Myeloid TGF-β signaling contributes to colitis-associated tumorigenesis in mice

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Myeloid cells have a critical role in maintaining intestinal homeostasis and regulating the development of inflammatory bowel disease and colitis-associated cancer (CAC). However, the signaling pathways that control the function of colonic myeloid cells in these pathological processes are still poorly defined. In this study, we demonstrate that transforming growth factor-β (TGF-β) signaling in colonic myeloid cells is significantly involved in the development of CAC. Myeloid TGF-β receptor II (Tgfbr2)-deficient mice showed reduced susceptibility to chemically induced colitis-associated tumorigenesis, as evidenced by decreases in number and size of tumors. Myeloid Tgfbr2 deficiency markedly decreased the production of interleukin-6 and tumor necrosis factor-α, two proinflammatory cytokines that are essential for colonic tumorigenesis; in addition, a marked increase in the proportions of Foxp3+CD4+ regulatory T cells was observed in the colonic lamina propria in the initial stage of CAC. Loss of myeloid Tgfbr2 was associated with a decrease in the presence of F4/80 positive macrophages and a downregulation of phosphorylated STAT3, proliferative cell nuclear antigen and cyclin D1 expression in colonic adenoma tissues. TGF-β enhanced macrophage recruitment at least in part, through modulating the expression of the chemokine (C-C motif) receptor 2 (CCR2) ligands in tumor environment and the CCR2 signaling in macrophages. Collectively, these results suggest that myeloid TGF-β signaling modulates intestinal inflammation and significantly promotes tumorigenesis in the development of colitis-associated colon cancer.

Introduction

Colon cancer is the third most common form of cancer in developed countries (1,2). Abundant evidence has suggested a physiological role of chronic inflammation in colonic tumorigenesis (3,4). Patients with inflammatory bowel diseases, such as Crohn’s disease and ulcerative colitis, are prone to developing colon cancer, and the prognosis of patients with colon cancer who have a background of inflammatory bowel disease is usually less favorable than that of patients with sporadic colorectal cancer (2–5). Myeloid cells are critical for the maintenance of intestinal immune homeostasis and the regulation of colorectal inflammation and cancer progression. Although several molecular events have been demonstrated for their function in intestinal myeloid cells during various pathological processes (6–9), the signaling mechanisms underlying myeloid cell-mediated regulation are still poorly defined.

Abbreviations: AOM, azoxymethane; BMDMs, bone marrow-derived macrophages; CAC, colitis-associated cancer; CBA, cytometric bead array; DSS, dextran sodium sulfate; IL-1, interleukin; KO, knock-out; LysM, lysozyme M; LPMCs, lamina propria mononuclear cells; M-CSF, macrophage colony-stimulating factor; mRNA, messenger RNA; PCNA, proliferative cell nuclear antigen; qRT–PCR, quantitative reverse transcription–PCR; TGF-β, transforming growth factor-β; Tgfbr2, TGF-β receptor II; Tgfbr2fl/fl; Tπ-α, tumor necrosis factor-α; Treg, regulatory T cells.

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Alterations in transforming growth factor-β (TGF-β) signaling have pronounced effects on the initiation and development of colon cancer. Enterocyte-restricted mutations in the components of the TGF-β signaling pathway have been found in patients with colon cancer (10,11). Attenuation of Tgfbr2 receptor II (Tgfbr2) or Smad4 function in T cells results in multifocal inflammatory diseases, including colon cancer (12,13). Emerging evidence reveals that the signals within different cell compartments may have dissimilar functions in regulating the development of colitis or colitis-associated cancer (CAC). For example, mice deficient in p38 in myeloid had less inflammation compared with control mice, whereas mice with colonic epithelial cell deletion of p38α displayed an increased progression of colitis (14). Cox-2 deletion in myeloid and endothelial cells rather than in epithelial cells aggravates colitis in mice (6). Recently, it has been shown that myeloid Tgfbr2-deficient mice are more resistant to tumor implantation (15). Therefore, although much attention has been paid to the effects of TGF-β signaling in colonic epithelial cells and T cells on the development of colon cancer, far less is known regarding whether TGF-β signaling in myeloid cells functions in this pathological process.

