MK2 contributes to tumor progression by promoting M2 macrophage polarization and tumor angiogenesis
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Chronic inflammation is a major risk factor for colorectal cancer. The p38/MAPKAP Kinase 2 (MK2) kinase axis controls the synthesis of proinflammatory cytokines that mediate both chronic inflammation and tumor progression. Blockade of this pathway has been previously reported to suppress inflammation and to prevent colorectal tumorigenesis in a mouse model of inflammation-driven colorectal cancer, by mechanisms that are still unclear. Here, using whole-animal and tissue-specific MK2 KO mice, we show that MK2 activity in the myeloid compartment promotes tumor progression by supporting tumor neoangiogenesis in vivo. Mechanistically, we demonstrate that MK2 promotes polarization of tumor-associated macrophages into protumorigenic, proangiogenic M2-like macrophages. We further confirmed our results in human cell lines, where MK2 chemical inhibition in macrophages impairs M2 polarization and M2 macrophage-induced angiogenesis. Together, this study provides a molecular and cellular mechanism for the protumorigenic function of MK2.

Significance

Colon cancer is the third most common cancer type and the second leading cause of cancer-related death. Chronic inflammation is a major contributor to colon cancer development and progression, but the molecular details of how inflammation contributes to colon cancer remain unclear. The p38/MAPKAP Kinase 2 (MK2) pathway is a central mediator of cell stress and inflammation. Here, we examine the importance of this pathway in an inflammation-driven model of colon cancer using whole-body and tissue-specific MK2 knockouts, in combination with measurements of macrophage polarization, endothelial proliferation, and morphogenesis. We demonstrate that the MK2 pathway promotes colon tumor development by regulating the polarization of macrophages into an M2 tumor-promoting state that modulates the tumor microenvironment and enhances tumor angiogenesis.


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multiple external stimuli into specific cellular responses. The p38α/MAPK/MAPKAP Kinase 2 (MK2) axis is a master regulator of cellular stress, responding to a plethora of external stress signals such as osmotic shock, heat, DNA damaging agents, inflammatory cytokines, and pathogen-associated molecular patterns (PAMPs) to drive specific cellular responses. Following inflammatory stimuli (i.e., Toll-like receptors, TNF-α, or IL-1 receptor activation), activation of the p38α-MK2 pathway posttranscriptionally up-regulates proinflammatory cytokines and mediators including IL-1β, IL-4, IL-6, IL-8, GM-CSF, IFN-γ, TNF-α, and COX2 (11). Consistent with its putative role in regulation of proinflammatory cytokine expression, genetic ablation of MK2 was recently reported to abrogate tumor formation in a mouse model of colitis-associated cancer, although the underlying mechanisms remain unclear (12).

Here, we show that MK2 function within myeloid cells is the most relevant activity necessary for tumor promotion under conditions of chronic inflammation. We observe that MK2 is crucial for M2 activation of tumor-associated macrophages and tumor neoangiogenesis. These findings support myeloid-specific blockade of MK2 as a chemopreventive strategy for IBD patients to prevent CRC progression.

Results
MK2 Promotes Tumorigenesis in a Murine Model of Colitis-Associated Cancer. To evaluate the role of MK2 in colitis-associated tumor development, we crossed our previously described mouse model for targeted and reversible MK2 inhibition (MK2 Cre-versible or CV) (13) with a β-actin–Cre driver mouse line to generate whole-body MK2 knockout (KO) mice (Fig. S1.A and B). Loss of MK2 protein was confirmed both by Western blot (WB) and immunohistochemistry on diverse murine tissues (Fig. S1.C and D).

Inflammation-driven colon tumorigenesis was modeled in these MK2 KO mice using the azoxymethane (AOM)/dextran sodium sulfate (DSS) protocol. This widely utilized procedure includes the administration of a DNA damaging agent, AOM, followed by cycling doses of DSS to promote colitis (14) (Fig. L4). This treatment generated visible tumors in the distal colon after 100 d. WT and MK2 KO mice were subjected to this treatment protocol, colonies were harvested, longitudinally opened, and the number of tumors were counted and tumor areas measured under the dissecting scope. In contrast to Ray et al. (12) who reported a complete absence of tumors in MK2-deficient mice after AOM/DSS treatment, we observed tumors in both the WT and MK2 whole-body knockout animals (Fig. 1B). However, MK2 depletion led to significantly smaller tumors and reduced total tumor burden in the MK2 KO mice compared with MK2-proficient mice (Fig. 1C), consistent with the proinflammatory role of MK2. There was also a trend toward fewer tumors in MK2 KO mice, although this failed to reach statistical significance. Tumors were processed for histology and blindly evaluated by an expert pathologist. Neoplasms were identified as low-grade adenomas in both groups (Fig. 1D).

Myeloid-Specific Depletion of MK2 Phenocopies Constitutional MK2 Loss in a Murine Model of Colitis-Associated Cancer. MK2 is ubiquitously expressed, and its activity has been demonstrated to be crucial for both epithelial and immune cell responses to multiple types of cellular stress, such as DNA damage, osmotic stress, heat, and inflammation. To gain additional insight into the mechanism through which MK2 promotes colorectal tumorigenesis, we next examined the contribution of MK2 signaling in specific cellular lineages by genetically engineering conventionally floxed MK2 conditional KO mice (Fig. S2.A–C). We generated tissue-specific knockouts by crossing this line to tissue-specific Cre driver mouse strains. We used Villin-Cre and LysM-Cre mice to specifically deplete MK2 in the intestinal epithelia (IEC-KO) and myeloid lineages (LysM-KO), respectively.

