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Linking tumor-associated macrophages, inflammation, and intestinal tumorigenesis: role of MCP-1

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Linking tumor-associated macrophages, inflammation, and intestinal tumorigenesis: role of MCP-1. Am J Physiol Gastrointest Liver Physiol 303: G1087–G1095, 2012. First published September 27, 2012; doi:10.1152/ajpgi.00252.2012.—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 ApcMin/+ or wild-type mice were euthanized at 18 wk of age and intestines were analyzed for polyp burden, apoptosis, proliferation, β-catenin, macrophage number and phenotype, markers for cytotoxic T lymphocytes and regulatory T cells, and inflammatory mediators. MCP-1 deficiency decreased overall polyp burden, apoptosis, proliferation, β-catenin, macrophage number and phenotype, markers for cytotoxic T lymphocytes and regulatory T cells, 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 and IL-23) and M2 macrophages (IL-13, CD206, TGF-β, and CCL17) (P < 0.05). MCP-1 knockout was also associated with increased cytotoxic T lymphocytes and decreased regulatory T cells (P < 0.05). In addition, MCP-1−/− offset the increased mRNA expression of IL-1β and IL-6 in intestinal tissue and IL-1β and 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 tumor growth and immune regulation that may serve as an important biomarker and/or therapeutic target in colon cancer.

colon cancer; inflammatory cytokines; macrophage phenotype

THERE IS A WELL-ESTABLISHED association between inflammation and cancer risk. In fact, inflammation has been suggested to represent the seventh hallmark of cancer (23). 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 (10, 24). Macrophages have recently emerged as major players in the connection between inflammation and cancer (27, 35); they represent up to 50% of the tumor mass and produce a wide array of inflammatory mediators with protumoral functions (27, 35). Furthermore, they have been associated with alterations in adaptive immune responses within the tumor microenvironment that may lead to reduced immune surveillance (35, 43). In general, high levels of tumor-associated macrophages (TAMs) are associated with poor prognosis in colon cancer (19–21, 27, 35). 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 (15, 22, 32). Using targeted gene disruption Lu et al. (22) created an MCP-1-deficient mouse and documented that MCP-1 is uniquely essential for monocyte recruitment in several inflammatory models. More recently in cancer models, MCP-1 has been suggested to be the most important chemokine for macrophage recruitment to the tumor microenvironment (4); both rodent and clinical studies have associated MCP-1 levels with TAM abundance and subsequent cancer progression (1, 2, 31). For example, we have shown that MCP-1 is correlated with abundance of large polyps in the ApcMin/+ mouse model of intestinal tumorigenesis (28). Similarly, Bailey et al. (1) have reported that MCP-1 expression is associated with TAM number and stage of colorectal cancer in humans. Furthermore, 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 (31). However, the specific role of MCP-1 in 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 effect of MCP-1 on macrophage number and phenotype (M1 and 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, and SOCS1) in the intestines and the tumor microenvironment in a genetic mouse model of intestinal tumorigenesis, and, furthermore, to relate these to tumor progression (polyp number and size). This was done by crossing the Apc<sup>Min/+</sup> mouse, the most widely used genetic mouse model for cancer studies that involve the gastrointestinal tract (9, 39), with an MCP-1 knockout mouse to develop an MCP-1-deficient model of intestinal tumorigenesis. Apc<sup>Min/−/−</sup> 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**

