Tumor-derived GM-CSF promotes inflammatory colon carcinogenesis via stimulating epithelial release of VEGF

Running title: GMCSF elicits VEGF

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Abstract

Chronic inflammation is a major driving force for the development of colitis-associated cancer (CAC). Elevated production of granulocyte macrophage colony-stimulating factor (GMCSF) has been observed in mucosa of patients with inflammatory bowel disease (IBD). Its actions in the progression from colitis to cancer, however, remain poorly understood. Herein, we demonstrated that colonic epithelial cells (CEC) were a major cellular source of GMCSF and its production was significantly augmented when CAC model was established by administration of azoxymethane and dextran sulfate sodium. Furthermore, we showed that GMCSF was a driver for VEGF release by CEC in autocrine and/or paracrine manners through extracellular regulated protein kinase (ERK)-dependent pathway. Blocking GMCSF activity in vivo significantly decreased epithelial release of VEGF, thereby abrogated CAC formation. In vitro treatment of transformed CEC with recombinant GMCSF dramatically augmented its invasive potentials, largely in VEGF-dependent fashion. Furthermore, commensal microbiota-derived lipopolysaccharides (LPS) was identified a trigger for GMCSF expression in CEC, as antibiotics treatment or toll like receptor 4 (TLR4) ablation considerably impaired its epithelial expression. Overall, these findings may have important implications for understanding of mechanisms
underlying CAC pathogenesis and the therapeutic potentials of regimens targeting GMCSF or VEGF in clinic.
**Introduction**

Chronic inflammation is thought to be a major driving force for the initiation and progression of cancer in many tissues (1). For example, in the patients suffering from ulcerative colitis (UC), one of the two major forms of inflammatory bowel diseases in human, the risk for colorectal cancer is elevated, which is ten-fold greater than in the general Western population (2,3). The risk of developing cancer increases strongly with the duration, extent, and severity of inflammatory disease (4). This process is well mimicked by a widely-utilized mouse model of colitis-associated colon cancer (CAC), which is induced by administration of azoxymethane (AOM) followed by repeated oral administration of dextran sulfate sodium (DSS). The molecular mechanisms underlying this neoplastic transformation are poorly understood, but a series of proinflammatory mediators produced by immune and non-immune cells are reported to contribute to tumor growth in chronic intestinal inflammatory settings (5).

New blood vessel formation (angiogenesis) is a fundamental event in the process of tumor growth and metastatic dissemination (6). Vascular endothelial growth factor (VEGF) has been regarded as major mediator of this process, which is mainly mediated through VEGFR2, a receptor tyrosine kinase that is expressed at elevated level by endothelial cells (7). Intriguingly, in addition to tumor growth, increasing evidence supports the involvement of angiogenesis and VEGF in the pathogenesis of chronic inflammatory disorders such as rheumatoid arthritis, psoriasis, and IBD (8). Of note, a recent study has shown that angiogenesis and VEGF signaling might pose an important link between inflammation and tumor development (9). Intestinal epithelial cells (IEC) from patients with CAC harbored activated VEGFR2 and responded to VEGF stimulation with augmented VEGFR2-mediated proliferation.
through STAT3-dependent pathway. Blockade of VEGF function with soluble decoy receptors suppressed tumor development, inhibited tumor angiogenesis, and blocked tumor cell proliferation in CAC model (9). The cellular source responsible for VEGF production and the details in the regulation of VEGF secretion, however, is largely unknown in the progression of colitis to cancer.

Granulocyte macrophage colony-stimulating factor (GMCSF) is a hematopoietic cytokine, whose levels are significantly elevated in the inflamed tissues of patients with IBD and rodents subjected to experimental colitis (10,11). In accord with this, GMCSF-/- mice were more susceptible to DSS-induced colitis, as shown by clinical and histological parameter and by augmented proinflammatory cytokine production (12). While, GMCSF administration in DSS-treated mice reduced colitis severity, which was associated with accelerated hyperproliferation of intestinal epithelium and mucosal healing following GMCSF treatment (13). However, the precise role of GMCSF in the development of CAC is not addressed to date. In this study, we aim to investigate the role of GMCSF signaling in CAC model using AOM and DSS. Furthermore, whether GMCSF is a driver for VEGF release in CAC milieu is addressed.
Material and methods

Mice

Balb/c wild type (WT) and Toll-like receptor 4 (TLR4)-/- mice were purchased originally from Jackson Laboratory (Bar Harbor, Maine). TLR4-/- mice were backcrossed for 12 generations onto the Balb/c background. Animals were housed in specific pathogen-free conditions with an alternating light/dark cycle. All experiments were performed using 6- to 8-week-old male mice. Care, use and treatment of mice in this study was in strict agreement with international guidelines for the care and use of laboratory animals and approved by Animal Ethics Committee of Institute of Basic Medical Sciences.

