CXCL1 Is Critical for Premetastatic Niche Formation and Metastasis in Colorectal Cancer
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Abstract
Emerging evidence suggests that the primary tumor influences the development of supportive metastatic microenvironments, referred to as premetastatic niches, in certain distant organs before arrival of metastatic cells. However, the mechanisms underlying the contributions of the primary tumor to premetastatic niche formation are not fully understood. Here, we demonstrate that colorectal carcinoma cells secrete VEGFA, which stimulates tumor-associated macrophages to produce CXCL1 in the primary tumor. Elevation of CXCL1 in premetastatic liver tissue recruited CXCR2-positive myeloid-derived suppressor cells (MDSC) to form a premetastatic niche that ultimately promoted liver metastases. Importantly, premetastatic liver-infiltrating MDSCs induced tumor cell survival without involvement of innate or adaptive immune responses. Our study provides the first evidence that primary malignant cell-secreted VEGFA stimulates tumor-associated macrophages to produce CXCL1, which recruits CXCR2-positive MDSCs to form a premetastatic niche to promote liver metastases. Our findings not only shed light on how the tumor microenvironment contributes to premetastatic niche formation at distant sites, but they also provide comprehensive insights into how MDSCs are recruited to other organs where they contribute to metastatic spread of disease. Moreover, our work also provides a rationale for development of CXCR2 antagonists to inhibit or prevent metastatic spread of disease. Cancer Res; 77(13); 3655–65. ©2017 AACR.

Introduction
Colorectal cancer is the second leading cause of cancer-related deaths and the third most common malignant neoplasm in the United States. Depending on the stage of disease at diagnosis, liver metastases occur in 20% to 70% of patients and lung metastases in 10% to 20% of patients. Unfortunately, distant metastases are the major cause of death for patients with advanced colorectal cancer because standard treatment options for widely metastatic colorectal cancer have not been as effective as needed, resulting in very low 5-year survival rates. Clearly, understanding the molecular mechanisms responsible for colorectal cancer metastases will lead to novel strategies for treatment of advanced disease.

The metastatic spread of carcinoma cells is a complex process. The observations that no new mutations occur in the metastatic tumors when compared with primary colorectal carcinomas in the same patient (1) and that no new mutations were found in primary tumors of colorectal cancer patients with or without distant metastases (2) suggest that new mutations are not required to enable tumor cells to leave the primary tumor and spread to secondary organs. Recent evidence suggests that tumor spread is facilitated by the formation of supportive metastatic microenvironments, referred to as premetastatic niches, which develop prior to primary tumor cell dissemination (3). Premetastatic niches support colonization, survival, and growth of metastasizing tumor cells. However, the mechanisms responsible for the formation of premetastatic niches are not well understood.

Primary tumor cells are thought to initiate premetastatic niche formation via secretion of proinflammatory cytokines, chemoattractants, and angiogenic factors that recruit and mobilize bone marrow–derived cells into future metastatic sites. For example, primary tumor–secreted VEGF or PIGF directly recruits bone marrow–derived VEGFR1+ hematopoietic progenitor cells into future metastatic sites to develop metastatic niches in mouse models of lung cancer and melanoma (4). Similarly, lysyl oxidase or CCL2 secreted from hypoxic mammary tumor cells induces premetastatic niche formation in lung via recruitment of bone marrow–derived cells and bone marrow–derived granulocytic myeloid-derived suppressor cells (MDSC), respectively (5, 6). Alternatively, primary tumor–secreted VEGFA, TGFβ, or TNFα induces A100A8/9 expression in resident cells of premetastatic lung, where A100A8/9 attracts MAC1+ myeloid cells to form premetastatic niches (7). These previous studies suggest that primary tumor cell–secreted factors either directly recruit bone marrow–derived cells to premetastatic tissues or interact with resident cells of premetastatic organs to generate a premetastatic niche. However, it is not clear whether the microenvironment in the primary tumor also contributes to premetastatic niche formation.