**Animals.** Apc<sup>Min/−/−</sup> and MCP-1<sup>−/−</sup> mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME). All experimental mice (Apc<sup>Min/−/−</sup> and Apc<sup>Min/−/−·MCP-1<sup>−/−</sup></sup>) were bred in the University of South Carolina’s Center for Colon Cancer Research (CCCR) Mouse Core Facility. Specifically, Apc<sup>Min/−/−</sup> male mice were bred with female C57BL/6 mice to generate Apc<sup>Min/−/−·MCP-1<sup>−/−</sup></sup> 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′-TGAGAAAAGACAGAAGTTA-3′ and antisense: 5′-TTC-CACCTTTGGCATAAGGC-3′. The Apc<sup>Min/−/−·MCP-1<sup>−/−</sup></sup> mouse was generated by introducing MCP-1<sup>−/−</sup> into the Apc<sup>Min/−/−</sup> model. This colony was maintained by breeding male Apc<sup>Min/−/−</sup>·MCP-1<sup>−/−</sup> mice with female MCP-1<sup>−/−</sup> mice. Offspring were genotyped for the mutant Apc allele as described above and for MCP-1. The primer sequences for MCP-1<sup>−/−</sup> were as follows: mutant GCCAGAGGC-CACTTGTTGATAG, wild-type forward TGACAGTCCCATCAGTC-ACA and common TCATTGGATCATCTTGTG. Male Apc<sup>Min/−/−</sup>, Apc<sup>Min/−/−·MCP-1<sup>−/−</sup></sup> and wild-type (n = 10/group) mice were maintained on a 12:12-h light-dark cycle in a low-stress environment (22°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.

**Tissue collection.** Body weight was measured prior to euthanasia (18 wk of age). Mice were euthanized for tissue collection as previously described (28). Sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, 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 (28), and therefore we included only section 3 for these analyses. Blood was collected from the inferior vena cava by 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 4,000 rpm for 15 min. Plasma was then stored at −80°C until assayed for IL-6 (R&D Systems, Minneapolis, MN).

**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).

**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 with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Antigens were unmasked using proteinase K (Millipore) and peroxidase activity was inhibited using BLOXALL (Vector Laboratories) for 10 min. Sections were incubated with rabbit polyclonal PCNA antibody (1:200; Abcam, Cambridge, MA) for 1 h at room temperature. For β-catenin and macrophage staining the HRP-DAB Cell and Tissue staining kit (R&D Systems) 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 room temperature. Color detection was visualized by exposing sections to 3,3′-diaminobenzidine (DAB). TUNEL-positive cells, PCNA-positive cells, and β-catenin were visualized by using the DAKO Chromavision Systems ACIS 3 system (36). 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<sup>Min/−/−·MCP-1<sup>−/−</sup></sup> mice. To quantify macrophages, F4/80-positive cells within each polyp (positive cells/μm<sup>2</sup>) and villi (10 villi/mouse) were counted (×40 magnification) and averaged for each animal (5, 6, 41).

**mRNA gene expression.** Quantification of gene expression for macrophage phenotype [IL-12 and IL-23 (M1 macrophage phenotype), IL-13, CD206, TGF-β, and CCL17 (M2 macrophage phenotype)], T cell markers (CD8 and Foxp3), and inflammatory mediators [IL-1β, IL-6, TNF-α, SOCS1 (suppressor of cytokine signaling 1)] were performed as previously described (28, 29). Briefly, mucosal tissue and polyps were homogenized under liquid nitrogen with a Polytron, and total RNA was extracted by using TRIzol reagent (Invitrogen). RNA was reverse transcribed into cDNA and quantitative RT-PCR analysis was done per manufacturer’s instructions (Applied Biosystems, Foster City, CA) with TaqMan Gene Expression Assays. Quantification of cytokine gene expression was calculated by the ΔΔC<sub>T</sub> method.

**Statistical analysis.** Analysis was done using commercially available software (SigmaStat, SPSS, Chicago, IL). For comparisons between Apc<sup>Min/−/−</sup> and Apc<sup>Min/−/−·MCP-1<sup>−/−</sup></sup> (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 means ± SE.

**RESULTS**

**Body weight.** Previous published data from our group has shown that Apc<sup>Min/−/−</sup> mice develop cachexia that is positively
correlated with polyp burden (7). As expected \(Apc^{Min/+}\) mice show a decrease in body weight compared with wild-type mice (22.2 ± 0.4 vs. 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.