MK2 loss in myeloid cells was confirmed by WB on magnetically isolated CD11b+ cells from the bone marrow of LysM-KO mice (Fig. S2.D). CD45+CD11b+ cells from blood, bone marrow, and spleen were additionally FACS sorted and processed for RNA isolation to measure MK2 expression by RT-PCR. As expected, we observed 80% and 90% loss of expression in blood and bone marrow-derived cells, respectively, but only a 60% inhibition in myeloid cells isolated from spleen (Fig. S2E). This pattern of MK2 inhibition is consistent with previous work on LysM-Cre mice, where depletion efficiency of 83–98% was observed in mature macrophages and near 100% in granulocytes (15). Partial depletion (16%) was detected in CD11c+ splenic dendritic cells, which are closely related to the monocyte/macrophage lineage (15). MK2 expression in intestinal mucosa was also measured by immunohistochemistry (IHC) on LysM-KO mice, where there was a clear down-regulation of MK2-positive infiltrated immune cells, with MK2 expression retained in intestinal epithelia and some subsets of lymphocytes (Fig. S2F).

MK2 loss in intestinal epithelial cells (IEC) was confirmed by Western blotting of isolated intestinal crypts from both small intestine and colon (Fig. S2F), as previously reported in the Villin-Cre mouse model (16). MK2-specific loss in gut epithelium was further confirmed by IHC on colon slice (Fig. S2G).

Remarkably, the colonic tumor phenotypes of these tissue-specific MK2 KOs differed substantially. MK2 loss in intestinal epithelia resulted in enhanced tumor incidence compared with controls, suggesting that MK2 activity in the epithelial compartment opposes tumor formation (Fig. 2A and B). We have previously demonstrated that the MK2 pathway plays an important role in maintenance of the G1/S and G2/M checkpoints after DNA damage, extending the time available for DNA repair (17, 18). When IEC MK2 KO animals were treated with AOM, and their colons were examined 24 h later, there was a notable increase in
the number of cells within the lower two-thirds of the intestinal crypts (where intestinal stem cells and transient amplifying cell populations are located) that stained positively for γH2AX, a marker of persistent DNA damage and double-strand breaks, compared with floxed controls (Fig. S7A). The number of apoptotic cells in these crypts, however, was similar between the KO and floxed controls (Fig. S7B), suggesting that increased amounts of DNA damage after AOM treatment in the amplifying cell population in the intestinal crypts of the IEC KO may contribute to the increased number of tumors that were observed in these animals.

In contrast to the intestinal epithelial cell-specific KO mice, myeloid-specific MK2 KO (LysM-Cre driven) mice developed significantly smaller tumors than littermate controls, recapitulating the global MK2 KO phenotype (Fig. 2 C and D). These findings suggest that MK2 activity in the myeloid cell lineages plays a dominant role in promoting tumor progression, and this myeloid-specific role was subsequently explored in more detail.

**MK2 Deficiency Impairs M2 Polarization in Tumor-Associated Macrophages.**

To better explore the cellular basis for the tumor-promoting role of MK2 in the myeloid cells, we investigated the recruitment of specific myeloid cell subsets to tumor stroma in MK2 KO mice. IHC was used to measure myeloid cell infiltration into tumors by staining for myeloid cell type-specific markers: pan-myeloid (CD11b), neutrophils (MPO), neutrophils/monocytes (Gr1), and macrophages (F4/80). No significant differences in broad categories of myeloid cell recruitment were observed between WT and MK2 global KO mice (Fig. S3). Furthermore, no significant differences in myeloid cell recruitment to the tumor stroma were observed in the myeloid-specific MK2 KO and intestinal epithelial-specific MK2 KO tumors (Fig. S4). However, we noted striking differences when we measured specific markers of classical (M1) or alternative (M2) activation of macrophages. AOM/DSS tumors recruited high numbers of arginase-1–expressing cells, which is associated with M2-like macrophages. Arginase-1–positive cells, however, were almost completely absent in MK2 KO mice tumors (Fig. 3A). Conversely, iNOS-positive cells (typical of M1 macrophages) were significantly higher in MK2 KO tumors compared with WT (Fig. 3B).

These observations suggest that MK2 is potentially involved in macrophage polarization. To directly explore whether MK2 is required for M1/M2 polarization in a cell-autonomous manner, we generated bone marrow-derived macrophage cultures from both MK2 WT and KO animals. Bone marrow-derived cells differentiated into macrophages with comparable efficiency in both the MK2 WT and KO, with ~70% CD11b+ F4/80+ cells (Fig. S4). This finding agrees well with the observation that similar numbers of F4/80+ cells were recruited to tumors in MK2 WT and KO mice (Fig. S3D).