TGF-β signaling has been recognized as a key pathway essential for establishing immune tolerance. Our study and others have demonstrated that mice with T cells deficient in sensing TGF-β signaling develop a fetal inflammatory syndrome and display compromised ability in the generation or maintenance of natural regulatory T (Treg) cells (16,17). On the other hand, recent evidence also revealed that TGF-β may greatly contribute to the development of inflammatory interleukin (IL)-9- or IL-17-producing T cells (18–20). These findings indicate the complexity of TGF-β in immune regulation within different contexts. To define whether myeloid TGF-β signaling plays a significant role in the development of CAC, we used mice carrying Tgfbr2 conditional alleles (21) and lysozyme M (LysM)-Cre to obtain a myeloid cell-specific deletion of Tgfbr2. By employing the azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced mouse colon cancer model, we provide evidence that myeloid Tgfbr2 functions efficiently in promoting CAC development.

Materials and methods

Mice

The generation of Tgfbr2fl/fl/Tgfbr22/2 (Tgfbr2+/+) mice has been described previously (21,22). The Tgfbr2+/+ mice were from the NCI Mouse Repository (NCI-Frederick, NIH) and kept in B6 background. The LysM-Cre+ mice (23) (B6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Pathogen-free mice were housed under specific pathogen-free conditions and fed regular food and water. All of the experiments involving mice were performed according to the institutional animal care and use committee of Shanghai and the National Research Council Guide for Care and Use of Laboratory Animals. Genotyping was performed by PCR using the proper primers. The primer sequences were as follows: Cre, 5′-GGGTGAAGGTGCGTAAGGGTGACG-3′ and 5′-CGAAGGCGAGATGTTGAGGACA-3′; Tgfbr2, 5′-GGTGAGTTGATGCGACCATACC-3′ and 5′-CGAAGGCGACATGGTTGAGGACA-3′.

Induction of CAC

CAC was induced as described previously (8). Briefly, 6- to 8-week-old female Tgfbr2-/-/LysM-Cre+ (knock-out [KO]) mice and Tgfbr2+/+/LysM-Cre+ (control littermates) were treated with 12.5 mg/kg body wt of AOM (Sigma–Aldrich, St Louis, MO) by intraperitoneal injection and maintained on a regular diet for 7 days. The mice then received 2.5% DSS (wt/vol) water (molecular weight 35 000–50 000 kDa; MP Biomedical, Solon, OH) for 5 days, followed by 16 days of normal water. This cycle was repeated, and then the mice were given 2% (wt/vol) DSS water for 4 days, followed by 10 days of normal water. Body weight, stool consistency and stool occult blood were monitored daily as described previously (24).

Mice were killed on days 14, 35 and 64. Colonos were removed, cut open longitudinally and examined for tumors with a dissecting microscope as described previously (25). After macroscopic assessment, some distal colonic...
tissues were fixed in 10% neutral-buffered formalin for histological analysis. The other tissues were frozen in liquid nitrogen until use.

Isolation of lamina propria mononuclear cells

After the colons were dissected, the mesentry was cleaned, the Peyers' patches were discarded, and the colon sections were cut longitudinally and then into small pieces that were incubated in Ca²⁺- and Mg²⁺-free Hank's-buffered salt solution containing 2 mM ethylenediaminetetraacetic acid (Sigma–Aldrich) and 5% (vol/vol) fetal bovine serum (Hyclone/Thermo Fisher Scientific, Logan, UT) and shaken at 250 r.p.m. for 20 min at 37°C. This process was repeated two additional times. The colon pieces were then washed in phosphate-buffered saline (PBS). The remaining intestinal tissues were minced and incubated (20 min with moderate shaking) in RPMI 1640 (Thermo Fisher Scientific) containing 5% fetal bovine serum and 1.5 mg/ml Collagenase type VIII (Sigma–Aldrich). The suspensions were passed through a 70 μm strainer, the cells were washed and resuspended in 40% Percoll (GE Healthcare Life Sciences, Waukesha, WI), and the cell suspension was layered over 75% Percoll. The lamina propria mononuclear cells (LPMCs) were removed from the 75-40% interfaces and washed twice in PBS.