Establishment of CAC model

CAC was induced according to classical protocols as described previously (14), with mild modification (Fig. 1A). On day 1, mice were injected intraperitoneally with AOM (10mg/kg; Sigma-aldrich) and maintained on regular diet and water for 5 days. Mice then received water with 2% DSS (MW 36, 000-50, 000; MP Biochemicals) for 1 week. After this, mice were maintained on regular water for two weeks and subjected to three more DSS treatment cycles. On day 35 (2 cycle of DSS) or 100 (4 cycle of DSS), mice were sacrificed. Macroscopic tumors were counted. The clinical
course of disease was followed daily by measurement of body weight and monitoring
for signs of rectal bleeding or diarrhea.

**Histological examination and immunohistochemistry**

Colons were removed from mice and fixed in 10% neutral-buffered formalin solution
and then embedded in paraffin, cut into tissue sections, and stained with hematoxylin
and eosin. Stained sections were examined for evidence of colitis according to the
criteria as previously described (15).

The protocol on immunohistochemistry is described in Supplemental materials
and methods.

**Detection and quantitation of apoptotic cells**

Experiments were carried out as described in Supplemental materials and methods.

**Isolation of colonic epithelial cells**

Colonic epithelial cells were fractionated as described previously (15) with the purity
> 80%, identified by CK-18 staining.

**Isolation of lamina præria immune cell subsets**

The protocol is described in Supplemental materials and methods.

**Immunoblotting**
Standard procedures for immunoblotting are described in Supplemental materials and methods.

**Whole colon culture**

200-300 mg of colon tissue was washed in cold PBS supplemented with penicillin and streptomycin. These segments were cut into small pieces (0.5×0.5 cm) and cultured (three pieces per mouse) in 24-well flat bottom culture plates in serum-free RPMI1640 medium at 37°C for 24 hr. Supernatants were centrifuged at 13,000 rpm at 4°C for 5 min and stored at -80°C until use.

**Cytokine analysis**

Cytokines (GMCSF, VEGF-A, GCSF, MCSF) were examined in whole colon culture supernatants by ELISA kits obtained from R&D Systems, according to the manufacturer’s instructions.

**Colon carcinoma cell line culture**

Human colon adenocarcinoma cell line HCT116 was purchased from American Type Culture Collection and maintained in McCoy’s 5A Medium (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. CT26, a murine colon cancer cell line, was kindly provided by Dr.
Zhihai Qin (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) and cultured in complete RPMI1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. CT26 or HCT116 cells were seeded in 6-well culture plates (1-3×10^6/ml/well) and stimulated with recombinant murine or human GMCSF, VEGF, IL-1β, IL-6 (Peprotech) or LPS (Sigma-Aldrich) at indicated concentration for 48 hr. In some settings, neutralizing monoclonal antibody to IL-6 or GMCSF (1 μg/ml; Biolegend), IL-1 receptor antagonists (IL-1Ra, 2 μg/ml; kindly gifted by Dr. Haiyan Hong at Beijing Proteomics Research Center) were added to the culture. To determine signaling pathways, cells were preincubated with MEK1/2 inhibitor (U0126; 1 μM), PI3K inhibitor (Wortmannin; 500 nM), and JAK2 inhibitor (AG490; 100 μM) alone or in combination for 30 min, followed by GMCSF stimulation. After incubation, cells were collected for analysis.

**Cell proliferation assays**

Cell growth was assessed by Sulforhodamine B(SRB) assay. Cells (5×10^3 cells per well in 100 μl medium) were seeded into 96-well plates in triplicate and exposed to recombinant human GMCSF (Peprotech) at titrated concentrations. After incubation for indicated time, 50 μl of 30% trichloroacetic acid was added and incubated for 60
min at 4°C. After washing and drying the plate, 100 μl of 0.4% SRB was added for 30 min. The plates were rinsed with 0.1% acetic acid and air-dried, after which 100 μl of Tris base (10 mM/L) was added, and the plates were shaken for 5 min. The SRB value was measured at a wavelength of 590 nm.