Macrophage polarization to the M2 phenotype was induced by IL-4 stimulation and assessed by flow cytometry. Expression of CD206, Arginase-1, and Resistin-like alpha (Retnla), which are hallmarks of murine M2 macrophages (19, 20), were significantly lower in MK2 KO macrophages compared with WT (Fig. 3C and D), suggesting a defect in M2 polarization in the absence of MK2. We also measured IL-10 expression, a hallmark M2 cytokine, and observed a trend toward lower levels of IL-10 in MK2 KO macrophages.

We next used LPS/IFN-γ stimulation to drive M1 polarization. This was assessed by CD68 surface expression and nitric oxide (NO) release, which are hallmarks of the M1 phenotype (21) (Fig. 3 E and F). MK2 depletion did not affect M1 polarization efficiency when assessed by CD68 and NO release. However, the mRNA levels of the canonical M1 effectors iNOS, TNF-α, and IL1-β were significantly lower in the MK2 KO compared with WT macrophages (Fig. 3G). These observations suggest that MK2 is not required for M1 polarization but regulates M1 cytokine expression, which is in good agreement with previous reports showing that MK2 promotes the synthesis of TNF-α downstream of TLR4 activation (22) and regulates the expression of IL-1β (23).

Curiously, we also observed IL-10 expression after M1 stimuli in both in WT and MK2 KO macrophages, even more than after M2 stimuli. This could be due to prolonged exposure to LPS, as reported by Chang et al. (24), although in both M1 and M2, polarized cells we found a trend toward reduced IL-10 expression with loss of MK2 (Fig. 3D).

Interestingly, myeloid-specific depletion of MK2 in vivo recapitulated these in vitro observations from macrophage cultures. Both the number of Arginase-1 and iNOS-positive cells were reduced in colonic tumors from LysM-KO mice compared with WT (Fig. 3H and I). Thus, combining these in vivo and in vitro findings, we conclude that MK2 broadly regulates macrophage biology. MK2 is required for efficient M2 polarization in a cell-autonomous manner, but is also important for optimal M1 macrophage function, since MK2 regulates the extent of production of several M1 cytokines.

**Myeloid MK2 Depletion Halts Tumor Angiogenesis.** We observed that global or myeloid-specific loss of MK2 resulted in a reduction in tumor size and a defect in macrophage M2 polarization. We next explored if these two phenomena were functionally connected. M2 macrophages are well-known tumor promoting agents (25). Among their tumor-promoting functions, they are major contributors to tumor angiogenesis because they secrete factors that help tumors to neovascularize and promote tissue remodeling that facilitates cell invasion and metastasis (9). We therefore examined the role of MK2 in tumor vascularization in vivo by immunostaining for the endothelial marker CD31. Interestingly, CD31-positive staining was significantly reduced in both whole-body and myeloid-specific KO mice tumors compared with their corresponding controls, suggesting a myeloid-driven role for MK2 in promoting tumor angiogenesis (Fig. 4B).
To further demonstrate a direct link between MK2-dependent myeloid secreted factors and angiogenesis, we established an in vitro protocol to evaluate endothelial responses to MK2-dependent macrophage secreted factors. In these experiments, the human monocytic cell line U-937 was differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA). Once cells were differentiated into M0 macrophages, M1 and M2 polarization was induced by stimulation with specific cytokine combinations, in the presence or absence of the MK2 inhibitor PF 3644022. Similar to what we observed in murine MK2 KO macrophages, M1 polarization efficiency of U-937 cells in response to LPS and IFN-γ was not significantly affected by the presence of the MK2 inhibitor, as assessed by the up-regulation of human M1 hallmark surface markers HLA-DR and CD86 (26) (Fig. S5A). The levels of M1 cytokine production, however, were reduced by the PF3644022 drug, exactly as observed with the murine MK2 KO macrophages (Fig. S5B). When M2 polarization was induced by IL4 and IL-13, M2 polarization efficiency was markedly reduced in the presence of MK2 inhibitor, as judged by both surface expression of the M2 marker CD163 (Fig. S5C) as well as by the pattern of M2 gene expression for TGM-2, IL-10, IL1RN, and PPARG, all of which are characteristic of M2 polarization in human macrophages (Fig. S5D) (21).

![Fig. 3.](image-url) MK2 deficiency impairs M2-like polarization of TAMs. (A) Representative pictures of Arginase-1 immunodetection in WT (Left) and whole-animal MK2 KO (Right) colon tumors. Stained slides were scanned and quantified in an automated manner using the positive nuclei algorithm in ImageScope and normalized by area analyzed in square millimeters. (B) Representative pictures of Nos2 immunodetection in WT (Left) and whole-animal KO (Right) colon tumors. Stained slides were scanned and quantified as in A. (C) Bone marrow-derived macrophages from WT and KO mice were polarized to M1 and M2 phenotypes. Twenty-four hours after polarization, surface marker expression was quantified by FACS. Percentages of CD206-positive cells were calculated out of total macrophages (CD11b+ F4/80+ cells). (D) Expression of hallmark M2 genes was measured by Q-PCR, actin was used for normalization, and fold change versus the corresponding unstimulated control was calculated. (E) Percentages of CD86-positive cells were calculated out of total macrophages (CD11b+ F4/80+ cells) by FACS. (F) Nitrite concentrations in the supernatants of MK2 WT and KO macrophages polarized to M1 or M2 as determined by the Griess reaction. (G) Expression of hallmark M1 genes was measured by Q-PCR. Actin was used for normalization and fold change versus the corresponding unstimulated control was calculated. (H) Representative pictures of Arginase 1 immunodetection in myeloid-specific KO tumors (Right) and littermate controls (Left). Stained slides were analyzed as in A. (I) Representative pictures of Nos2 immunodetection in myeloid-specific KO tumors (Right) and littermate controls (Left). Stained slides were analyzed as in B. (Scale bars: 200 μm.) *P < 0.05, **P < 0.01, ****P < 0.0001; Student’s t test (A, B, H, and I), two way-ANOVA. Data in C–G is from at least three independent experiments. Mouse images courtesy of Clker/Barretto.)

