exercise on macrophage chemoattractants. MCP-1 is a major player in

macrophage chemotaxis in the *Apc*$^{Min/+}$ mouse (86). In fact, we recently reported a link between macrophages and MCP-1 in this mouse model (86). Although our data indicate an exercise-induced decrease in the expression of macrophage associated markers in the mucosal tissue, we did not find a significant decrease in MCP-1. Therefore, we also examined Fetuin A and CXCL14, both of which have been implicated in macrophage recruitment. Fetuin A is a recently characterized macrophage chemoattractant that is also known to play a role in macrophage polarization (93). While our results show a decrease in the expression of Fetuin A that is consistent with our macrophage findings, this did not reach statistical significance. Similarly, there was no effect of exercise on CXCL14, a chemoattractant for activated tissue macrophages. It is important to point out that only a subset of macrophage chemoattractants were measured in this study, thus it is possible that exercise may have impacted other chemokines.

One of the most important effector mechanisms of anti-tumor immunity is the activities of CTLs (33, 34). For example, growth of B16 melanoma cells can be controlled in mice following the transfer of CD8+ cells (35). On the other hand, Tregs have been linked to accelerated tumor growth and immune evasion due to their inhibitory actions on CTLs and helper T cells (36, 37). The findings of the current study show an increased expression of CD8, and conversely, a decreased expression of Foxp3 in the mucosal tissue following exercise training. While this is the first report of a favorable effect of exercise on CTLs and Tregs in a model of colon cancer, the evidence supporting exercise-induced alterations in immune function is far reaching. In general, regular moderate exercise is thought to enhance immune function; however, these effects are likely dependent on a multitude of factors including individual characteristics, the type,

intensity and duration of exercise, and the stage of cancer. Although these findings support a positive effect of exercise on the T cell profile in a cancer model, it is important to point out that whether this is due to a direct effect of the exercise on these cell populations or an indirect effect that results from the reduction in large polyp number resulting from an entirely different mechanism could not be determined from this study.

Consistent with previous reports, we show a benefit of exercise training on reducing large polyp number in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis. This was associated with alterations in the expression of immune markers in the mucosal tissue including a reduction in markers associated with M1 and M2 macrophages, an increase CD8 and a decrease in Foxp3. Overall this data provides important new information on immune regulation as a possible mechanism for the benefits of exercise training on reducing colon cancer progression.