Transwell migration and invasion assays

In vitro cell migration and invasion assays were performed using transwell chambers with polyethylene terephthalate membrane (24-well inserts, 8.0 μm; Corning). For the migration assay, 2.5×10⁴ cells were added to the top chambers. For the invasion assays, 5×10⁴ cells were seeded to the top chambers coated with Matrigel (BD Biosciences). Complete medium alone or with addition of recombinant human GMCSF at indicated concentrations was added to the bottom wells to stimulate migration or invasion. In some cases, monoclonal antibody to human VEGF (bevacizumab, Roche) or isotype (2 μg/ml) was added into the bottom chamber. After incubation for 16 hr, cells were stained with 0.4% Sulforhodamine B. Quantification was performed by determining SRB value at a wavelength of 590 nm.

Flow cytometry

The protocol is described in Supplemental materials and methods.

In vivo neutralization

Mice subjected to CAC induction received intraperitoneally monoclonal antibody to murine GMCSF (MP1-22E9) or VEGF (2G11-2A05) at a dose of 0.25 mg/mouse on
day -1, 0, 2, 5 and then every 5 days until animals were sacrificed. The isotype controls were administrated with the same dosage and periodicity. All neutralizing antibodies and isotypes were purchased from Biolegend.

**Commensal depletion**

6-week-old mice were treated with ampicillin (1 g/L), vancomycine (500 mg/L), neomycin sulfate ((1 g/L), and metronidazole (1 g/L) in drinking water for 4 weeks. Stool was collected from antibiotic-treated and untreated mice and the efficacy of commensal depletion was confirmed by staining of SYBR green solution as described previously (16). During CAC induction, administration of cocktail of antibiotics was continued until mice were sacrificed. In some settings, flora-depleted mice were injected intraperitoneally with LPS (10 mg/kg).

**Quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR)**

Experiments were carried out as described in Supplemental materials and methods. The primers for real-time were listed in Supplemental table.

**Statistical analysis**
Data are presented as mean±SD. The student t test (2-tailed) was used to determine significance, with P < 0.05 considered significant. Statistics were performed using SPSS 10.0 for Macintosh, and graphs were made on Deltagraph (SPSS, Chicago, IL).
Results

GMCSF is produced substantially by neoplastic colonic epithelial cells

Firstly, we collected the colon samples of mice suffering from CAC, and isolated the epithelium. Quantitative PCR analysis showed that the neoplastic epithelial cells were one of major sources for GMCSF and produced high levels of this cytokine (Fig. 1B), which is paralleled by elevated contents of GMCSF in the colonic tissues compared to controls (Fig. 1C). Also, as reported by previous studies (17), mononuclear phagocytes (Gr-1<sup>lo/-</sup>CD11b<sup>+</sup>) residing in lamina propria expressed considerably this cytokine (Fig. 1B). Of note, augmented production of GMCSF in diseased tissues was observed at the early stage of CAC development (Fig. 1C), in sharply contrast to the patterns of expression of other putative colitis-associated cytokines, for example GCSF and MCSF (Fig. 1D). These results indicate during the progression of intestinal inflammation to tumor, irritated colonic epithelial cells produce large amounts of GMCSF, which may be a key event linking inflammation and cancer.

GMCSF drives VEGF expression in malignant colonic epithelial cells

VEGF has been regarded as a critical factor involved in inflammation-associated colorectal cancer (9). Accordingly, we detected its expression in the colons of CAC-bearing mice and found significantly increased production of VEGF at the early and
late stage of CAC development (Fig. 2A). The dissection of cell types responsible for VEGF production showed that colonic epithelial cells were one of major cellular sources for VEGF (Fig. 2B). Intriguingly, mononuclear phagocytes residing in LP also expressed this cytokine substantially and the expression was augmented in the CAC-bearing mice (Fig. 2B). Nevertheless, the identity of driver of VEGF secretion is still elusive. Given the same patterns of expression of GMCSF and VEGF, we hypothesized that GMCSF was a stimulator for epithelial VEGF expression, as colonic epithelial cells expressed GMCSFRα and the level of this receptor expression was elevated after CAC formation (Fig. S1A). To address this issue, GMCSF was used to stimulate a mouse colon carcinoma cell line who expressed GMCSF receptor (Fig. S1B). Indeed, GMCSF could induce considerable expression of VEGF (Fig. 2C), in a dose-dependent manner. Notably, GMCSF appeared to be a major factor for forcing neoplastic epithelial cells to express VEGF, as IL6 or IL1β alone was unable to induce VEGF expression (Fig. 2D), considering their tumor-promoting role in colitis-associated cancer (16,17), although CT26 virtually expressed the receptor of these cytokines (Fig. S2). Therefore, although GMCSF stimulation also induced IL6 and IL1β expression in cancer cells (Fig. S3), it seemed to be unlikely that GMCSF-driven VEGF expression was indirect and via inducing IL6 or IL1β expression. This
conclusion was further confirmed by the fact that blocking IL6 or IL1 activity did not affect GMCSF-elicited VEGF expression (Fig. 2E).