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Fig. 4. MK2 loss in the myeloid compartment halts tumor angiogenesis. (A) Representative pictures of blood vessels detection by CD31 staining in MK2 whole-animal KO (Upper) and myeloid-specific KO (Lower) and their corresponding controls. Insert in Upper Left shows typical blood vessel morphology when viewed under higher magnification. Stained slides were scanned and automated quantified using the positive pixel algorithm in ImageScope. Strong positive pixels counts were normalized by area of tumor analyzed. *P < 0.05, Student’s t test. (B) ECIS cell impedance (resistance) of confluent HUVEC cultures over time. Wounds were induced by electroablation as indicated, and confluency recovery was monitored over time in the presence or absence of macrophage-derived conditioned media. Data represent three independent experiments. (C) Rate of recovery for the first 25% of confluence was calculated per experimental group. *P < 0.05; **P < 0.01; ****P < 0.0001, one-way ANOVA. (D) HUVEC tubule formation morphogenesis assays were performed in the presence or absence of various types of macrophage-derived conditioned media. (E) Number of nodes, junctions, segments, and mesh area were measured in an automated manner using the angiogenesis analyzer plug-in in ImageJ. Data were normalized to values from untreated control (no conditioned media), which represents 100%. *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA. (Scale bars: A, 200 μm; D, 400 μm.) Mouse images courtesy of Clker/Barreto.
A proposed model for MK2-dependent macrophage function in inflammation-driven colonic tumorigenesis. Early inflammation is dominated by classically activated M1 macrophages. Persistent chronic inflammation allows rise of oncogenic mutations, which can be eliminated by M1 macrophages. However, when the lesions progress, macrophages in the tumor microenvironment acquire an M2-like phenotype. M2-like macrophages promote tumor neovascularization and angiogenesis. This macrophage shift from the M1 to M2 state is also known as “the angiogenesis switch.” MK2, as a key regulator of macrophage biology, is required for that angiogenesis switch in TAMs; therefore, MK2 inhibition leads to poor tumor vascularization and delayed tumor progression. Mouse images courtesy of Clker/Barretto. Tumor angiogenesis, macrophages, and carcinoma images adapted from Servier Medical Art.

Fig. 5. A proposed model for MK2-dependent macrophage function in inflammation-driven colonic tumorigenesis. Early inflammation is dominated by classically activated M1 macrophages. Persistent chronic inflammation allows rise of oncogenic mutations, which can be eliminated by M1 macrophages. However, when the lesions progress, macrophages in the tumor microenvironment acquire an M2-like phenotype. M2-like macrophages promote tumor neovascularization and angiogenesis. This macrophage shift from the M1 to M2 state is also known as “the angiogenesis switch.” MK2, as a key regulator of macrophage biology, is required for that angiogenesis switch in TAMs; therefore, MK2 inhibition leads to poor tumor vascularization and delayed tumor progression. Mouse images courtesy of Clker/Barretto. Tumor angiogenesis, macrophages, and carcinoma images adapted from Servier Medical Art.

described above, with or without the MK2 inhibitor, for 24 h, then washed and recultured in cytokine/inhibitor-free media for an additional 24 h to generate macrophage-conditioned media. This media was then tested for its effects on endothelial cell proliferation, migration, and blood vessel formation. Endothelial cell proliferation and migration was assessed in HUVEC wound healing assays using an electric cell-substrate impedance system (ECIS) device, following standard published procedures (27–29). This system enables accurate dynamic tracking of transepithelial electrical resistance as a readout of cellular confluency and permeability of tight junctions. In brief, HUVEC cells were grown to confluency, and a radial clear zone of defined size (354 μm diameter) was created by electroablation. The closure of the wound was followed over time (Fig. 4B). Media from U-937-derived M2 macrophages significantly enhanced the rate of closure relative to media from the parental nonpolarized cells (purple versus black line), in agreement with a proangiogenic role for M2 macrophages. In contrast, media from M1-polarized U-937 macrophages markedly impaired the ability of HUVEC cells to proliferate, migrate, and heal the wound (orange versus black line). The presence of the MK2 inhibitor during the first 24 h of polarization resulted in a small but reproducible statistically significant decreased rate of recovery, suggesting that MK2 signaling regulates secreted factors that promote endothelial cell proliferation. Inhibition of MK2 signaling in M1 cells resulted in an even further impairment of their conditioned media to drive endothelial wound healing relative to controls (Fig. 4B, pink and yellow lines). The rate of wound healing during the first 25% of closure was quantified. As shown in Fig. 4C, the wound closure velocity was significantly higher when HUVEC cells were in the presence of M2-derived media, whereas M1 media slowed down recovery. MK2 inhibition decreased the rate of monolayer recovery in both cases.