CXCL1 levels are elevated in colorectal cancer, and increased levels are associated with tumor size, advancing stage, depth of invasion, and patient survival (8, 9). Our previous work revealed that the CXCL1 receptor, CXCR2, is required for recruitment of
granulocytic MDSCs (gMDSC) into colonic mucosa and colitis-associated tumors from the circulatory system (10). Indeed, the levels of MDSCs in the blood and/or tumor tissue correlated with clinical cancer stage, metastatic tumor burden, or poor survival in patients with colon, esophageal, gastric, or pancreatic cancers (11–17). MDSCs have been shown to contribute to cancer immune evasion by suppressing effector T-cell activation, proliferation, trafficking, viability, inhibition of natural killer (NK) cells, and activation/expansion of regulatory T cells (Treg ref. 18). Our previous study also showed that MDSCs promoted inflammation-associated tumorigenesis by inhibiting CD8+ T-cell cytotoxicity against tumor cells (10). Emerging evidence reveals that MDSCs accumulate in premetastatic sites (5, 19, 20). However, the underlying mechanisms responsible for the recruitment of MDSCs into premetastatic organs and the contribution of MDSCs to colonization, survival, and outgrowth of metastasizing tumor cells remain elusive.

In this study, we investigate whether the molecular interactions of malignant cells with associated stromal cells in the primary tumor contribute to the formation of premetastatic niches and how these premetastatic niches facilitate metastasis.

Materials and Methods

Cell culture and reagents

HCT-116 and LS-174T cell lines were obtained from the ATCC in 2014. HCT-116, HCT-116/GFP-Luc, HCT116/vector, and HCT-116/shVEGFA, LS-174T, LS-174T/GFP-Luc, LS-174T/vector, and LS-174T/shVEGFA cells were cultured in McCoy’s 5A medium with 10% FBS. All colorectal cancer cells were used between passages 1 and 2. Human colonic epithelial cells (HCoEpiC) were obtained from ScienCell Research Laboratories. HCoEpiC cells were used between passages 2 and 5. Human colonic epithelial cells (HCoEpiC) were obtained from ScienCell Research Laboratories in 2014 and cultured in colonic epithelial cell medium (ScienCell Research Laboratories). HCoEpiC cells were authenticated before the experiment according to ATCC STR database.

Primary carcinoma cells isolated from human colorectal cancer specimen were cultured in Chang medium with 10% FBS. All colorectal cancer cells were used derived from human primary colorectal cancer specimen were injected into the cecal wall of male NSG mice at the age of 8 weeks. After cecal injection, mice bearing HCT-116 tumors were sacrificed in 4 or 7 weeks for the experiments of premetastatic phase or metastatic stages. Similarly, mice bearing LS-174T or human primary carcinoma tumors were sacrificed in 7 or 11 weeks for the experiments of premetastatic phase or metastatic stages. A total of 2 × 10^5 pfu of adeno-cre in 100 µL PBS were administrated into the distal colon of wild-type (WT) or Apc^fl/fl;LSL-Kras^G12D^/C0 male mice aged 3 months, and the mice were sacrificed at the age of 12 months. For CXCR2 antagonist (SB225002, Sigma-Aldrich) treatment, male NSG mice injected with HCT-116 cells were treated with SB225002 at 400 µg/100 µL PBS per mouse or vehicle by daily intraperitoneal injection for 4 or 7 weeks after injection. Similarly, male NSG mice injected with LS-174T cells were treated with same dosage of SB225002 for 7 or 11 weeks after cecal injection.

Isolation of immunocytes from organs

Liber was excised after perfusion of portal vein with cold PBS, and lung was excised after flushing with cold PBS. Excised liver, lung, cecal tumor, and normal cecum were weighted, minced, and digested by digestion buffer (3 mg/mL dispase II, 127.5 U/mL collagenase A, and 25 U/mL DNase I in HBSS) with 5% FBS for 1 hour at 37°C under slow rotation. Digest II and DNase I were purchased from Roche, whereas collagenase A was obtained from Gibco. Discontinuous (44% and 67%) percoll (GE Healthcare) separation method was used to enrich immunocytes. Whole blood was collected and bone marrow was isolated from the right ventricle and femurs. The red blood cells (RBC) in blood and bone marrow were lysed with RBC lysis buffer (eBioscience). A total of 1 × 10^6 immunocytes isolated from liver, lung, blood, bone marrow, cecal tumor, and normal cecum were subjected to the flow cytometry analyses.