Polyp incidence. At 18 wk of age, mice \((Apc^{Min/+}\) and \(Apc^{Min/+}/MCP-1^{-/-}\) were euthanized, 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 vs. 54.2 ± 2.5) \((P < 0.05)\) (Fig. 1A). To examine polyp size (Fig. 1B), we classified polyps as being large (>1 mm in diameter), medium (1–2 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 vs. 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 (Fig. 1C) and found that polyp number was decreased in \(Apc^{Min/+}/MCP-1^{-/-}\) by ~40% in section 1 (3.3 ± 0.5 vs. 5.6 ± 0.5) \((P < 0.05)\), ~15% in section 4 (37.7 ± 2.7 vs. 45.1 ± 2.6), and ~45% in section 5 (1.8 ± 0.4 vs. 3.4 ± 0.6) \((P < 0.05)\).

Apoptosis. Apoptosis, or programmed cell death, is typically evaded by tumors and leads to uncontrolled tumor growth (17). TUNEL staining was used to quantify apoptotic cells within polyps (Fig. 2A). This analysis was done only in section 4, a region of high polyp incidence, 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 \((P < 0.05)\).

Proliferation. Sustaining proliferative signaling is one of the hallmarks of cancer. PCNA staining was done in section 4 to quantify proliferating cells (Fig. 2B). There were no differences detected in the percentage of the polyp area staining positive for PCNA (1.0 ± 0.2 vs. 1.2 ± 0.1 for \(Apc^{Min/+}\) and \(Apc^{Min/+}/MCP-1^{-/-}\) mice, respectively).

\(\beta\)-Catenin. Immunohistochemical staining for \(\beta\)-catenin, a central molecule of the Wnt-signaling pathway that is involved in cellular replication and that has been correlated with poor prognosis in colon cancer, was also performed (5, 6, 41). 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 \(\beta\)-catenin (1.0 ± 0.1 vs. 1.0 ± 0.1 for \(Apc^{Min/+}\) and \(Apc^{Min/+}/MCP-1^{-/-}\) mice, respectively) (Fig. 2C).

Macrophage number and 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 (Fig. 2D) (10 villi were counted per mouse) as well as the polyps (Fig. 2E) (positive cells/μm²) 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 vs. 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 vs. 92.2 ± 7.4) \((P < 0.05)\). Furthermore, gene expression of macrophage phenotypic markers [IL-12 and IL-23 (M1 macrophage phenotype), IL-13, CD206, TGF-β, and CCL17 (M2 macrophage phenotype)] were examined in the mucosal scrapings (Fig. 3C) of intestinal tissue and in the polyps (Fig. 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 \(Apc^{Min/+}/MCP-1^{-/-}\) vs. \(Apc^{Min/+}\) mice \((P < 0.05)\).
T cell markers. We also performed gene expression analysis of markers associated with CTLs (CD8) and Tregs (Foxp3) (Fig. 4). In the mucosal tissue (Fig. 4A), there was no difference in mRNA gene expression of CD8 across the groups. There was, however, a significant increase in Foxp3 in the ApcMin/MCP-1 KO mouse, and this effect was blunted in the ApcMin/MCP-1 KO group. For the polyps (Fig. 4B), we found a significant increase in mRNA expression of CD8 in the

Fig. 2. MCP-1 deficiency increases the number of apoptotic cells and decreases macrophage number but does not affect proliferation or β-catenin. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were assessed in polyps located in intestinal section 4 of ApcMin+/− and ApcMin+/−/MCP-1−/− mice (n = 10/group) at 18 wk of age (A). Polyps were stained for PCNA-positive cells in ApcMin+/− and ApcMin+/−/MCP-1−/− mice (n = 10/group) (B). β-Catenin was examined in section 4 of the intestines in ApcMin+/− and ApcMin+/−/MCP-1−/− mice (n = 10/group) (C). The number of F4/80-positive cells was counted in the villi (D) and polyps (E) from intestinal section 4 of ApcMin+/− and ApcMin+/−/MCP-1−/− mice (n = 10/group) (arrows indicate representative macrophages). Values are means ± SE. Data are representative of 3 experimental blocks. *Significantly different (P < 0.05).
ApcMin/+/MCP-1−/− group (P < 0.05). And, similar to the mucosal data, there was a decrease in Foxp3 expression in the polyps of ApcMin/+/MCP-1−/− mice (P < 0.05).