Flow cytometry analysis and antibodies

The LPMCs were stained with surface antibodies against CD3, CD4, CD8 (BD Biosciences, San Jose, CA), CD11b, CD11c, F4/80 and Ly6G (eBioscience, San Diego, CA) for 30 min at 4°C and analyzed with a FACS caliber flow cytometer (BD Biosciences). Intracellular staining with antibodies against tumor necrosis factor-α (TNF-α), FOXP3, IL-6 (eBioscience) and IL-17A (BioLegend) was performed after stimulation of the LPMCs with 40 ng/ml phorbol 12-myristate 13-acetate (Sigma–Aldrich) and 1 μg/ml ionomycin in the presence of brefeldin A (eBioscience) for 4 h. The percentages of TNF-α, IL-6- and IL-17A-producing cells were then analyzed. Additional cells were stained in parallel with the respective control isotype antibodies.

Bone marrow-derived macrophages, migration and invasion assay

Bone marrow cells were cultured in complete RPMI 1640 supplemented with macrophage colony-stimulating factor (M-CSF, 20 ng/ml; Peprotech). Fresh medium supplemented with 60 ng/ml M-CSF was added on day 5. Cells were harvested with cold PBS containing 15 mM ethylenediaminetetraacetic acid (Sigma–Aldrich) on day 7. The procedure for the examination of bone marrow-derived macrophages (BMDMs) migration and invasion has been described previously (7). Migration assays were performed using 24-well chambers with inserts (8 μm pores; BD Biosciences). Medium with CCL2 (100 ng/ml; Peprotech) and M-CSF (100 ng/ml; Peprotech) lacking serum was placed in the lower chamber, and BMDMs (1 × 10⁶) suspended in the medium without CCL2 or M-CSF but with serum were added in the top chamber. The plates were incubated at 37°C in a 5% CO₂ incubator for 22 h. The non-migratory cells were removed from the top of the insert membrane and the underside of each membrane was fixed and stained with the crystal violet. For invasion assay, the insert membrane was coated with matrigel and the procedure was similar to that of migration assay. The number of cells that migrated completely through the 8 μm pore was determined in four random high-power fields (×40 objective).

Immunohistochemistry

Sections were deparaffinized and quenched with 3% H₂O₂. Antigen unmasking was performed by heating (30 min) in 0.01 M sodium citrate buffer in a microwave oven. The slides were incubated with primary antibodies against p-STAT3 (Cell Signaling), cyclin D1 (Cell Signaling), proliferative cell nuclear antigen (PCNA; Cell Signaling) and F4/80 (Abcam). Appropriate secondary antibodies were then added and the slides were incubated at room temperature for 30 min. The color was developed using a diaminobenzidine substrate kit (Thermo Scientific) followed by a hematoxylin counterstain. Controls without primary antibody showed low or no background staining in all cases.

RNA analysis

Total RNA was isolated from colons and macrophages using RNA extraction kits (Tiangen, Beijing, China) and converted to single strand complementary DNA using Superscript™ III RT (Invitrogen) according to the manufacturer’s protocol. Amplification was performed with the ABI PRISM 7300 Quantitative PCR System (Applied Biosystems, Foster City, CA) using Premix Ex Taq™ (Takara, Dalian, China). The genes of interest were quantitated according to the Kaplan–Meier method and compared by the log-rank test. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). A paired, two-tailed Student’s t-test was used to determine the significance between two groups. The survival curves were plotted according to the Kaplan–Meier method and compared by the log-rank test. A P value < 0.05 was considered statistically significant.