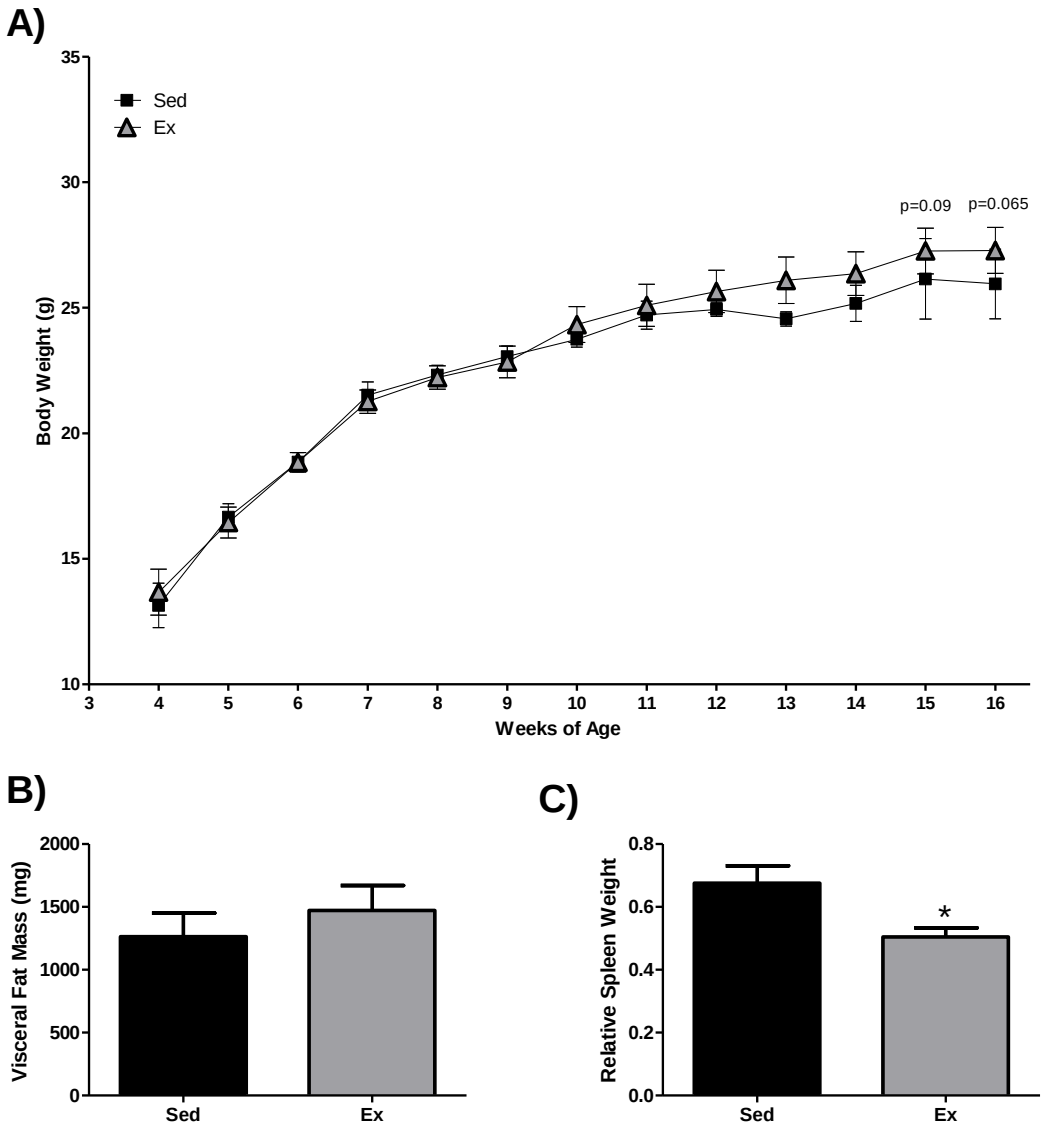

Figure 3.1. Effects of exercise on body weight, visceral fat mass and spleen weight in *Apc*$^{Min/+}$ mice. Differences in body weight (A), visceral fat mass (B) and spleen weight (C) were examined in sedentary (Sed) and exercised (Ex) *Apc*$^{Min/+}$ mice (n= 6-9/group) at 16 weeks of age. Values are means ± SEM. *Significantly different (P<0.05).