Flow cytometry analysis and sorting

For multicolor flow cytometry immunotypic analyses, immunocyte cells were incubated with the appropriate combination of the following antibodies in staining buffer (BD Biosciences) at the following dilution: CD45-PB (1:200), CD11b-FITC (1:11), Ly6C-PE (1:80), Ly6G-Alexa700 (1:40), CD11c-PE-Cy7 (1:80), F4/80-PE TexasRed (1:80), and/or CXCR2-FITC for 30 minutes on ice. After the cells were washed twice with 1 mL of the labeling buffer, they were analyzed on a Gallios flow cytometer (Beckman Coulter). For analysis of CXCL1 expression on immune cells, immunocyte cells were stained with cell surface markers as described above. Then, the cells were fixed and permeabilized by using a Cytofix/Cytoperm Kit (BD Biosciences), followed by intracellular staining with primary rabbit anti-mouse CXCL1 antibody (5 µg/mL) and second anti-rabbit-IgG-APC or anti-rabbit-IgG-FITC (1:1,000, BioLegend) in permeabilization buffer for 30 minutes on ice. After the cells were washed twice with 1 mL of the permeabilization buffer, they were analyzed on a Gallios flow cytometer (Beckman Coulter). The flow cytometric profiles were analyzed by counting 20,000 events using Kaluza software program (Beckman Coulter). CD11b^Ly6C^ high cells from blood and liver were sorted by Gallios flow cytometer (Beckman Coulter).
Total proteins were extracted from normal cecum, cecal tumors, livers, and lungs by homogenizing and subsequently sonicating in antiprotease buffer (50 mmol/L Hepes, 150 mmol/L NaCl, and 1 mmol/L EDTA) containing protease inhibitor cocktail tablets (Roche). The levels of human and mouse CXCL1, VEGFA, and other factors in mouse tissues and cell-free supernatants were measured by using Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions.

Lentivirus production and stable transfection

HCT-116 and LS-174T cells were transduced with pLV-TH-GFP-Luc vector. VEGFA shRNA and its vector were purchased from GE Dharamcon. Lentivirus production and stable transfection were performed according to the manufacturer’s instructions.

Apoptosis assays

The HCT-116 cells (2.5 × 10^5 /each well) were plated in the lower chamber of 6-transwell plates (0.4 µmol/L, Corning). After culture overnight, the cells were washed twice with PBS, and then MDSCs were placed in the upper chamber at ratios (gMDSCs: HCT-116 = 0:1, 0.5:1, 1:1, 2:1, or 4:1) for 3-day culture. The percent of apoptotic tumor cells was determined by flow cytometry using TACS Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s instructions (R&D Systems). The combination of Annexin V-FITC and propidium iodide allowed for the differentiation between early apoptotic cells (Annexin V-FITC positive), late apoptotic cells (Annexin V-FITC and propidium iodide positive), necrotic cells (propidium iodide positive), and viable cells (both negative).

Statistical analysis

Each in vitro experiment was done at least 3 times, and each in vivo experiment was conducted at least twice. Data are presented as mean ± SEM. Comparisons among multiple groups were performed by factorial ANOVA, followed by Bonferroni test. Comparisons between two groups were performed with Student t test or Mann–Whitney U test where appropriate. Fisher’s exact test was used for categorical variables. P < 0.05 was considered statistically significant.