**Inflammatory modifiers.** Gene expression of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and SOCS1 was examined in the mucosal scrapings of intestinal tissue as well as the polyps (Fig. 5). In the mucosal tissue (Fig. 5A), IL-1β and IL-6 were increased in ApcMin+/ 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 ApcMin+/ mice (P < 0.05) but there was no difference in SOCS1 expression between wild-type mice and ApcMin+/+/MCP-1−/− mice. In the polyps (Fig. 5B), there was a decrease in mRNA expression of IL-1β and TNF-α in ApcMin+/+/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 ApcMin+/ mice compared with ApcMin+/+/MCP-1−/− mice (P < 0.05).

**Complete blood count and 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 1) (8). We found that overall WBC counts were increased in ApcMin+/ mice (13.6 m/mm^3 ± 1.8) compared with wild-type (5.7 m/mm^3 ± 0.6) (P < 0.05) and MCP-1 deficiency blunted this response (7.1 m/mm^3 ± 0.9) (P < 0.05). In general, these findings were consistent across all cell types (lymphocytes, monocytes, and granulocytes). For RBCs, ApcMin+/ mice showed an overall large decrease (2.6 m/mm^3 ± 0.2) vs. wild-type mice (7.9 m/mm^3 ± 0.2) (P < 0.05), and MCP-1 deficiency offset this effect (3.9 m/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 ApcMin+/ mice (40.0 ± 6.3 pg/ml) compared with wild-type mice (0.8 ± 0.8 pg/ml) (P < 0.05) and report that this effect was blunted.
in the MCP-1-deficient ApcMin/− mice (19.3 ± 1.9 pg/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 (10, 24–27, 35). MCP-1 has been identified as the most important chemokine for recruitment of macrophages to the tumor microenvironment (4). However, the link between MCP-1, macrophages, inflammation, and tumorigenesis in colon cancer has not yet been established. Through the use of the ApcMin/+ /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 ApcMin/+ /MCP-1−/− mice. Furthermore, 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 (1, 2, 31). However, there have been relatively few studies that have specifically linked MCP-1 to colon cancer progression. Here we show that ApcMin/+ 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...

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### Table 1. Circulating levels of white blood cells, red blood cells, and IL-6 are altered in ApcMin/+ mice deficient for MCP-1

<table>
<thead>
<tr>
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<th>WT</th>
<th>ApcMin/+</th>
<th>ApcMin/+ /MCP-1 KO</th>
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</thead>
<tbody>
<tr>
<td>White blood cells, m/mm³</td>
<td>5.7 ± 0.6</td>
<td>13.6 ± 1.9*</td>
<td>7.1 ± 0.9†</td>
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<td>Lymphocytes, m/mm³</td>
<td>4.4 ± 0.4</td>
<td>11.3 ± 1.6*</td>
<td>5.9 ± 0.8†</td>
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<td>Monocytes, m/mm³</td>
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<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0†</td>
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<td>Granulocytes, m/mm³</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.2†</td>
<td>0.9 ± 0.1‡</td>
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<tr>
<td>Red blood cells, m/mm³</td>
<td>7.9 ± 0.2</td>
<td>7.6 ± 0.2*</td>
<td>3.9 ± 0.2*‡</td>
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<td>Hct, %</td>
<td>34.9 ± 1.2</td>
<td>46.4 ± 0.8*</td>
<td>20.0 ± 1.2*,†</td>
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<tr>
<td>Hb, g/dl</td>
<td>12.2 ± 0.3</td>
<td>3.9 ± 0.3*</td>
<td>6.4 ± 0.3*,†</td>
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<tr>
<td>Plasma IL-6, pg/ml</td>
<td>0.8 ± 0.8</td>
<td>40.0 ± 6.3*</td>
<td>19.3 ± 1.9*,‡</td>
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*Values are means ± SE. Data are representative of 3 experimental blocks. 
†Significantly different from wild type, P < 0.05; †significantly different from ApcMin/+, P < 0.05; ††trend to be different from wild type, P < 0.1.
plays a larger role in progression of growth as opposed to initiation of development. Although the current investigation was limited to one euthanasia time point (18 wk), given that \textit{Apc}\textsuperscript{Min}\textsuperscript{-/-} mice develop most of their polyps by 12 wk of age, it is likely that an even larger disparity in polyp growth, but not number, would be observed if mice had been euthanized 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 (28). Similarly, a recent rodent study showed a decrease in the size of tumors in an MCP-1 receptor-deficient mouse model of colitis-associated colon cancer (31). Furthermore, it has been reported that ablation of D6, a promiscuous decay receptor that scavenges inflammatory chemokines, increases colitis, and results in more severe malignancies in colitis-associated cancer (40). Our findings are also confirmed by the only available clinical study reporting an association between MCP-1 expression and stage of colorectal cancer (1).