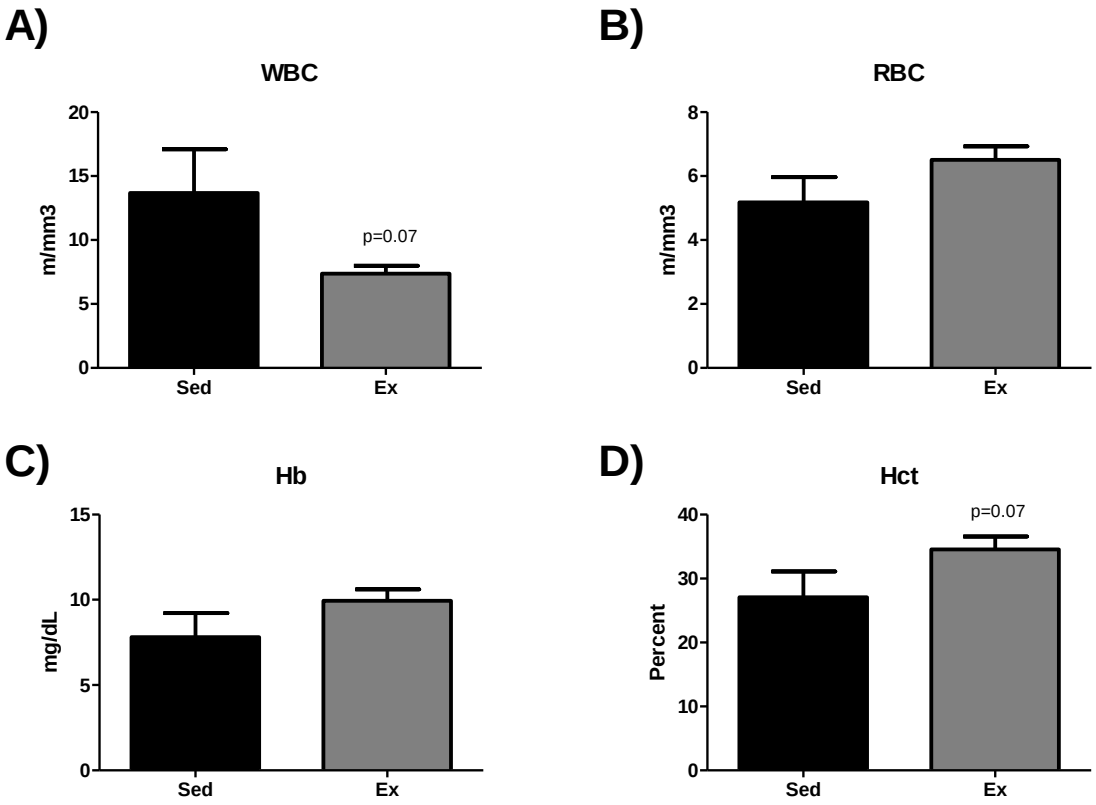

Figure 3.2. Effects of exercise on WBCs, RBCs, Hb and Hct in *Apc*$^{Min/+}$ mice. Differences in WBC count (A), RBC count (B), Hb (C) and Hct (D) were examined in sedentary (Sed) and exercised (Ex) *Apc*$^{Min/+}$ mice (n=6-9/group) at 16 weeks of age. Values are means ± SEM. **Trend for significant difference (P<0.1).

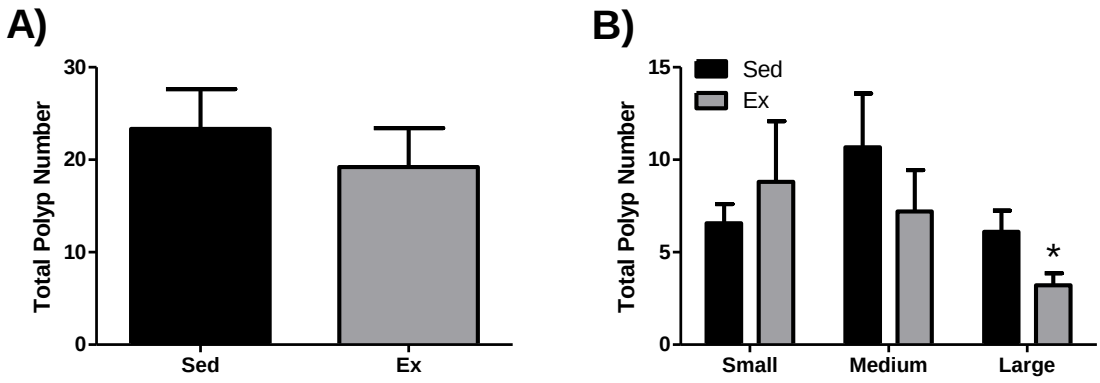

Figure 3.3. Effects of exercise on polyp number and size in *Apc*$^{Min/+}$ mice. Differences in total polyp number (A) and polyp size (B) were examined in sedentary (Sed) and exercised (Ex) *Apc*$^{Min/+}$ mice (n=6-9/group) at 16 weeks of age. Values are means ± SEM. *Significant difference (P<0.05).