It is well known that the binding of GMCSF to its receptor activates at least three signaling pathways: Janus kinase/signal transducer and activation of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) (18,19). Thus, we detected the activation of these pathways in colon carcinoma cell line upon GMCSF stimulation. The result showed that the activation of STAT5 was not seen in GMCSF-pulsed cells (Fig. 2F), and the PI3K/AKT signaling also was not disturbed (data not shown). In contrast, the phosphorylation of MAPK/ERK was visible in CT26 cells upon stimulation with GMCSF (Fig. 2F). Furthermore, to define the signaling pathways required for GMCSF-induced VEGF production, appropriate inhibitors alone or in combination were added. Blockade of JAK/STAT or PI3K/AKT pathway had no effect on GMCSF-driven VEGF expression (Fig. 2G). While, VEGF production was impaired dramatically upon the addition of ERK inhibitor or combination of three inhibitors (Fig. 2G), indicating a nonredundant role of MAPK/ERK signaling pathway in GMCSF-elicited VEGF production.

GMCSF blockade reduces tumor load and improves colonic injury
To investigate the function of GMCSF on CAC development, we injected intraperitoneally with neutralizing monoclonal antibody to mouse GMCSF into mice subjected to CAC induction. Blocking GMCSF activity remarkably decreased incidence of CAC and improved the severity of intestinal damage (Fig. 3A and B). Consistent with these macroscopic changes, increased apoptosis of colonic epithelial cells (CEC) was visible in GMCSF mAb-treated mice compared to controls (Fig. 3C). This may be due to reduced activation of ERK MAPK (Fig. 3D) as well as lower contents of Bcl-XL of anti-GMCSF-treated CEC (Fig. 3E), an antiapoptotic protein that is reported to mediate CEC survival (20). Moreover, the proliferation of CEC was limited after GMCSF mAb administration, as reflected by reduced number of Ki-67-positive colonic epithelial cells (Fig. S4A) and down-regulated expression of several cell cycle genes in CEC (Fig. S5A). These data support a tumor-promoting role of GMCSF in CAC formation.

Neutralization of GMCSF activity down-regulates VEGF production and reduces angiogenesis

We asked whether the effect of anti-GMCSF on CAC development was associated with reduced VEGF expression and angiogenesis. To this end, VEGF expression in CEC of isotype or GMCSF antibody-treated mice subjected to AOM+DSS was
examined. Indeed, GMCSF blockade led to significant decrease of VEGF expression in CEC (Fig. 4A). This was paralleled with reduction in intratumoral vessel density (Fig. 4B). Furthermore, the amounts of phosphorylated STAT3 in CEC were detected. The results showed that the phosphorylation of STAT3 in anti-GMCSF-treated CEC was pronouncedly inhibited (Fig. 4C). In accord with this, NF-κB activity in epithelium of anti-GMCSF-treated mice was restrained, as shown by reduced phosphorylation of p65 subunit (Fig. 4C).

To directly evaluate the association of VEGF expression with CAC development, a monoclonal antibody recognizing VEGF was utilized. We found that blocking VEGF activity did repress tumor growth and attenuate intestinal damage as well as reduce angiogenesis (Fig. 4D-F). In addition, the protumor effect of GMCSF-VEGF axis during CAC development was partially attributed to its direct regulation of the growth of CEC, as murine CEC virtually expressed VEGFR2 (9, Fig. S2) and anti-GMCSF treatment reduced VEGFR2 activation in CEC (Fig. 4G). As described above, VEGF blockade resulted in increased apoptosis of CEC (Fig. 4H) and reduced expression of Bcl-XL (Fig. 4I). Furthermore, restricted activation of STAT3-dependent pathway in anti-VEGF-treated CEC was observed (Fig. 4J). In accord with this, the proliferation of epithelial cells was scarcely visible in anti-VEGF-treated mice (Fig. S4B), which may be due to reduced expression of several cell cycle genes.
in CEC (Fig. S5B). Taken together, these results suggest that GMCSF-elicited VEGF expression in CEC may play a crucial role in CAC formation.