Results

Primary tumor induces a accumulation of MDSCs in premetastatic liver

As orthotopic mouse models of colorectal cancer allow us to distinguish human cancer cell–secreted factors from mouse host stromal cell–secreted factors, we were able to identify factors that directly attract bone marrow–derived cells into premetastatic organs that were secreted from human malignant cells and/or from mouse host stromal cells. To precisely separate the premetastatic phase from metastatic stages in this model, human colorectal carcinoma HCT-116/GFP-Luc and LS-174T/GFP-Luc cells, which stably express GFP and luciferase, were injected into the cecal wall of NSG mice. After injection, cecal tumor growth, liver metastasis, and lung metastasis were monitored by bioluminescence. For HCT-116 cells, cecal tumors formed in 1 week as judged by examining histologic sections and luciferase activity. After injection, cecal tumor growth, liver metastasis, and lung metastasis were monitored by bioluminescence. For HCT-116 cells, cecal tumors formed in 1 week as judged by examining histologic sections and luciferase activity. As no luciferase activity in liver and lung was detected and no GFP cells were found in liver and lung at 4 weeks, all experiments for the “premetastatic” phase were performed within 4 weeks after injection. Similarly, all experiments with injection of LS-174T cells for the premetastatic phase were performed within 7 weeks after injection.
We first evaluated whether the presence of a primary tumor affected immune cell profiles in premetastatic liver and lung of NSG mice. As shown in Fig. 1A and B, the presence of cecal tumors from HCT-116 or LS-174T cells induced accumulation of CD11b⁺Ly6GhighCD11c⁻/F4/80⁻ cells in premetastatic liver and lung as compared with normal cecal tissues injected with HCoEpiC as a control. NSG mice injected with HCoEpiC cells did not develop cecal tumors. Moreover, CD11b⁺Ly6GhighCD11c⁻/F4/80⁻ cells isolated from premetastatic livers were able to inhibit the proliferation and IFNγ production of activated CD8⁺ T cells in vitro, demonstrating that these cells are MDSCs (Supplementary Fig. S1A-B). Interestingly, MDSCs isolated from premetastatic liver inhibit CD8⁺ T-cell activity more effectively than bone marrow–derived MDSCs (Supplementary Fig. S1A and S1B). Cecal tumors also induced infiltration of macrophages (CD11b⁺F4/80⁻Ly6G⁻CD11c⁺) in only premetastatic livers (Fig. 1A and B). In contrast, the presence of cecal tumors did not affect accumulation of dendritic cells (DC; CD11c⁻Gr1⁺F4/80⁻) or neutrophils (CD11b⁺Ly6G⁺CD11c⁻F4/80⁻), but not CD11b⁺Ly6GhighCD11c⁻F4/80⁻; Fig. 1A and B; Supplementary Fig. S1A). RT-PCR analyses further confirmed that no luciferase gene was detected in liver or lung tissues, demonstrating that the presence of a cecal tumor alters immune cell profiles in premetastatic liver and lung. In mice, MDSCs are broadly defined as CD11b⁺Gr1⁺ cells that suppress the proliferation and the cytokine production of T cells. Mouse MDSCs are further divided into two subsets, granulocytic (CD11b⁺Ly6GhighLy6Clow) and monocytic (CD11b⁺Ly6C⁺) MDSCs (21). However, gMDSCs isolated from livers of NSG do not express low levels of Ly6C. In most experimental tumor models, gMDSCs are more markedly expanded than monocytic MDSCs (10, 21). Indeed, 90% of MDSCs expressed both CD11b and Ly6Ghigh markers, whereas 10% of MDSCs expressed both CD11b and Ly6C but not Ly6G, especially in premetastatic liver (Supplementary Fig. S1C and C).
S1D). Furthermore, human primary colon carcinoma cell–derived cecal tumors only induced an infiltration of gMDSCs in premetastatic liver and lung (Fig. 1C). We further examined whether colonic tumors also altered immune cell profiles in premetastatic liver and lung in a mouse model of spontaneous colorectal cancer, Apc^{−/−}/Kras^{G12D} mice. Indeed, colonic tumors only promoted an accumulation of gMDSCs in premetastatic liver of Apc^{−/−}/Kras^{G12D} mice as compared with WT mice (Fig. 1D). As we only found gMDSC accumulation in premetastatic liver in the four animal models we tested, we concluded that primary tumors preferentially induce an infiltration of gMDSCs into premetastatic liver.

The CXCL1–CXCR2 axis is required for recruitment of MDSCs into premetastatic liver and liver metastasis

As CXCR2 has been shown to be required for homing of MDSCs into colonic mucosa and tumors from the circulatory system (10), we postulated that CXCR2 ligands, such as CXCL1, CXCL2, and/or CXCL5, are able to recruit circulatory MDSCs into local organs via CXCR2. Indeed, HCT-116, LS-174T, and human primary colon carcinoma cells secreted higher levels of CXCL1 than HCoEpiC (Fig. 2A). Unexpectedly, human CXCL1 was not detected in premetastatic liver and lung, although the levels of both human and mouse CXCL1 were elevated in cecal tumors as compared with normal cecal tissues injected with HCoEpiC as a control (Fig. 2B). These results demonstrate that primary tumor cell–secreted factors do not directly attract gMDSCs into premetastatic liver. Moreover, mouse CXCL1 was also increased in colonic tumors and premetastatic livers in Apc^{−/−}/Kras^{G12D} mice (Fig. 2E). Collectively, these results indicate that mouse CXCL1 secreted from primary tumor stromal cells is responsible for attracting circulatory gMDSCs into premetastatic liver in our models.