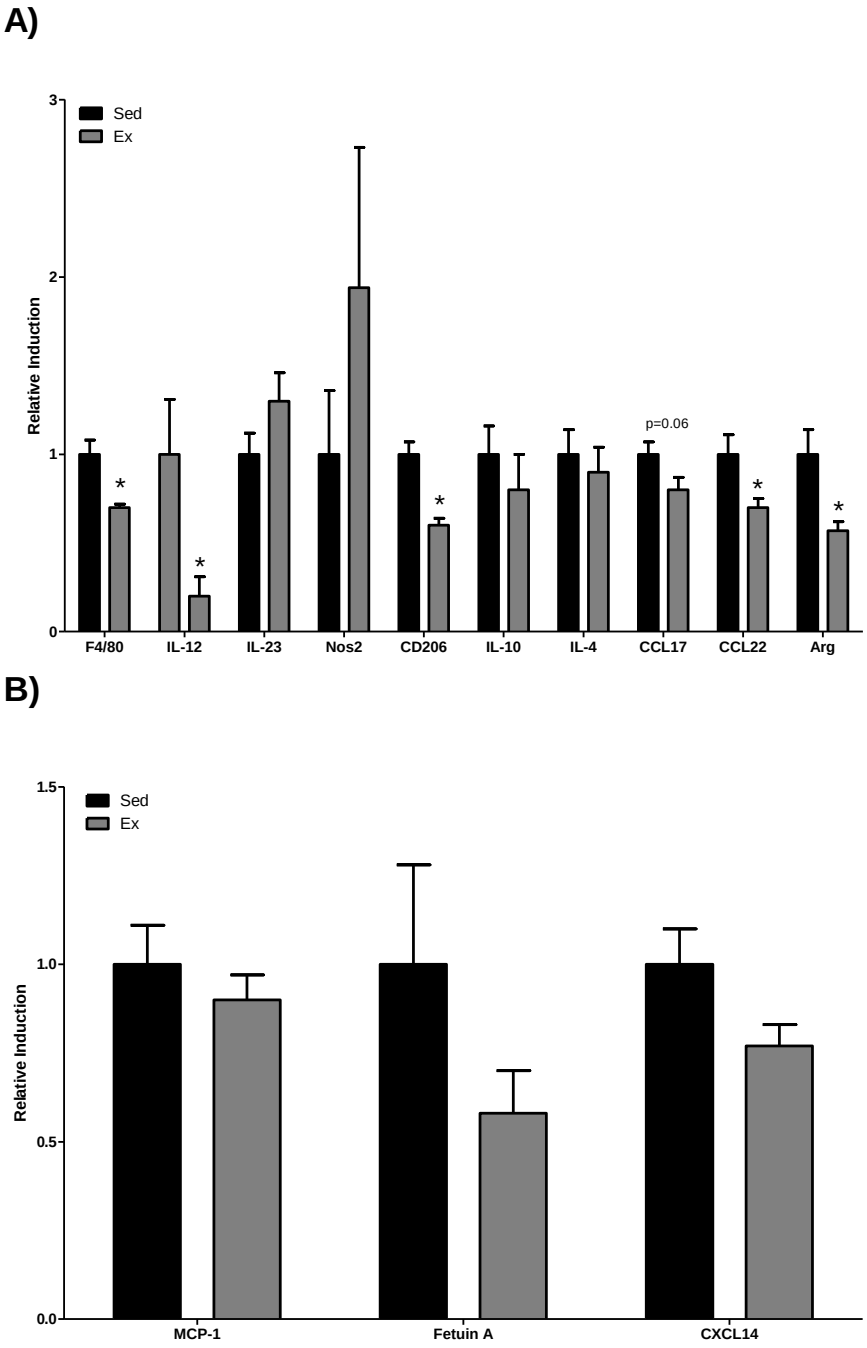

Figure 3.4. Effects of exercise on gene expression of M1 and M2 associated phenotypic macrophage markers in *Apc*$^{Min/+}$ mice. Differences in gene expression of F4/80 (A), M1 macrophage markers (IL-12 (B) and IL-23 (C)), and M2 macrophage markers (CD206 (D), IL-10 I, IL-4 (F), CCL17 (G) and CCL22 (H)) were examined in sedentary (Sed) and exercised (Ex) *Apc*$^{Min/+}$ mice (n=6-9/group) at 16 weeks of age in mucosal scrapings. Values are means ± SEM. *Significant difference (P<0.05). **Trend for significant difference (P<0.1).

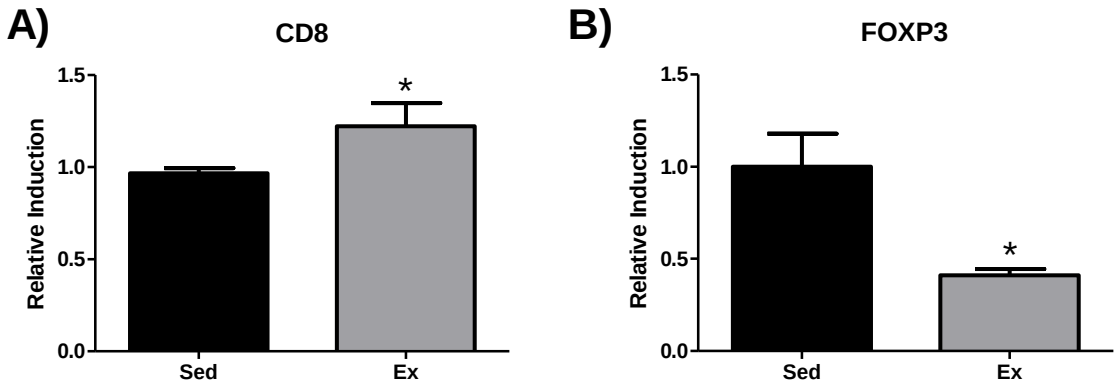

Figure 3.5. Effects of exercise on gene expression of CTL and Treg cell markers in *Apc*$^{Min/+}$ mice. Differences in gene expression of CD8 (A), a marker for CTLs and Foxp3 (B), a marker for Tregs were examined in sedentary (Sed) and exercised (Ex) *Apc*$^{Min/+}$ mice (n=6-9/group) at 16 weeks of age in mucosal scrapings. Values are means ± SEM. *Significant difference (P<0.05).