**Stimulation with exogenous GMCSF augments the invasive potentials of CEC via inducing VEGF production**

Next, we sought to determine the autocrine/paracrine effect of GMCSF expression on the outgrowth and invasion of malignant colon epithelial cells HCT116, which express GMCSF receptor (Fig. S1C), we found that stimulation with GMCSF did not modify the growth of HCT116 (Fig. 5A). The migration and invasion of HCT116, however, was significantly enhanced when exposed to exogenous GMCSF, in dose-dependent manners (Fig. 5B and C). Furthermore, in agreement with the data shown in Fig. 2D, GMCSF stimulation did lead to VEGF expression in HCT 116 cell lines (Fig. S6A) and this effect was dependent on the activation of ERK pathway, given that inhibition of this pathway dramatically repressed GMCSF-driven VEGF expression (Fig. S6B and C). Therefore, we aimed to determine the relationship between VEGF release and GMCSF-mediated invasion of HCT116, which virtually expressed the receptors of VEGF (21,22). Blocking VEGF using neutralizing specific antibody dramatically impaired the potentials of GMCSF to promote migration and
invasion of HCT116 (Fig. 5D and E). These data indicate that GMCSF could enhance the invasive behavior of transformed CEC by up-regulating its expression of VEGF.

**CEC challenged by Commensal flora-derived LPS produces GMCSF**

Commensal translocation is thought to be a key event for initiation of intestinal inflammation and CAC development (23). Thus, we hypothesize that the attack of luminal microbiota is required for GMCSF expression in CEC. To address this issue, bacteria inhabiting the gut were eradicated by antibiotics treatment followed by AOM+DSS. Indeed, depletion of commensal microbiota reduced tumor load and attenuated intestinal damage (Fig. 6A and B). Of importance, deletion of colonic microorganism partially suppressed GMCSF expression in the colon (Fig. 6C), and in CEC after CAC induction (Fig. 6D). This suggests commensal flora as a trigger for GMCSF expression in CEC.

To further dissect this issue, Toll-like receptor 4 (TLR4)-/- mice were subjected to CAC induction. As described previously (24), TLR4 knockout protected against CAC development (Fig. 7A). In parallel with macroscopic changes, the absence of TLR4-mediated signaling led to a considerable reduction of GMCSF expression in CEC after CAC induction (Fig. 7B). As CEC expressed TLR4 (Fig. S7A and B), we speculated that the outcome was directly due to loss of TLR4 in CEC. To address this,
CT26 cells, that virtually expressed TLR4 identified by FACS analysis (Fig. S7C), were stimulated with TLR4 ligand LPS. Consequently, LPS challenge was sufficient to induce GMCSF and VEGF expression in transformed CEC (Fig. 7C). To further investigate the link of CEC-derived GMCSF expression to LPS challenge in vivo, we injected LPS into commensal flora-depleted mice. The result showed that LPS administration efficiently triggered GMCSF expression in CEC (Fig. 7D). Overall, these findings indicate an important role of commensal flora-derived LPS-mediated signaling in promoting GMCSF expression in CEC during CAC tumorigenesis.
Discussion

Recent studies have demonstrated that GMCSF plays a nonredundant role in facilitating epithelial cell proliferation and ulcer healing in response to epithelial injury during intestinal inflammation (13,25). Its function, however, on the development of colitis-associated cancer remains unclear. In the present study, we provide evidence for the first time that GMCSF is a key regulator to promote the development of CAC and that neoplastic colonic epithelial cells are an important producer for this cytokine. Given that GMCSF administration has been proven to be effective for treatment of IBD (13,26), our data highlight that it may be necessary to rigorously assess these therapeutic regimens.