We further determined whether the CXCL1–CXCR2 axis is required for recruitment of gMDSCs into premetastatic liver. As CXCR2 is mainly expressed on Ly6G^+ cells, including neutrophils and MDSCs, in mice (22), we first confirmed whether gMDSCs express CXCR2 in our model. Almost all circulating gMDSCs expressed CXCR2 regardless of the presence or absence of cecal tumors (Fig. 2F). In contrast, almost all circulating mMDSCs did not express CXCR2 regardless of the presence or absence of cecal tumors (Supplementary Fig. S1E). In addition, cecal tumors derived from HCT-116 and LS-174T cells increased gMDSC populations in blood but not in bone marrow.
(Supplementary Fig. S2A). As our previous study demonstrated that Cxcr2-deficient gMDSCs have lost their ability to migrate to colonic mucosa and tumors (10), we postulated that inhibition of CXCR2 signaling attenuated primary tumor–induced infiltration of gMDSCs into premetastatic liver and inhibited liver metastasis. Indeed, CXCL1 attracted gMDSCs isolated from blood of NSG mice, but did not affect the expansion of gMDSCs isolated from the premetastatic livers (Supplementary Fig. S2B and S2C). Importantly, a CXCR2 antagonist (SB225002) inhibited CXCL1-induced chemotaxis (Supplementary Fig. S2D). These results demonstrate that CXCR2 is required for CXCL1 induction of gMDSCs in premetastatic livers (Fig. 2G) and reduced overall burden of metastatic disease in the liver (Fig. 2H). In addition, the CXCR2 antagonist significantly decreased infiltration of gMDSCs into premetastatic livers without affecting other immune cells, such as DCs and macrophages (Fig. 2G), demonstrating that the CXCR2 antagonist specifically targets gMDSCs. Importantly, treatment with the CXCR2 antagonist resulted in the accumulation of gMDSCs in the circulatory system (Supplementary Fig. S2E), demonstrating that inhibition of CXCR2 by its antagonists blocks the recruitment of gMDSCs into premetastatic livers from the circulatory system. Interestingly, the CXCR2 antagonist did not affect cecal tumor growth in both the premetastatic stage (Supplementary Fig. S2F, left) and metastatic stage (Supplementary Fig. S2F, right). Similarly, the CXCR2 antagonist also inhibited primary tumor–induced infiltration of gMDSCs in premetastatic livers (Supplementary Fig. S2G) and reduced liver metastatic tumor burden (Supplementary Fig. S2H) in mice injected with LS-174T cells without affecting cecal tumor growth (Supplementary Fig. S2I). Collectively, these results demonstrate that elevation of mouse CXCL1 in premetastatic liver recruits CXCR2-expressing gMDSCs from the circulatory system into premetastatic liver, which in turn promotes liver metastasis.

VEGF is required for induction of CXCL1 and infiltration of MDSCs in premetastatic liver as well as promotion of liver metastasis

CXCL1 has been shown to be upregulated by proinflammatory cytokines TNFα and IL1β or by bacterial infection in colorectal carcinoma cells (23). However, the levels of TNFα were undetectable in both LS-174T and HCT-116 cell culture supernatants, whereas IL1β levels were undetectable in LS-174T cells and cecal tumors derived from HCT-116 cells. These results demonstrated that TNFα and IL1β were not likely to be tumor cell–secreted factors that upregulate CXCL1 expression in our models. In contrast, VEGFA levels were significantly higher in cell culture supernatants of LS-174T, HCT-116, and primary colon carcinoma cells than HCoEpiC cells (Fig. 3A). Both human and/or mouse VEGFA were also elevated in cecal tumors of NSG mice (Fig. 3B) and colon tumors found in Apc−/−/KrasG12D mice (Fig. 3C). In contrast, human VEGFB, -C, and -D were not detectable in cecal tumors. However, primary tumors did not induce mouse VEGFA expression in premetastatic livers of both NSG and Apc−/−/KrasG12D mice (Fig. 3D). As mentioned earlier, human VEGF is not detectable in premetastatic livers of NSG mice. These results demonstrate that human VEGFA secreted from human cancer cells and mouse VEGFA secreted from tumor stromal cells are not responsible for recruitment of gMDSCs. As VEGF has been shown

Figure 4. VEGF is required for induction of CXCL1, infiltration of MDSC in premetastatic liver, and promotion of liver metastasis. A, The protein levels of human VEGFA (left) and CXCL1 (right) in the cell culture supernatants of indicated cells. B, The protein levels of human VEGFA, human CXCL1, and mouse CXCL1 in cecal tumors of NSG mice injected with HCT-116/ vector or HCT-116/shVEGFA (left), as well as LS-174T/vector or LS-174T/ shVEGFA cells (right) without developing liver metastasis. C, The protein levels of mouse CXCL1 in premetastatic livers of NSG mice injected with indicated cells as described in B. D, The numbers of gMDSCs in premetastatic livers of NSG mice injected with indicated cells as described in B. Error bar, ± SEM. *, P < 0.05.
Figure 5.