CONCLUSION

Epidemiological evidence links colon cancer to chronic intestinal inflammation (54). Further support for a role of inflammation in colon cancer comes from studies using mouse models that have been shown to be responsive to treatment with anti-inflammatory agents, including both anti-inflammatory dietary supplements as well as non-steroidal anti-inflammatory drugs (NSAIDs) (39). Despite this, the timing and magnitude of the inflammatory cytokine response in relation to tumorigenesis has not been characterized. The first study used an established mouse model of intestinal tumorigenesis to examine this response in the $Apc^{Min/+}$ mouse. We show here for the first time the timing and magnitude of the inflammatory cytokine response in intestinal tissue across time in the $Apc^{Min/+}$ mouse. These findings may have important implications for colon cancer. Firstly, these results may contribute to the future development of biomarkers to assess colon cancer progression. Our data shows a strong relationship between polyp progression and MCP-1 in a mouse model of colon cancer. Future studies are necessary to fully evaluate the potential of these inflammatory cytokines as prognostic indicators for colon cancer. Secondly, these data provide important new information that can be used for the determination of appropriate timing of effective treatments that can be used in the design of future investigations that target inflammatory processes in mouse models of colon cancer. These data contribute to the growing evidence on the association between inflammation and colon cancer and provide

important new data that could be used in the development of biomarkers as well as in the design of future investigations of anti-inflammatory treatments.

Macrophages play a key role in tumorigenesis; they are a major player in the inflammatory response that contributes to cellular transformation, promotion, apoptosis, proliferation, invasion, angiogenesis, and metastasis (1, 4, 14, 15, 73, 74). MCP-1 has been identified as the most important chemokine for recruitment of macrophages to the tumor microenvironment (22). However, the link between MCP-1, macrophages, inflammation, and tumorigenesis in colon cancer has not yet been established. Through the use of the $Apc^{Min/+}$/MCP-$1^{-/-}$ mouse bred in our laboratory, we report the novel finding that MCP-1 is a link between macrophages, inflammation, and tumorigenesis in colon cancer. In summary, we report that MCP-1 is an important regulator of macrophages, T cells, and inflammatory responses in the tumor microenvironment that can lead to increased polyp burden in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis. Using an $Apc^{Min/+}$/MCP-$1^{-/-}$ mouse that we developed in our laboratory, we show a decrease in overall polyp number and large polyp abundance that was consistent with a reduction in macrophage number, an alteration in T cell markers that are associated with improved immune surveillance, and a decrease in inflammatory processes in the polyp tissue and surrounding intestinal tissue as compared to MCP-1 sufficient $Apc^{Min/+}$ mice. Given the important regulatory role of MCP-1 in these facets of cancer, development of effective pharmacological or antibody approaches to inhibit MCP-1 may have important implications for the prevention and/or treatment of colon cancer.

There is an inverse relationship between physical activity and colon cancer risk (89). A multitude of mechanisms, including immune function dysregulation, have been

implicated in this response. Macrophages and T cells play a significant role in the pathogenesis of colon cancer and exercise can influence the actions of these cells; however, there is very little evidence on the benefits of exercise on macrophage and T cell responses in the settings of colon cancer. We examined the effects of exercise on markers associated with macrophages and select T cell subsets in a mouse model of intestinal tumorigenesis in relation to polyp characteristics. We show a benefit of exercise training on reducing large polyp number in the $Apc^{Min/+}$ mouse. This was associated with alterations in the expression of immune markers in the mucosal tissue including a reduction in markers associated with M1 and M2 macrophages, an increase CD8 and a decrease in Foxp3. Overall this data provides important new information on immune regulation as a possible mechanism for the benefits of exercise training on reducing colon cancer progression.

Overall, these data provide new information on the progression of inflammation in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis as well as the importance of macrophages and cytokines in this process. We have also shown a possible mechanism for the benefits of exercise on tumorigenesis in this mouse model.

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