The molecular actions of GMCSF in promoting the progression of colitis to cancer are still unknown. In this report, we provided clues that transformed epithelial cell-derived GMCSF elicited large release of VEGF by itself in an autocrine and/or paracrine manner, thereby facilitated uncontrolled proliferation of CEC and intratumoral angiogenesis. This regulation of VEGF production and angiogenesis by GMCSF is in agreement with recent studies in tumor and non-tumor models (27,28). The dissection of signaling pathways involved in this process revealed that ERK-mediated pathway was required for GMCSF-elicited VEGF expression. Indeed, ERK
signaling is one of the major pathways that are responsible for VEGF expression induced by various stimuli in transformed or non-transformed cells (29, 30). Of note, activation of ERK signaling appears to increase translocation of hypoxia-inducible factor 1α (HIF-1α) from cytoplasm to nuclei, consequently it binds to the VEGF promoter and initiates the transcription of the growth factor (31). Considering elevated contents of HIF-1α are a universal feature in tumor cells, including colon carcinoma cells (22), it is plausible that GMCSF-elicited VEGF expression in CEC in chronic inflammatory microenvironment is attributed to, at least in part, increased HIF-1α translocation. Of note, MPs (including macrophage and DC) residing in lamina propria are also cellular sources of GMCSF and VEGF in the setting of CAC. So it is likely that MP-derived GMCSF/VEGF also has a role in malignant transformation of CEC during intestinal inflammation and that there is crosstalk between CEC and MPs. The details need further investigation in the future.

Our data demonstrated that GMCSF stimulation in malignant colon epithelial cells enhanced their motility. VEGF was a key player in this process, which is in line with previous reports showing that VEGF represents one of proinvasive factor for human colon cancer cells (32). Of note, GMCSF had no ability to enhance proliferation of CEC in vitro. Thus, in the context of CAC, other proinflammatory factors may be involved in hyperproliferation of epithelium, for example IL-6.
Blockade of GMCSF in vivo down-regulates the expression of these factors thereby represses CAC formation. In fact, we found that blocking GMCSF did decrease IL-6 expression in LP mononuclear phagocytes (data not shown). Therefore, GMCSF has ability to promote the growth and invasion of malignant CEC via acting on epithelium itself and/or modulating protumor function of stromal cells in the surroundings.

GMCSF is secreted by a variety of cell types in vitro in response to proinflammatory stimuli (33). In CAC model, DSS administration disrupts the epithelial barrier and led to translocation of luminal microbiota into mucosa that triggers intestinal inflammatory reaction. Insult of invasive microorganisms and their components such as LPS is sufficient and necessary to GMCSF expression in CEC, in that eradication of commensal flora or TLR4 deficiency resulted in substantial reduction of GMCSF expression in CEC after CAC induction. Other factors such as tumor necrosis factor-α (TNF-α) and interleukin-1 that have potentials to induce GMCSF (34), however, may be involved in this process, because these factors play a crucial role in the pathogenesis of CAC (16,35). Of note, the expression of these proinflammatory mediators in CAC microenvironment is regulated by commensal flora (36). Thus, it is reasonable that commensal microbiota and their components (e.g. LPS) induce GMCSF expression in CEC directly and indirectly in the milieu of intestinal inflammation.
Our study proposed a schematic model of GMCSF function on CAC carcinogenesis as following: oral administration of DSS leads to disruption of colonic epithelial barrier and commensal translocation into mucosa. Challenge of microbiota elements such as LPS induces high-level expression GMCSF in CEC. GMCSF has a positive feedback on overexpression of GMCSF and elicits VEGF release by CEC, the latter promotes hyperproliferation of CEC and angiogenesis thereby enhances its tumorigenic capacity. Notably, besides an immune-independent effect of GMCSF on CAC development, its influence on immune cells (e.g. myeloid suppressor cells) in the microenvironment of inflammation is deserved to be addressed, as shown by a recent study that tumor-derived GMCSF potently induced the development of myeloid suppressor cells that suppressed anti-tumor immune response (37). Indeed, in CAC milieu, we found increased accumulation of Gr-1+ myeloid suppressor cells in the lesions and that blocking GMCSF activity dramatically decreased the number of this population (our unpublished data). Thus, further investigations on the immune-dependent effect (particularly myeloid suppressor cells) of GMCSF on CAC development are warranted. Overall, our findings may have implications for understanding of mechanisms underlying CAC pathogenesis. Targeting GMCSF or VEGF may have therapeutic potentials to the treatment of CAC in clinic.
References


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Figure legends

Figure 1. Elevated expression of GMCSF in the colonic epithelial cells of CAC-bearing mice. (A) Schematic overview of CAC regimen. (B) On day 100 after initiation of CAC induction, colonic epithelium and several immune cell subsets in LP were isolated and GMCSF expression in these cells was examined by quantitative RT-PCR. (C) On day 35 (2 cycle of DSS) and 100 (4 cycle of DSS), colon explants were cultured ex vivo for 24 hr. GMCSF contents in the supernatants were determined by ELISA. (D) Samples were collected as described in C, GCSF and MCSF contents in the supernatants were determined by ELISA. The data were pooled from four independent experiments. Each group consists of 6-8 mice. **, \( P<0.01 \); ***, \( P<0.001 \) vs naïve controls.