We also studied the ability of these conditioned media to directly promote blood vessel formation using tubule formation assays, as described by Kubota et al. (30). In these experiments, HUVEC cells were seeded onto matrigel, which induces their differentiation into capillary-like structures (Fig. 4D) (30). In agreement with a proangiogenic role of M2 macrophages (31, 32), M2-derived conditioned media significantly increased the number of junctions, nodes, segments, and total mesh area in the tube networks that were generated relative to media from nonpolarized controls. Interestingly, M1-conditioned media also promoted the morphogenic capacity of HUVEC cells, albeit to a lesser extent (Fig. 4E), in contrast to the inhibitory effect seen previously on cell proliferation (Fig. 4B and C). Interestingly, conditioned media from MK2-inhibited M2 macrophages, but not from MK2-inhibited M1 macrophages, showed significantly less morphogenic potential than their corresponding uninhibited controls, as evidenced by lower numbers of junctions, nodes, segments, and total mesh area following MK2 inhibition. Taken together, these results demonstrate an important role of MK2 in macrophage M2 polarization, and its subsequent effects on endothelial cell proliferation, migration, and blood vessel morphogenesis.

**Discussion**

In this study, we have identified MK2 as a regulator of M1/M2 polarization in tumor-associated macrophages. MK2 regulates several aspects of macrophage biology, including both regulating M2 polarization and M1 cytokine production. In agreement with a proangiogenic role of M2-like macrophages, tumors developed in MK2 KO mice showed markedly less vascularization and reduced tumor size in a colitis-associated cancer model. Our results are in agreement with previous studies that link either p38 or MK2 to inflammation-driven tumorigenesis (12, 33, 34). Here, however, we have established the underlying molecular and cellular mechanisms by selective ablation of MK2 activity in myeloid versus epithelial cellular linages and identified the myeloid compartment as most relevant for tumor promoter activities of MK2 in the colon.

The AOM/DSS model is based on the DNA alkylation function of the mutagenic molecule AOM, and the tumor promoting effects of chronic inflammation as a result of cyclic colonic damage induced by DSS. Because of its high reproducibility and potency, as well as the simple and affordable mode of application, the AOM/DSS has become a well-accepted model for studying colon carcinogenesis and a powerful platform for chemopreventive intervention studies (35). Both MK2 whole-body and myeloid-specific KO mice developed significantly smaller tumors, which indicates an epithelial-independent promoting role of MK2 in colonic tumorigenesis. Conversely, specific MK2 loss in epithelial cells resulted in an increased number of adenomas. We have previously shown that MK2 is involved in DNA damage repair and maintenance of cell cycle checkpoints in cancer cells (13, 36, 37). It is plausible then, that after AOM-induced DNA damage, MK2 is required for DNA damage repair, cell cycle arrest, and/or apoptosis in intestinal cells, influencing the number of mutated cells that will progress into adenomas. In agreement with this, the frequency of DNA double-strand breaks after AOM insult was significantly higher, with no corresponding
increase of apoptotic cells, in IEC-KO mice than in controls (Fig. S7). This imbalance of damaged cells and apoptosis could plausibly lead to the accumulation of mutated cells that have the potential of becoming an adenoma after chronic inflammation. We focused here on the mechanism by which myeloid MK2 inhibition delays tumor progression as a potential therapeutic strategy for cancer prevention in IBD patients.

In vivo MK2 loss led to decreased infiltration of arginase-positive cells in tumors, which are normally identified as M2 macrophages. However, myeloid suppressor cells also express arginase (38). We therefore cannot exclude the possibility that at least some of the Arginase-1-positive cells are MDSCs. Our in vitro experiments however, in both murine and human systems, demonstrated a clear role of MK2 in broad macrophage biology, including polarization into M2 macrophages and macrophage production of proangiogenic factors, consistent with the proposed mechanism for the observed mouse phenotypes. A functional role for MK2 in other myeloid cell types, including neutrophils and dendritic cells (39–41), could also contribute to the observed phenotype.