Results

Deletion of Tgfb2 in myeloid cells impairs the development of colitis-associated tumor in mice

To investigate the role of myeloid TGF-β signaling in colitis-associated tumorigenesis, we generated mice lacking Tgfb2 specifically in myeloid cells using LysM-Cre mice and Tgfb2fl/fl mice. The floxed Tgfb2 alleles in isolated Ly6G+ and F4/80+ cells were specifically deleted as confirmed by DNA-PCR (data not shown). We treated Tgfb2fl/fl/LysM-Cre+ (hereafter termed Tgfb2 KO) mice and Tgfb2fl/fl/LysM-Cre- control littersmates with the AOM/DSS regimen, a well-established protocol for the CAC model. We observed a dramatic decrease of 50% in tumor incidence in myeloid Tgfb2-deficient mice compared with controls (Figure 1A). The tumor size in the KO mice was also reduced (Figure 1B). Moreover, the tumor load, a sum of the diameters of all tumors in a given mouse (25), was significantly lower in the KO mice (Figure 1C), and myeloid Tgfb2-deficient mice exhibited a lower incidence of larger adenomas than control mice (Figure 1D). Notably, myeloid-specific deletion of Tgfb2 had no apparent impact on the development of the intestine in a steady state, as evaluated by histological examination (Figure 1E).

In AOM/DSS-treated mice, hyperplasia of the middle to distal colon was found 35 days after treatment (Figure 1E), and adenomatous lesions were observed after 64 days (Figure 1E and F). These observations demonstrate the critical role of myeloid TGF-β signaling in the development of CAC in mice.

Myeloid Tgfb2 deficiency modulates experimental colitis and local proinflammatory cytokine expression

Numerous data have demonstrated the importance of colonic inflammation in the initiation of CAC in mice. We hypothesized that myeloid TGF-β signaling might regulate intestinal inflammation in the mice upon DSS treatment in the early stage of CAC development and thereby affect the tumor incidence. In the CAC model, we found an increase in the survival of myeloid Tgfb2 KO mice compared with control mice (Figure 2A). After the first round of DSS treatment, control mice transiently lost more body weight than Tgfb2 KO mice (Figure 2B). We observed the clinical features of the mice in the initial stage of CAC development. On day 14 after AOM/DSS treatment, we found that control mice suffered from more severe weight loss, diarrhea and rectal bleeding than Tgfb2 KO mice and exhibited higher clinical scores (Figure 2C). Consistent with the varied clinical features, control mice displayed greater shortening of the colon than KO mice (Figure 2D). Collectively, these results demonstrate that myeloid TGF-β signaling contributes to chemically induced colitis in mice.

We then considered that the impairment in the severity of colitis in myeloid Tgfb2-deficient mice might be associated with alterations in the expression of inflammation-related factors. We harvested the colon tissues from the mice 2 days after the first DSS cycle and examined the levels of several proinflammatory cytokines. Tgfb2 KO mice exhibited a substantial decrease in the production of IL-6, TNF-α and a subtle increase in IL-10 expression in the colon tissues as examined by CBA (Figure 3A and B). To characterize the cellular sources of inflammation-related factors in greater detail, we isolated colonic lamina propria cells from the mice on day 14 after AOM/DSS treatment. Intracellular cytokine expression analysis revealed that in the initial stage of CAC, lamina propria CD4+ cells produced less TNF-α in Tgfb2 KO mice than in control mice (Figure 3C).
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Also, a significant decrease in the expression of IL-6 and TNF-α was observed in lamina propria CD11c+ cells derived from myeloid Tgfbr2 KO mice. Moreover, we found that Tgfbr2 deficiency resulted in a downregulation in the expression of IL-6 in F4/80+ macrophages (Figure 3C). The percentages of IL-17- and interferon-γ-producing cells in CD4+ T from Tgfbr2 KO mice exhibited no difference compared with those of controls (Figure 3C). Further analysis revealed that CD103+ dendritic cells produced less TNF-α and IL-6 in Tgfbr2