Figure 2. GMCSF potently drives VEGF expression in CEC. (A) On day 35 (2 cycle of DSS) 100 (4 cycle of DSS), pieces of colon tissues were cultured ex vivo for 24 hr and the supernatants were collected. VEGF-A contents were detected by ELISA. (B) On day 100 after CAC induction, CEC and several immune cell subsets in LP were isolated and VEGF expression was examined by quantitative RT-PCR. (C) CT26 cells were exposed to GMCSF at indicated doses for 48 hr. cells were collected and VEGF expression was examined by real-time RT-PCR. (D) CT26 cells were
stimulated with IL6, IL1β, VEGF or GMCSF at the same doses (20 ng/ml) for 48 hr, respectively. VEGF expression was examined by quantitative RT-PCR. (E) CT26 cells were stimulated with GMCSF (50 ng/ml) alone or with monoclonal antibody against IL6 (1 μg/ml) and/or IL-1R antagonist (2 μg/ml) for 48 hr. VEGF expression was detected by quantitative RT-PCR. (F) CT26 cells were stimulated with GMCSF (50 ng/ml) for 15 min. The protein was extracted and phosphorylation of STAT5 and ERK1/2 was examined by immunoblotting. As positive controls, murine macrophage (RAW264.7) was used. (G) CT26 cells were preincubated with JAK2 inhibitor (AG490, 100 μM), ERK1/2 inhibitor (U0126, 1 μM), PI3K inhibitor (wortmannin, 500 nM) or in combination for 30 min respectively, and stimulated with GMCSF (50 ng/ml) for 48 hr. VEGF expression was determined by quantitative RT-PCR. The experiments were repeated 3-4 times with similar results. Each group consists of 6-8 mice. n.s, no significance; *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 3. Blockade of GMCSF activity reduces CAC loads. Mice suffering from CAC induction received neutralizing antibody to mGMCSF or isotypes according to the regimen as described in Materials and methods, the following parameters were evaluated on day 100. (A) Tumor number in colon and rectum was counted. (B) Histological examination with H&E staining was performed. Colitis score was shown.
Scale bar: 200μm. (C) Apoptosis of colonic epithelial cells was detected by TUNEL methods. Quantitation of apoptotic cells was shown. Scale bar: 100μm. (D,E) Epithelial cells were isolated as described in Materials and methods and proteins were extracted. ERK MAPK phosphorylation (D) and Bcl-XL contents (E) were determined by Western blotting. The data were pooled from two or three independent experiments. Each group consists of 5-8 mice. **, *P* <0.01; ***, *P* <0.001 vs isotype controls.