Cancer is associated with a marked increase in the number of macrophages, particularly M2, which has previously been shown to correlate with metastasis and poor prognosis of the disease (42). It has been proposed that a gradual switching of TAM polarization takes place, from M1 to M2, an early tumorigenesis that may contribute to elimination of cancer cells, to M2-like as the tumor progresses and becomes a “wound that does not heal” (43). TAMs influence tumor progression at different levels. TAMs influence the intrinsic properties of tumor cells, as well as those of the tumor microenvironment, for example, by producing growth factors, such as EGF, which stimulates cancer cell proliferation. TAMs also produce proteolytic enzymes that digest the extracellular matrix to promote tumor cell dissemination and metastasis. Furthermore, TAMs promote angiogenesis and lymphangiogenesis, as well as tissue remodeling by, for example, stimulating the deposition of fibrous tissue. All of these processes can support tumor development and progression (32). AOM/DSS tumors also accumulate M2 macrophages as they progress (44), and clodronate-mediated depletion of macrophages significantly decreases tumor burden in this model (45). Consistent with the reduced number of M2 macrophages, we observed a decrease in CD31 staining in tumors, which labels both blood and lymph vessels, both in systemic and myeloid-specific MK2 KO. Therefore, we hypothesized that MK2 depletion leads to inefficient macrophage-driven angiogenesis and a consequent reduction in tumor size. We tested our hypothesis on isolated vitro systems, where we could interrogate the MK2 macrophage-specific contribution to endothelial cells proliferation, migration and morphogenesis. We demonstrated that macrophages secrete proangiogenic factors that are MK2 dependent. Indeed, MK2 regulates the expression of IL-8, IL-6, VEGF, COX-2, IL-1α, and IL-1β (23, 46–48), all cytokines with angiogenic capacities. The exact identity and specific mechanism of regulation of proangiogenic factors by MK2 in our system are not yet known.

We also looked at the adaptive immune components of the tumor microenvironment in both the myeloid-specific and intestinal epithelial-specific MK2 KO animals. Interestingly, we saw opposite effects of MK2 inhibition in these two KO genotypes. In the LysM-KO versus WT macrophages, however, and myeloid-specific control animals, there were no differences in tissue type. In the LysM-KO versus floxed controls, there were no differences in tissue type. Interestingly, we saw opposite effects of MK2 inhibition in these two KO genotypes. In the LysM-KO versus WT macrophages, however, no significant differences in T-cell recruitment into the tumor stroma, but there was decreased B-cell recruitment (Fig. S4 D–H). In contrast, the IEC-KO tumors showed an increased number of CD3-positive T cells and reduced numbers of CD8 and FoxP3-positive T cells compared with the floxed littermate controls, while B cell numbers were not affected (Fig. S4 D–H). These observations further indicate that MK2 has opposing roles in the epithelial and myeloid compartments, particularly with respect to modulation of the adaptive arm of the immune system, in addition to its role in myeloid-driven tumor neoangiogenesis.

Blockade of the p38/MK2 axis has been proposed as a mechanism to treat diverse chronic inflammatory disorders, including IBD (49). Given the unacceptable side effects of systemic p38 inhibition, MK2 inhibitors are currently under active development, encouraged by the full viability and increased resistance to endotoxic shock and collagen-induced arthritis seen in MK2 KO mice (48). Our whole-body and tissue-specific KO results are in agreement with recent studies testing the efficacy of MK2 inhibition on regression of colon tumors in vivo (50). Our study, together with others, encourages the potential use of MK2 inhibition not only to prevent chronic inflammation but also for cancer prevention and treatment in high cancer risk IBD patients.

Methods

Mice. To generate MK2 null mice, the MK2fl/fl mice previously generated in our laboratory (which are fully MK2 proficient in the absence ofCre recombinase activity; ref. 13), were crossed with β-actin promoter-driven Cre mice (51), in which Cre is ubiquitously expressed in all tissues. Mice with a germ-line inversion of exon 2 were obtained, and the Cre driver gene subsequently bred out of the colony, resulting in a mouse line with a stable exon 2 inversion, and a complete loss of MK2 expression in all tissue types, as verified by PCR, WB, and IHC. Examples of selected tissues from these whole-body MK2 KO animals are shown in Fig. S1 C and D. Animals were genotyped at birth using a strategy developed by our laboratory (43). Primers are internal: 5′-GAC-3′; P2: 5′-GAGCTCTCCACCATCGA-3′; P3: 5′-GCAGACAGCCCATATGGAT-3′; P4: 5′-AGTCTTCCCTCGT-AGAC-3′. Both mice strains were backcrossed 10 generations to C57/BL6N (Taconic) strain.

To generate mice carrying conditional alleles of MK2, we constructed a targeting vector based on the pBluescript variant pKS-DTA (generously provided by A. Ventura, David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA). The Bac clone (BAC clone number 12812) (Bacillus Resource Center) spanning the entire genomic focus encoding MK2 served as the template for PCR-directed cloning. The final targeting construct consists of 2.7 kb of the 5′ flanking sequence before exon 2, a LoxP site, exon 2, followed by a second LoxP site, a FRT-Pgk::Neo-FRT cassette to select for integration of the targeting construct into ES cells, and exons 3–10. The targeting construct was linearized using Ascl, which cuts at a unique site in the pBluescript parent backbone of the targeting construct, and electroporated in 129/E1 hybrid ES cells. Transfected ES cell clones were selected by growth in media containing neomycin for 1 wk. Proper integration was monitored using Southern blot analysis from Spel genomic DNA digests to monitor the 5′ integration event using a 300-bp fragment from the genomic sequence in intron 1–2 that lies outside the targeting construct as probe A (Fig. S2 A and B). The probe was generated by PCR amplification following digestion with Spel and priming. In the WT genomic MK2 sequence, the probe sites in intron 1 and intron 10 gives rise to a 16.3-kb band, while correct 5′ integration of the targeted allele gives rise to a 11-kb band due to the presence of a Spel site within the Pgk::Neo cassette. The 3′ integration event was monitored by Southern blotting using a 300-bp probe B corresponding to the 3′ flanking region outside the targeting construct. The targeted allele gives rise to a 7.2-kb band (Fig. S2 A and B).