Fig. 1. Myeloid Tgfbr2-deficient mice are resistant to colitis-associated tumorigenesis. (A) Numbers of tumors in the colons of Tgfbr2+/+ LysM-Cre+ (control) and Tgfbr2 flop/LysM-Cre+ (KO) mice after completion of the CAC challenge on day 64. The data points represent individual mice with the indicated medians and are pooled from four independent experiments (control mice, n = 27; KO mice, n = 29; ***P < 0.001). (B) Mean tumor size in the mice at the end of the CAC challenge (day 64). The data shown are representative of two independent experiments and are depicted by the mean ± standard error (SE; n ≥ 8; ***P < 0.001). (C) The average tumor load was determined by summing all tumor diameters in a given mouse. The results are shown as the mean ± SE (n ≥ 8; ***P < 0.001). (D) The histogram shows the size distribution of the tumors. The proportion of tumors larger than 2 mm in diameter on day 64 is shown. Each value represents the mean ± SE (n ≥ 8, ***P < 0.001). (E) Hematoxylin and eosin staining of colon sections on days 0, 35 and 64 after AOM/DSS. Scale bar, 50 μm. (F) Tumors in the CAC model. Nine weeks after AOM/DSS treatment, mice were killed and colons were cut open longitudinally. Red arrows indicate polypoid lesions.
KO mice than in control mice (Figure 3C). The decreases in IL-6 and TNF-α expression in colon tissues with myeloid Tgfbr2 deficiency were consistently present at the messenger RNA (mRNA) level (Figure 3D). Notably, we did not find a significant difference in the expression of these cytokines between untreated control and Tgfbr2 KO mice (Figure 3D). As the altered proinflammatory cytokines are also procarcinogenic in colon cancer, these results suggest that myeloid Tgfbr2 may not only contribute to the induction of colonic inflammation but also promote the initiation of inflammation-associated tumorigenesis as well.

**Myeloid Tgfbr2 regulates the immune cell composition in mouse colonic lamina propria during the early stage of CAC development**

To further analyze the infiltration by immune cells and its association with colitis, we isolated LPMCs from the colons of control and myeloid Tgfbr2-deficient mice. Flow cytometry analysis revealed that the proportions of T-cell subsets, CD11c+ F4/80+ and Ly6G+ cells were not significantly different in the splenic cells or LPMCs between control and Tgfbr2 KO mice on day 14 after AOM/DSS treatment (Figure 4A and B). Considering the function of CD4+ Foxp3+ Treg cells in the control of colonic inflammation in the CAC model (26,27), we also examined the composition of Treg cells in the colonic LPMCs of control and Tgfbr2 KO mice. Intriguingly, a significant increase in the percentage of Treg cells was found in the colons of myeloid Tgfbr2-deficient mice on day 14, though the proportion of CD4+ Foxp3+ Treg cells in the spleen was similar in these two groups (Figure 4C).

**Myeloid Tgfbr2 deficiency decreases the abundance of macrophages in colon adenoma**

Accumulating evidence indicates a functional role of macrophages in tumor progression. We next evaluated whether myeloid Tgfbr2 could regulate macrophage infiltration in the colonic adenomas. We induced BMDMs from control and myeloid Tgfbr2 KO mice and determined their mobility toward the chemoattractants M-CSF and CCL2, two chemokines well known for the recruitment of macrophages. After 22 h of incubation, a significant reduction in the migration ability of these cytokines between untreated control and Tgfbr2 KO mice was observed in BMDMs with Tgfbr2 deficiency compared to wild-type BMDMs. Consistently, flow cytometry analysis confirmed that myeloid Tgfbr2 enhances macrophage recruitment to the tumor region. The decreases in IL-6 and TNF-α expression in colon tissues with myeloid Tgfbr2 deficiency caused an increase in the expression of IL-10 mRNAs (Figure 5D). Furthermore, we also isolated colonic LPMCs from adenoma tissues at day 64 of the CAC regimen and stimulated them with phorbol 12-myristate 13-acetate plus ionomycin for 4 h. The results indicated that in the end stage of CAC, no significant difference was observed in the percentages of TNF-α- and IL-6-expressing F4/80+ macrophages within CD4+ T cells between the control mice and Tgfbr2 KO mice. The difference in the percentages of TNF-α- and IL-6-expressing F4/80+ macrophages was subtle between KO mice and control mice (Supplementary Figure S1, available at Carcinogenesis Online). Together, these results indicate that the impairment in the presence of macrophages may largely contribute to the alteration in the production of the proinflammatory cytokines and significantly involve in the regulation of proliferation of the neoplastic epithelial cells.