**Figure 4. Decreased expression of VEGF in CEC following GMCSF neutralization is involved in CAC tumorigenesis.** (A) On day 100 after CAC induction, CEC from mice administrated by neutralizing antibody to GMCSF or isotype was isolated and VEGF expression was examined by quantitative RT-PCR. (B) Angiogenesis in tumor tissues was detected by staining with anti-CD31 antibody. Representative images from three independent experiments were shown. The arrows denote vessels. Scale bar: 50μm. Staining density was measured and quantified with ImagePro plus 7.0 Software using images (left panel). Fifteen to twenty histologically fields randomly selected from each slide for analysis of staining density. (C) CEC was isolated from mice treated with anti-GMCSF or isotype and protein was extracted. Phosphorylation of NF-κB p65 subunit was examined by immunoblotting. (D-K)
Mice subjected to CAC induction were injected with neutralizing antibody to mVEGF-A or isotype according to the protocol as described in Materials and methods. (D) Tumor number was counted. (E) Histological examination of colon tissues was performed by H&E staining. Colitis score was shown. Scale bar: 200μm. (F) Angiogenesis in tumor tissues was detected by staining with anti-CD31 antibody. Representative images from three independent experiments were shown. The arrows denote vessels. Scale bar: 50μm. Staining density was measured and quantified with ImagePro plus 7.0 Software using images (left panel). Fifteen to twenty histologically fields randomly selected from each slide for analysis of staining density. (G) On day 35 after initiation of DSS drinking, CEC of anti-GMCSF or isotype-treated mice were isolated and VEGFR2 phosphorylation in CEC was determined by immunoblotting. The experiments were repeated 3-4 times with similar results. (H) Apoptosis of CEC was detected by TUNEL method. Quantitation of apoptotic cells was shown. Scale bar: 100μm. (I,J) The protein of CEC was extracted and Bcl-XL (I) contents and phosphorylation of STAT3 (J) were detected by immunoblotting. The data were pooled from three independent experiments. Each group consists of 6-8 mice. **, $P<0.01; ***, P<0.001$ vs isotype controls.
Figure 5. CEC exposed to GMCSF renders enhanced motility in vitro. (A) The capacity of proliferation of HCT116 with rhGMCSF stimulation at indicated concentrations was examined by SRB assays. (B,C) HCT116 was stimulated with rhGMCSF at indicated concentrations for 16 hr. the ability of HCT116 to migration (B) and invasion (C) was detected by transwell assays. Scale bar: 200μm. (D,E) HCT116 was stimulated with rhGMCSF (50 ng/ml) for 16 hr. Monoclonal antibody to VEGF (bevacizumab, 2μg/ml) or isotypes was added in the culture. The ability of HCT116 to migration (D) and invasion (E) was detected by transwell assays. Scale bar: 200μm. The experiments were repeated five times. **, P<0.01; ***, P<0.001.

Figure 6. GMCSF expression in CEC is dependent on commensal flora. Bacteria in the gut were eradicated by antibiotics treatment, followed by CAC induction. On day 100 after initiation of DSS drinking, the following parameters were evaluated. (A) Tumor number was calculated. (B) H&E staining of colon tissues was performed. Colitis score was shown. Scale bar: 200μm. (C) Pieces of colon tissues were cultured for 24 hr and GMCSF contents in the supernatants were examined by ELISA. (D) CEC was isolated and GMCSF expression was detected by quantitative RT-PCR. The data were pooled from three independent experiments. Each group consists of 5-8 mice. **, P<0.01; ***, P<0.001 vs untreated controls.
Figure 7. LPS/TLR4 signaling triggers GMCSF expression in CEC. (A,B) TLR4-/- and WT littermates were subjected to CAC induction. (A) On day 100, tumor number in the colon and rectum was counted. (B) GMCSF expression in CEC was examined by quantitative RT-PCR. (C) CT26 cells were challenged by LPS at indicated doses for 24 hr. GMCSF and VEGF expression was detected by real-time RT-PCR. (D) Commensal flora was eradicated by antibiotics treatment as described in Materials and methods, followed by injection of LPS (10mg/kg) or PBS intraperitoneally. CEC were isolated at 12 hr after LPS challenge and GMCSF expression was examined by real-time RT-PCR. Each group consists of 6-8 mice. The experiments were repeated 3 times. **, P<0.01; ***, P<0.001 vs WT littermates or untreated controls.
Figure 1

A

AOM  DSS  DSS  DSS  DSS  DSS  sacrifice

day 35  day 100

B

Relative level of GMCSF

IEC  neutrophil  αβT  γδT  MPS

naive  CAC

C

GMCSF (ng/ml)

naive  2 cycle  4 cycle  CAC

***  ***

D

GCSF (ng/ml)

naive  2 cycle  4 cycle  CAC

MCSF (ng/ml)

naive  2 cycle  4 cycle  CAC

***  ***
Figure 2

A

VEGF (pg/ml)

naive 2 cycle 4 cycle

---

B

Relative level of VEGF

IEC neutrophil αvβ3 VEGF MPs

---

C

Relative level of VEGF

GMCSF (ng/ml)

0 1 20 50 100

---

D

Relative level of VEGF

medium IL6 IL-1β VEGF GMCSF

---

E

Relative level of VEGF

GMCSF IL6 mAb IL-1Ra

---

F

GMCSF (50 ng/ml)

P-STAT5 STAT5 P-ERK1/2 ERK1/2

CT26 RAW264.7

---

G

Relative level of VEGF

GMCSF wortmannin U0126 AG490

---
Figure 5
Figure 6

A

B

C

D
Figure 7
Tumor-derived GM-CSF promotes inflammatory colon carcinogenesis via stimulating epithelial release of VEGF

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