One ES cell clone containing the integrated MK2 floxed (FL) allele was injected into host albino FBNB blastocysts and implanted into pseudopregnant mice. Chimeric mice were crossed to C57-FLPe mice to eliminate the FRT-Pgk::Neo-FRT cassette. The offspring was genotyped by PCR using primers flanking exon 2 and spanning the second LoxP site (P1 and P2) (Fig. S2C).

MK2 FL/FL mice were then crossed with Villin-Cre (16) and Lyz2tm1(cre)Ifo (15) strains to generate the specific depletion of MK2 on intestinal epithelia (IEC-KO) and myeloid lineage (LysM-KO), respectively. IEC-KO mice and control littermates were maintained in a mixed C57/BL6N × 129SvJ background, whereas LysM-KO and control littermates were backcrossed five generations to C57/BL6N. Animals containing tissue specific knockouts were always compared with their matched littermate controls.

Murine Colitis-Associated Cancer. Eight- to 12-wk-old male mice were administered 2.5% DSS (MP Biochemicals) in the drinking water for 5 d every 21 d, for a total of five cycles to induce chronic inflammation. Colon tumors were generated by i.p. administration of 10 mg/kg AOM (Sigma) before chronic DSS administration (14). Colons were harvested and tumors were
examined 100 d after AOM administration under a dissecting scope. Tumor images were taken and tumor size was measured using ImageJ (S2).

All mouse studies described here were approved by the Massachusetts Institute of Technology Institutional Committee for Animal Care and conducted in compliance with the Animal Welfare Act regulations and other federal statutes relating to animals and experiments involving animals and adhere to the principles set forth in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996 (Institutional Animal Welfare Assurance No. A-3125-01).

Intestinal Crypt Isolation. Eight- to 10 wk-old male mice were CO2 euthanized, and both small intestines and colons were collected and washed in cold PBS before incubation in 4 mM EDTA, 1 mM DTT PBS solution with rocking for 45 min at 4 °C. After incubation, tissue was transfused to fresh PBS and shaken manually for 2 min to release intestinal crypts. The muscle layer was removed and the released crypts in PBS were filtered through a 70-μm filter to remove villi. Intestinal crypts were lysed in RIPA buffer containing a commercial protease inhibitor mixture (Roche) for protein extraction studies.

Immunohistochemistry. Colon sections were dewaxed and rehydrated before heat-mediated antigen retrieval in citrate buffer (pH 6). Anti-MK2 (CST D4E11, 1:200), anti-F4/80 (Thermo Scientific MF48000, 1:100), anti-MPO (ThermoScientific D4D115, 1:100), anti-CD11b (eBioscience 14-0011-1200, 1:200), anti-Arg1 (CST 93668S, 1:100), anti-iNOS (CST 131205, 1:400), anti-CD31 (Abcam, ab28364, 1:200), anti-iCD3 (Abcam ab56900, 1:200), anti-FoxP3 (eBioscience 14-0808-30, 1:200), anti-CD4 (eBioscience 14-9766-80, 1:200), anti-CD8 (14-5773-80, 1:200), anti-B220 (eBioscience 16-0452-85), anti-CD4 (eBioscience 14-9766-80, 1:200), anti-CD86 (eBioscience 14-0808-30, 1:200), anti-CD3 (CST 97/18, 1:400), anti-CC3 (CST 9661, 1:400) antibodies were used for immunohistochemistry. Impact DAB (Vector) secondary antibodies were used, and samples were hematoxylin counterstained before mounting. Slides were scanned in a Leica slide scanner before analysis in Aperio Imagescope software.

CD11b+ Cells Isolation. Mice were CO2 euthanized and femurs and tibias were collected, cleared of adherent muscle, and flushed with a 21G syringe to collect bone marrow in cold PBS containing 2% FBS. Red blood cells (RBCs) in the sample were lysed in RBC lysis buffer (eBioscience) for 5 min at room temperature. Biotin-labeled CD11b antibody (Miltenyi Biotec) was used to isolate myeloid cells using CELLection Biotin Binder Kit (Thermo Scientific) following the manufacturer's instructions. Both CD11b+ and CD11c+ cells were then processed for Western blotting to measure MK2 expression.

Blood was collected by cardiac puncture, and leukocytes were recovered after RBC lysis. Spleens were collected and mechanically dissociated between frosted glass slides. Spleenocytes were recovered after RBC lysis and processed for cell sorting. CD11b+ cells were isolated using a MoFlo sorter and processed for RNA extraction to measure MK2 expression.

Q-PCR. RNA was extracted using TRIzol reagent (Ambion) according to the manufacturer's instructions, and 1–2 μg of total RNA was used for reverse transcription using the SuperScript III First-Strand Synthesis Kit (Invitrogen) and oligo-dT priming as per the manufacturer's instructions. For qPCR, cDNA was amplified using SYBR green PCR mastermix (Applied Biosystems) according to the manufacturer's cycling conditions for 40 cycles on a Bio-Rad C1000 Thermal Cycler. Data were analyzed using the delta-delta Ct method as described previously (53) and plotted as fold change versus control. Primers used are listed in Table S1.