To further address whether any difference exists in the migration and invasion capabilities between the macrophages with or without Tgfbr2, we induced BMDMs from control and myeloid Tgfbr2 KO mice and determined their mobility toward the chemoattractants M-CSF and CCL2, two chemokines well known for the recruitment of macrophages. After 22 h of incubation, a significant reduction in the migration ability to CCL2 was observed in BMDMs with Tgfbr2 deficiency compared with that of control BMDMs. Unexpectedly, M-CSF-mediated chemotraction, however, was not significantly compromised by Tgfbr2 deficiency. Analysis of macrophage invasion yield similar results (Figure 5E and F). The results clearly demonstrate that the
Fig. 3. Expression of inflammation-related factors in the colon tissues during the initial stage of the CAC development. (A) The supernatant cytokine levels from the colon tissues obtained on day 14 were evaluated by CBA. Representative plots show the cytokine levels in control mice and mice with myeloid-specific deletion of Tgfbr2 during the initial stages of the CAC regimen. (B) The supernatants of the colon tissues from control and KO mice obtained on day 14 after treatment were assessed using the CBA. The data shown are the mean ± SD of the fold difference (*P < 0.05; n = 5). (C) The colons were collected on day 14, and LPMCs were pooled and stimulated with phorbol 12-myristate 13-acetate plus ionomycin for 4 h (n = 3). After stimulation, the cells were stained for cell marker CD103, CD11c, CD4, F4/80 and the intracellular cytokines IL-6, TNF-α, interferon-γ and IL-17. The percentages of cells producing IL-6, TNF-α, interferon-γ and IL-17 are shown (*P < 0.05, **P < 0.01). (D) The expression of inflammatory gene mRNAs in the colon tissues of mice on day 14 was quantitated by real-time PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA. The data represent the mean ± SD (*P < 0.05, n ≥ 7).
strength of the chemokine (C-C motif) receptor 2 (CCR2) signaling is impaired in macrophages with Tgfbr2 deficiency. We further examined the levels of the transcripts of CCR2, M-CSF receptor and other molecules related to the migration of macrophages. Interestingly, we found that the expression of CCR2 and fibroentin-1 was reduced in BMDMs with Tgfbr2 deficiency (Figure 5I). Lastly, we examined the expression of CCR2 ligands (CCL2, CCL7 and CCL12) and M-CSF in the colon tumor tissues from KO and control mice by real-time RT–PCR. The results showed that myeloid Tgfbr2 deficiency considerably impaired the expression CCL2 and CCL12 (Figure 5J). Together, our observation suggests that myeloid TGF-β enhances macrophage recruitment, at least in part, through modulating the expression of CCR2 ligands in tumor environment and the activities of CCR2 signaling in macrophages.

Myeloid Tgfbr2 regulates the proliferation of neoplastic colonic cells

In addition to decreased tumor incidence, a reduction in tumor size was observed in myeloid Tgfbr2-deficient mice treated with the CAC regimen, suggesting a role for myeloid Tgfbr2 in regulating the proliferation of neoplastic colonic cells. We analyzed the cell proliferation levels in control and myeloid Tgfbr2-deficient tumors by PCNA staining and qRT–PCR, and we found that PCNA was substantially decreased in myeloid Tgfbr2-deficient adenomas (Figure 6A–C). The expression of cyclin D1 was also lower in myeloid Tgfbr2-deficient tumors by immunohistochemical analysis (Figure 6A and B) and qRT–PCR (Figure 6C), indicating decreased growth capacity of the adenoma cells in myeloid Tgfbr2-deficient mice. Moreover, downregulation of STAT3 phosphorylation, a downstream effector of IL-6 signaling, was observed in the myeloid Tgfbr2-deficient adenomas (Figure 6A and B). These results suggest that myeloid Tgfbr2 regulates the proliferation of neoplastic epithelial cells in the CAC model.