Tumor cell–secreted VEGF stimulates TAMs to produce CXCL1. A and B, The percentage (left) and numbers (right) of DCs, gMDSCs, macrophages (Mφ), and neutrophils (Neu) in normal cecum of NSG mice injected with HCoEpiC cells and cecal tumors of NSG mice without developing metastasis after injection of HCT-116 cells (A) or LS-174T cells (B). C, Left, data represent the percentage of CXCL1⁺ macrophages, CXCL1⁺ gMDSCs, and CXCL1⁺ DCs in total macrophages, gMDSCs, and DCs in normal cecum and cecal tumors of NSG mice as described in A, right, the numbers of CXCL1⁺ macrophages in normal cecum and cecal tumors of NSG mice as described in A. D, Left, data represent the percentage of CXCL1⁺ macrophages, CXCL1⁺ gMDSCs, and CXCL1⁺ DCs in total macrophages, gMDSCs, and DCs in normal cecum and cecal tumors of NSG mice as described in B. Right, the numbers of CXCL1⁺ macrophages in normal cecum and cecal tumors of NSG mice as described in B. E and F, Data represent the percentage of CXCL1⁺ macrophages in total macrophages (left) and numbers of CXCL1⁺ macrophages (right) in cecal tumors of NSG mice without developing metastasis after injection of HCT-116/vector or HCT-116/shVEGFA cells (E) as well as LS-174T/vector or LS-174T/shVEGFA cells (F). G, Protein levels of mouse CXCL1 in the supernatants of mouse BMDMs treated with indicated dose of recombinant human VEGFA. Error bar, ± SEM. *, P < 0.05.
to induce CXCL1 expression in human lung carcinoma epithelial cells and endothelial cells (24, 25), we first examined whether cancer cell–secreted VEGF regulated CXCL1 expression. Knockdown of VEGF-A in HCT-116 and LS-174T cells reduced the levels of VEGF-A and CXCL1 in supernatants (Fig. 4A). Similarly, the levels of human VEGF, human CXCL1, and mouse CXCL1 were significantly reduced in cecal tumors derived from HCT-116/shVEGFA and LS-174T/shVEGFA cells as compared with vector controls (Fig. 4B). Importantly, knockdown of VEGF-A in HCT-116 and LS-174T cells significantly decreased mouse CXCL1 levels and gMDSC numbers in premetastatic livers as compared with controls (Fig. 4C and D). Finally, mice bearing cecal tumors derived from VEGF knockdown cells developed fewer liver metastatic lesions than that seen from vector cells (Fig. 4E and F). In contrast, knockdown of VEGF-A did not affect cecal tumor growth during the premetastatic stage (Supplementary Fig. S3A) and metastatic stage (Supplementary Fig. S3B). These results demonstrate that human VEGFA secreted from primary tumor cells is required for induction of mouse CXCL1 in primary tumors and premetastatic liver as well as recruitment of gMDSCs into premetastatic liver and liver metastasis.

Tumor cell–secreted VEGF stimulates tumor-associated macrophages to produce CXCL1

As human VEGF is not detectable in premetastatic liver, human VEGF induction of mouse CXCL1 must occur in cecal tumors. To determine which primary tumor–associated stromal cells express CXCL1, flow cytometry analyses were performed. Cecal tumor–associated fibroblasts and endothelial cells did not express CXCL1. We further analyzed immune cell profiles in cecal tumor specimens and found that the majority of immune cells were macrophages following injection of HCT-116 cells (Fig. 5A), whereas the majority of immune cells were comprised of macrophages and gMDSCs following injection of LS-174T cells (Fig. 5B). Mouse CXCL1 was mainly expressed in tumor-associated macrophages (TAMs) (Fig. 5C and D). Knockdown of VEGFA in HCT-116 or LS-174T cells significantly reduced CXCL1-expressing macrophages in cecal tumors (Fig. 5E and F), indicating that VEGFA secreted from tumor cells stimulates TAMs to produce CXCL1. In addition, TAMs mainly expressed VEGFR1 in the cecal tumors derived from HCT-116 cells (Supplementary Fig. S3C). We further examined whether VEGFA is able to directly induce CXCL1 expression in macrophages. Treatment of mouse BMDCs with human VEGFA resulted in induction of CXCL1 expression (Fig. 5G), demonstrating that human VEGFA can directly stimulate mouse macrophages to produce mouse CXCL1. Collectively, these results demonstrate that VEGFA secreted from primary tumor cells stimulates primary tumor–infiltrating macrophages to produce CXCL1.