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. Although 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 on other cancer models. For example, knockout of MCP-1 has been shown to enhance tumor cell apoptosis in mammary carcinoma cells (18), and MCP-1 inhibited apoptosis in PC-3M prostate cancer cells (34). However, we did not detect an effect of MCP-1\textsuperscript{-/-} 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 (44). 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 \(\beta\)-catenin with MCP-1 knockout. In contrast, a recent study reported less nuclear \(\beta\)-catenin accumulation in an MCP-1 receptor-deficient mouse model of chronic colitis (31). 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 (2, 4). These macrophages can represent up to 50% of the tumor mass producing a wide array of inflammatory mediators with protumoral functions (27, 35). Furthermore, abundance of TAMs has been associated with poor prognosis in colon cancer (1, 19–21, 27, 35). 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 (31). Furthermore, in a clinical study it was documented that macrophage accumulation within the tumor was correlated with stage of progression (1). Consistent with these reports, our MCP-1 deficient \textit{Apc}\textsuperscript{Min}\textsuperscript{-/-} 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) (35), 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 protumoral functions (35). 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-\(\beta\), and CCL17) macrophage phenotypic markers in the tumor environment as well as surrounding intestinal tissue in the \textit{Apc}\textsuperscript{Min}\textsuperscript{-/-} mouse. Although 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 (38, 42). For example, adoptive transfer of CD8\(^{+}\) cells has been shown to control the growth of B16 melanoma in mice (14). 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 (11, 33). 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 (12, 13). 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-1\(\beta\), IL-6, and TNF-\(\alpha\) have been associated with poor outcome in colon cancer (10, 24). 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, 15, 30). Our study in \textit{Apc}\textsuperscript{Min}\textsuperscript{-/-}/MCP-1\textsuperscript{-/-} 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 \(\beta\)-catenin accumulation in SOCS1-deficient mice, suggesting its role as an anti-oncogene that prevents inflammation-mediated carcinogenesis (16). Our data show a reduction in SOCS1 expression in the polyps and surrounding intestinal tissue in \textit{Apc}\textsuperscript{Min}\textsuperscript{-/-} 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 (37). 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 inflam-
Inflammatory mediators is mirrored by elevations in protein concentrations (28).

Changes in both WBC and RBC counts are characteristic in colon cancer and have been show to occur in the Apc\textsuperscript{Min+/+} mouse (8). We therefore also performed a complete blood count to determine the role of MCP-1 on these parameters in the Apc\textsuperscript{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\textsuperscript{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\textsuperscript{Min+/+} mouse model of intestinal tumorigenesis. Using an Apc\textsuperscript{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 compared with MCP-1-sufficient Apc\textsuperscript{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.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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