Discussion

Chronic inflammation greatly contributes to the development and progression of malignant diseases. Typically, chronic inflammatory bowel disease represents an extreme example of chronic inflammation in initiating colon carcinogenesis. However, the cellular components of the immune system that are involved and the mechanisms for their regulation in the pathogenesis of the disease are still obscure. Here, we have shown that myeloid TGF-β signaling significantly regulates the development of CAC in mice. We demonstrate that the absence of Tgfbr2 in myeloid cells results in a significant decrease in tumor number and size when the mice were subjected to AOM/DSS-induced CAC model. Our observations regarding the effect of deficiency of TGF-β signaling in myeloid cells on CAC development is different from the influence of colon epithelial or T cell-restricted inactivation of TGF-β signaling (11,12). These findings emphasize the complexity of TGF-β signaling in the regulation of CAC development and suggest a cell context-dependent impact of TGF-β signaling on the pathological intestinal milieu.

Myeloid cells within the intestine during chronic inflammation display proinflammatory phenotypes, including the expression of TNF-α, IL-6, IL-23, Cox-2 and inducible nitric oxide synthase. These cytokines or molecules are most likely tumor promoting and regulated by various events (9,28–31). For example, in the CAC model, deletion of the Ikβ kinase and Hif-2α in myeloid cells results in a significant decrease in tumor size and impaired expression of proinflammatory cytokines/chemokines that either initiate inflammation or directly stimulate the proliferation of transformed enterocytes (7,8). We have observed that in the initial stage of CAC, the colons derived from myeloid Tgfbr2-deficient mice produced less inflammatory cytokines, IL-6 and TNF-α, than that of control mice. IL-6 expression in F4/80+ macrophages, and the expression of TNF-α and IL-6 in CD11c+ cells, was significantly impaired in the lamina propria of myeloid Tgfbr2 KO mice, whereas CD4+ T cells and CD11c+ cells
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Fig. 5. Alterations in macrophage presence in the myeloid Tgfr2-deficient colon adenoma. (A) F4/80 immunostaining in adenomas. Arrows point to F4/80+ macrophages. T, tumor area; S, stalk; scale bars = 20 μm. (B) Qualitative observations of macrophage infiltration were analyzed. Columns represent the mean from tumor areas corresponding to 5–8 fields at x200 magnification from five mice, respectively. The data indicate the mean ± SE (*P < 0.05), unpaired two-tailed Student’s t-test. (C) The percentage of F4/80+ cells was analyzed in the spleen, colonic lamina propria and colon adenomas by flow cytometry on day 64. The data represent the mean ± SE (*P < 0.05; n = 5). (D) IL-10, IL-6 and TNF-α expression was determined by real-time PCR analysis in colonic tissues from control and KO mice on day 64 after AOM injection. The data represent the mean ± SE (*P < 0.05; n = 5). (E) Bone marrow cells from control and KO mice were isolated and differentiated into BMDMs. The migration capacity of BMDMs toward the CCL2 or M-CSF was determined by employing a barrier polyethylene terephthalate membrane. Representative images are shown. Scale bars: 100 μm. (F) The invasion capacity of BMDMs toward the CCL2 or M-CSF was determined. Representative images are shown. Scale bars: 100 μm. (G) Quantification of migrated macrophages. Data were representative of three separate experiments with similar results (**P < 0.001). (H) Quantification of invaded macrophages. Data were representative of two separate experiments with similar results (*P < 0.05). (I) Expression of the related chemokine receptors and molecules was determined by real-time PCR in BMDMs from control and KO mice (n = 3; *P < 0.05, **P < 0.01, ***P < 0.001). (J) The expression of CCL2 and CCL12 in tumor tissues from control and KO mice was determined by real-time PCR (n = 3; *P < 0.05).
also produced lower level of TNF-α compared with that in wild-type mice. In addition, we also found that CD103+CD11c+ cells produced less TNF-α and IL-6 in Tgfbr2 KO mice than that in control mice. However, of note, the decrease in TNF-α expression did not occur in the macrophages, indicating that myeloid Tgfbr2 signaling functions directly or indirectly via an array of interactions in the colonic inflammatory microenvironment. Consistently, a recent study has shown that myeloid deletion of Tgfbr2 can inhibit tumor growth and cause a downregulation of IL-6 expression in mice model of subcutaneous implantation of the tumor cells (15). On contrary, the alteration of TNF-α expression due to myeloid Tgfbr2 deletion in their study differs from the observation in this study. The discrepancy may be because of the dramatic difference between the tumor models employed in these two studies, in which the inflammatory settings, the sites for tumor development are apparently different.