MDSCs in premetastatic liver promote cancer cell survival

As NSG mice lack mature T cells, B cells, and functional NK cells, we questioned how MDSCs in premetastatic liver enhanced liver metastatic tumor formation and growth without involvement of innate and adaptive immune responses. To address this question, we determined whether MDSCs promote cancer cell survival by acting directly on cancer cells. Indeed, gMDSCs isolated from premetastatic livers of NSG mice bearing HCT-116 cecal tumors (Fig. 6A) or LS-174T cecal tumors (Fig. 6B) inhibited HCT-116 cell apoptosis induced by serum deprivation in cell culture without the cell–cell interaction of malignant cells and gMDSCs. The gMDSC enhancement of tumor cell survival was ratio dependent. Interestingly, bone marrow–derived gMDSCs from NSG mice were not able to promote cancer cell survival (Fig. 6), indicating that only MDSCs located in the premetastatic niche were able to promote cancer cell survival. These results reveal that liver-infiltrating gMDSC-secreted factors are able to promote cancer cell survival without involvement of innate and adaptive immune responses. Taken together, these results demonstrate that primary tumor cell–secreted VEGFA stimulates primary tumor–infiltrating macrophages to produce CXCL1 that recruits CXCR2-expressing MDSCs from the circulatory system into premetastatic liver. These MDSCs then promote metastatic tumor formation and growth via modulation of cancer cell survival (Fig. 7).

Discussion

A growing body evidence demonstrates that premetastatic niches are critical for facilitating homing, survival, and growth of circulating tumor cells in secondary organs. However, the cellular and molecular mechanisms by which primary tumor cells influence the formation of premetastatic niches in secondary organs are not well understood. Our study not only reveals a novel mechanism by which the primary tumor facilitates premetastatic niche formation that promotes metastasis, but also may offer CXCR2 as a new target in treating or preventing advancement of colorectal cancer disease.

Previous studies have shown that primary tumor cell–secreted factors either directly recruit BMDCs to premetastatic lesions or interact with resident cells in the premetastatic sites to generate premetastatic niches. However, our observation that primary malignant cell–secreted factors were undetectable in premetastatic liver in our mouse model of colorectal cancer liver metastasis indicates that these factors could not directly recruit MDSCs to premetastatic lesions or interact with resident cells in the premetastatic sites to generate a premetastatic niche. Moreover, we provide the first evidence that malignant cell–secreted VEGFA stimulates TAMs to produce CXCL1 that directly recruits circulatory MDSCs into premetastatic liver, which in turn promotes liver metastasis (Fig. 7). Our results demonstrate for the first time that primary tumor stromal cells contribute to the formation of premetastatic niches.
TAMs are recognized as a poor prognostic sign in various tumors, including colorectal cancer (26, 27). Multiple lines of evidence indicate that TAMs promote cancer progression and metastasis through supporting tumor-associated angiogenesis, enhancing tumor cell migration, invasion, and intravasation, and suppressing immunosurveillance (28). For example, VEGFA secreted from TAMs located in a primary tumor induces angiogenesis by binding to VEGFR1 and VEGFR2 on vascular endothelial cells (29). In addition, TAMs suppress CD8\(^+\) T-cell cytotoxic activity by either expressing immune checkpoint receptor ligands, such as PD-L1 and B7-H4 or recruiting Treg cells (30, 31). However, it was unknown whether TAMs located in the primary tumor play a role in the formation of premetastatic niches. Our data reveal that CXCL1 secreted from TAMs is required for recruitment of MDSCs into premetastatic liver to form premetastatic niches. Our data reveal that CXCL1 secreted from TAMs is required for recruitment of MDSCs into premetastatic liver to form premetastatic niches. Although VEGFR1 and VEGFR2 are mainly expressed in vascular endothelial cells, VEGFR1 is also expressed in hematopoietic, monocytes/macrophage, and smooth muscle cells (32). For example, bone marrow–derived VEGFR1\(^+\) hematopoietic progenitor cells are required for premetastatic niche formation (4). Our data showed that TAMs expressed higher levels of VEGFR1 than macrophages in normal cecum, indicating that VEGFA induces CXCL1 expression in TAMs via VEGFR1. Although VEGFA has been shown to induce CXCL1 via PKD2 in endothelial cells (25) and to induce CXCL1 via JNK and PI3K pathways in human lung carcinoma epithelial cells (24), further research is needed to determine molecular mechanisms underlying VEGF-A induction of CXCL1 in TAMs. Unexpectedly, knockdown of VEGFA in cancer cells did not affect primary tumor growth, although VEGFA knockdown indeed inhibited liver metastasis. Previous results are consistent with our observation. Ablation of macrophages by deletion of colony-stimulating factor 1 (CSF1) significantly attenuates lung metastasis without affecting primary tumor growth in the PyMT model of breast cancer (33). Similarly, deletion of SRC1 reduces CSF-1 production in tumor cells and TAM numbers in primary tumor, which inhibits lung metastasis without affecting PyMT primary tumor formation (34). These studies indicate that TAMs are required for metastasis, but may not be involved in primary tumor growth.