Western Blotting. For Western blotting, 15 μg of total protein was resolved by 10% SDS-PAGE before immunoblotting. Primaries antibodies were used as follows: anti-MK2 (CST D4E11, 1:2000), anti-GAPDH (Sigma GB796, 1:2000), anti-i-tubulin (Sigma T7816, 1:2000). Fluorescently labeled secondary antibodies were used for protein detection using an Odyssey fluorescence imaging system.

Murine Bone Marrow-Derived Macrophage Differentiation and Polarization. Bone marrow cells were isolated from the femurs and tibias of MK2 WT and MK2 KO mice by flushing with IMDM (10% FBS with antibiotics) or by briefly centrifuging the bones at 15,000 × g for 15 s at 4 °C. Eppendorf tube. After RBC lysis in RBC lysis buffer (eBioscience), 2 × 106 cells per mL were seeded on 10-cm bacterial plates in IMDM supplemented with 10 ng/mL murine M-CSF. An equal volume of M-CSF–supplemented media was added on top of the existing media 72 h after seeding. On day 7 after seeding, cells were detached with 5 mM EDTA (in PBS without Ca and Mg) and replated on 24-well tissue culture plates at 200,000 cells per well and/or on 6-cm tissue culture plates at 4 million cells per plate in IMDM (10% FBS supplemented with 1% antibiotics). Cells were cultured for 4 days (18-29). Cells were then washed twice with saline. HUVEC cells were purchased from ATCC and cultured in EGM-2 media supplemented with endothelia specific growth factors (EGM-2, Bulletkit; Lonza), 37 °C, 5% CO2. For macrophage differentiation, 2 × 106 cells were seeded in six-well plates and stimulated with PMA for 48 h. After macrophage differentiation, cells were stimulated with 100 ng/mL LPS (Sigma) and 50 ng/mL human IL-4 (Gibco) (M1) or with 10 ng/mL murine IL-4 (Gibco) (M2) and harvested 48 h after stimulation. Where indicated, 10 μM of the MK2 chemical inhibitor PF3644022 (Tocris) was added.

FACS Analysis of Macrophage Populations. Cells were detached by incubation in PBS containing 5 mM EDTA for 5–10 min. Single-cell suspensions were incubated for 10 min at room temperature with Fc blocking (CD16/32) antibody (eBioscience) before staining with fluorochrome-conjugated antibodies against the following antigens: CD11b-APC-Cy7, F4/80-FITC, CD86-PE (eBioscience). Cells were then fixed and permeabilized (eBioscience) before CD206-APC staining. Aqua Live/Dead dye (Invitrogen) was used to exclude dead cells. Human U937 cells were processed similarly but staining panel consisted of the following antibodies: CD11b-APC-Cy7, HLA DR-FITC, CD86-PE (eBioscience), CD163-PerCy7. DAPI was used to exclude dead cells. Multicarriable analysis was performed on a LSR Fortessa (BD), and bidimensional dot plots were generated using FlowJo software.

ECIS Wound Healing Assay. ECIS plates (96W1E+; Applied Biophysics) were cleaned and stabilized by incubating with 200 μL per well of 10 mM l-cysteine at room temperature for 15 min. Wells were washed twice with saline before coating with 200 μL per well of 1% gelatin for 30 min. After coating, wells were washed two times with saline. HUVEC cells were purchased from ATCC and cultured in EGM-2 media supplemented with endothelia specific growth factors (EGM-2, Bulletkit; Lonza), 37 °C, 5% CO2. HUVEC cells were seeded in 300 μL per well on ECIS plates and grown to confluence. Impedance levels were used to verify that confluence was achieved and maintained. Once cells reached confluence, 150 μL of HUVEC media was removed from each well and replaced with 150 μL of conditioned media collected from polarized U-937 cells. The cells were then allowed to acclimatize to the fresh media for 30 min before undergoing two wounding cycles (6,000 Hz, 20 s, 400 uA) (27–29). ECIS resistance readings were collected and plotted against time after wounding. Linear regression was used to fit the data points, and the slope was used to determine the time required for each group to recover to 25% of prewounding cell confluency. The rate of recovery was then calculated from the time taken for the cells to cover 23.71 μm.

Tubule Formation Assay. Twenty-four thousand HUVEC cells were seeded on top 120 μL of matrigel (Corning) in complete cell culture media (EGM-2 Bulletkit; Lonza) and 15% of conditioned or control media. Tubules were allowed to form overnight and analyzed by phase contrast microscopy. Images were automatically analyzed in ImageJ with the angioiogenesis analyzer plugin. Plates were also fixed and stained with Diff Quick stain kit (Thermo Scientific) for bright-field pictures acquisition, following manufacturer's instructions.

Statistical Methods. Unless stated otherwise, all data were plotted and analyzed in GraphPad Prism software, using Student’s t test or ANOVA followed by the Bonferroni post hoc analysis. Data represent the mean ± SEM.
Illustrations. Mouse illustrations were adapted from freely distributed clipart obtained from www.clker.com. Images used in Fig. 5 were adapted from SMART server medical art under a creative commons license.

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