CD4+Foxp3+ Treg cells are critical in maintaining mucosal tolerance. Adoptive transfer of Treg cells effectively alleviates or prevents symptoms of colitis and reduces the incidence of colon tumors in mice (26,32). Our present data indicate that the absence of myeloid TGF-β signaling is associated with a substantial increase in the proportion of Treg cells in the colonic lamina propria during the early stage of CAC development. A reciprocal regulation exists between Treg and Th17
cells, and the skewed balances between these two cell types have often been observed in a variety of autoimmune diseases (33,34). Th17 cells are also significantly involved in colonic inflammation. In this study, however, we did not observe a significant difference in the presence of Th17 cells between control mouse and Tgfb2 KO mice at day 14 of the CAC regimen. Obviously, the exact mechanisms underlying the phenomenon remain unclear and warrant intensified exploration.

In the late stage of the CAC model, we observed the reduction in macrophage presence in the colonic adenomas of myeloid Tgfb2 KO mice. In line with this observation, our in vitro experiments also revealed that the migration and invasion of BMDMs toward CCL2 were remarkably compromised by Tgfb2 deficiency. The levels of the transcripts of CCR2 and fibronectin-1 in BMDMs with Tgfb2 deficiency were reduced. Fibronectin-1 is a secreted protein that can enhance cell migration by binding to integrin receptors and heparin and fibrin (7,35). Importantly, we also found that the mRNA expression of CCR2 ligands CCL2 and CCL12 was significantly downregulated. As tumor-associated macrophages produce an array of factors, such as IL-6, TNF-α, IL-1β and CCL-2, to promote the proliferation of premalignant cells and angiogenesis (36–38), we speculate that the decrease in number of macrophages in neoplastic environment may result in a relative shortage of the tumor-promoting factors, and thereby impairs tumor growth in myeloid Tgfb2-deficient colon. Indeed, we found that mice deficient in myeloid Tgfb2 persistently exhibited reduced IL-6 expression in colon tissues. In agreement with this result, we also found that phosphorylated STAT3 was significantly downregulated in mice lacking myeloid Tgfb2. Given the previously known role of IL-6–STAT3 axis in the survival and cell cycle progression of premalignant enterocytes (39,40), the deregulation of this pathway may account for the decreased proliferation of colonic neoplastic cells and the reduced size of the tumors in myeloid Tgfb2-deficient mice.

In conclusion, our studies demonstrate that myeloid Tgfb2 plays a crucial role in inflammation-induced colon cancer. Myeloid Tgfb2 deficiency significantly reduces colitis and decreased colonic tumor incidence in mice. This study provides evidence for understanding the signaling pathways that control function of colonic myeloid cells in the processes of intestinal inflammation and inflammation-associated colon cancer.

Supplementary material
Supplementary Table 1 and Figure S1 can be found at http://carcin.oxfordjournals.org/

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