It has been well established that CXCL1 promotes tumor growth via induction of angiogenesis. Recent evidence demonstrated that CXCL1 enhanced tumor growth via recruitment of neutrophils into the tumor microenvironment. For example, tumor cell–secreted CXCL1 promoted lung cancer growth via recruitment of neutrophils into tumor tissue in vivo (35). TNF\(\alpha\) stimulates mesenchymal stromal cells to produce CXCR2 ligands.

**Figure 7.**
A novel mechanism underlying the contribution of primary tumor to premetastatic niche formation and liver metastasis. Primary malignant cell–secreted VEGFA stimulates primary tumor–associated macrophages to produce CXCL1 that recruits CXCR2–positive MDSCs from circulatory system into premetastatic liver. MDSCs in premetastatic liver promote liver metastases via induction of cancer cell survival in a mouse orthotopic model of colorectal cancer.
that recruit CXCR2+ neutrophils into primary tumors in a mouse model of breast cancer (36). Infiltration of CXCR2+ neutrophils into primary tumor promotes lung metastasis. Inhibition of CXCR2 suppresses metastases by targeting Ly6G+ cells in mouse models of pancreatic cancer (37). Moreover, our group and others have demonstrated that the CXCR2 ligand–CXCR2 axis is required for infiltration of MDSCs into colonic mucosa and tumors (10, 38). In this study, we extended the scope of our research to reveal that this axis is also essential for formation of premetastatic niches and liver metastasis.

It is well established that CXCL1 can directly recruit circulatory CXCR2-expressing neutrophils and MDSCs into inflammatory sites and tumor tissues. As CXCL1 has also been shown to indirectly mobilize hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) into the peripheral blood from bone marrow via MMP9 release from neutrophils (39, 40), CXCL1 may indirectly mobilize HSC and HPC into premetastatic liver, in which they differentiate into monocytes or granulocytes, such as neutrophils and MDSCs. In addition, it remains unclear mechanistically how CXCL1 accumulated in premetastatic liver at higher concentrations than in the systemic circulation if CXCL1 produced in colonic tumor diffuses to premetastatic liver tissue via the circulatory system. It is possible that VEGF produced by the primary colonic tumor directs TAMs to localize in premetastatic liver and produce CXCL1. Further studies are needed to investigate the precise mechanisms regulating CXCL1 levels and CXCL1-induced accumulation of MDSCs in premetastatic liver.

Although substantial evidence demonstrates that primary tumors induce the accumulation of bone marrow–derived immature myeloid cells in secondary organs before arrival of metastatic tumor cells, the functions of these cells in the premetastatic niche have been poorly characterized. One study showed that accumulation of MDSCs inversely correlated with suppression of NK-cell cytotoxicity and maturity in premetastatic lung (5). However, the immunosuppressive function of MDSCs in premetastatic niches has not been investigated. One possible reason could be that T cells have not been directly linked to the premetastatic niche formation. Thus, the central question is how MDSCs in premetastatic niches promote metastatic tumor cell colonization, survival, and proliferation without involvement of NK and T cells. One study reported that MDSCs in premetastatic lung tissue might enhance mammary tumor cell extravasation by remodelling blood vessels via MMP9 (19). Moreover, emerging evidence notes that MDSCs in premetastatic liver promote metastasis via directly targeting these cells (41, 42). Here, we provide the first evidence showing that MDSCs in premetastatic liver promote metastasis via directly enhancing metastatic tumor cell survival. This finding reveals a novel function of MDSCs in promotion of cancer cell survival without the involvement of innate and adaptive immune responses.

In summary, VEGFA secreted by primary tumor cells stimulates primary tumor–associated macrophages to produce CXCL1 that recruits CXCR2-positive MDSCs to form premetastatic niches promoting liver metastases. MDSCs in premetastatic niches promote cancer cell survival by directly targeting cancer cells. These findings not only shed light on how primary tumor microenvironment contributes to premetastatic niche formation at distant sites, but also provide comprehensive insights into how MDSCs are recruited to secondary organs and how MDSCs contribute to metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Wang, H. Sun
Writing, review, and/or revision of the manuscript (i.e., for all authors of the article): D. Wang, H. Sun, R.N. DuBois
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Wang
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