SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment

Running title: mast cells remodel tumor microenvironment

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Abstract

Despite the evidence for the role of inflammation in cancer initiation, promotion, and progression, the precise mechanism by which the inflammation within tumor is orchestrated by inflammatory cells remains to be determined. Here we report that tumor-infiltrating mast cells remodel tumor microenvironment and promote tumor growth. Mast cell infiltration and activation in tumor were mainly mediated by tumor-derived stem cell factor (SCF) and its receptor c-Kit on mast cells. Low concentration of SCF efficiently induced the chemotactic migration of mast cells. Tumor-infiltrating mast cells, activated by higher concentration of SCF, expressed multiple proinflammatory factors and increased IL-17 expression in tumor. The activity of NF-κB and AP-1 in tumor cells was intensified in mast cell-remodeled inflammatory microenvironment. SCF-activated mast cells also exacerbated tumor immunosuppression by releasing adenosine and increasing Treg cells, which augmented the suppression of T cells and NK cells in tumor. These findings emphasize that the remodeling of tumor microenvironment can actually be initiated by tumor cell-released SCF, and suggest that mast cell is not only a participator but also a critical regulator of inflammation and immunosuppression in tumor microenvironment.
Introduction

Chronic inflammation, a “promoting force” in tumor microenvironment, has long been known to be commonly braided with the initiation, promotion, and progression of tumorigenesis.\textsuperscript{1–5} To date, however, it is still incompletely understood how the inflammation in tumor microenvironment is orchestrated by inflammatory cells. Recently, mast cells (MCs) were highlighted as not only a major participator but also an important regulator of inflammation,\textsuperscript{6,7} and their accumulation in tumors has also been well documented,\textsuperscript{8–13} implying that mast cells may possibly play an important role in orchestrating the inflammation in tumor.

Tumor microenvironment is regarded as a “smoldering” inflammation site in which a lot of cytokines, chemokines, and enzymes mediate the inflammatory process and drive malignant progression.\textsuperscript{14,15} Among them, TNF-\(\alpha\), IL-6, VEGF, iNOS, Cox-2, and MMP-9 are of particular interest.\textsuperscript{15–18} Coincidently, all of them can be produced by mast cells. On the other hand, tumor microenvironment is also characterized by its immunoediting from immnosurveillance to immunosuppression.\textsuperscript{19} Mast cells have been found to play a critical role in the suppression of immune reactions.\textsuperscript{20} They not only produce inhibitory cytokine IL-10,\textsuperscript{21} but also are essential for the immune tolerance mediated by regulatory T cells.\textsuperscript{22} Thus, mast cell infiltration into tumor may possibly remodel tumor microenvironment and profoundly influence tumor behavior by participating and regulating inflammatory and immune reactions. However, although some studies have shown that mast cells promote tumor angiogenesis and tumor growth due to their properties as inflammatory cells,\textsuperscript{23–25} the roles of mast cells in tumor progression have been incompletely understood so far. Several key questions remain unclear, especially how mast cells are recruited into tumor site and whether they can remodel tumor microenvironment.
Mast cell migration to tumor site and the following activation may be the prerequisite for their promoting-effect on tumor. In this regard, stem cell factor (SCF) is possibly involved, since SCF triggers c-Kit signaling pathway for the differentiation, migration, maturation, and survival of mast cells. In the present study, we investigated the relationship of mast cells and SCF in tumor progression, and showed that SCF recruited and activated mast cells; the activated mast cells remodeled tumor microenvironment by intensifying inflammation and immunosuppression; tumor cell NF-κB and AP-1 activities were augmented and the suppression of T cells and NK cells was exacerbated in such remodeled microenvironment. These findings provide a new insight into the role of mast cells in tumor and the relationship among inflammation, immunosuppression, and tumor.

Materials and methods
Animals and cell lines

BALB/c and C57BL/6 mice, 6 to 8-week-old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) and Center of Experimental Animals of Chinese Academy of Medical Science (Beijing, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. Mouse tumor cell lines H22 (hepatocarcinoma), colon-26 (colon cancer), 4T1 (breast cancer), RM1 (prostate cancer), B16 (melanoma), and LLC1 (lung cancer), and human tumor cell lines HepG2 (liver cancer), SNU1 (gastric cancer), Caco2 (colon cancer), A549 (lung cancer), MCF7 (breast cancer), SKOV3 (ovarian cancer), and EC9706 (esophageal cancer) were purchased from the American Type Culture Collection (ATCC, Manassas VA) and China Center for Type Culture Collection (CCTCC, Wuhan, China), and cultured according to their guidelines.

Generation of bone marrow-derived mast cells

Bone marrow cells were harvested from femurs of mice and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in the presence of 10 ng/ml of IL-3 (PeproTech, Rocky Hill, NJ), and the nonadherent cells were passaged every 3 days. 4 weeks later, the cells were used as mast cells for experiments and referred to as bone marrow-derived mast cells (BMMCs).

Tumor growth experiment

BALB/c mice were inoculated with H22 tumor cells by subcutaneous injection of $1 \times 10^5$ cells to the left flank. 12 days later, the mice (n=8 per group) with tumor size of $\sim 5 \times 5$ mm$^2$ received $1 \times 10^5$ BMMCs by intra-tumor injection or $5 \times 10^6$ BMMCs by i.v. injection. When
indicated, the mice received i.p. injection of 100 µg of goat-anti-mouse SCF neutralizing antibody (IgG, R&D Systems, Minneapolis, MN) or goat IgG isotype control 24 h and 1 h before BMMCs injection, or received the i.v. injection of BMMCs mixed with 50 µg of rat anti-mouse c-Kit blocking antibody (eBioscience, San Diego, CA) or rat IgG2b isotype control. Tumor growth was monitored by measuring the length (L) and width (W) of tumors. The volume of tumor (V) was calculated by the formula: \( V = \frac{L \times W^2}{2} \).

**Analysis of the infiltration of mast cells in tumor tissues**

BALB/c mice were inoculated with H22 tumor cells as above. 12 days later, 5×10^6 CFSE-labeled BMMCs, with or without antibodies as above, were injected into tumor-bearing mice via tail vein. The peripheral tumor tissues were surgically excised from mice 24 h after the injection, and frozen sections were prepared and analyzed by fluorescence microscopy.

**In vitro migration assay**

Fresh H22 tumor tissues were set in lower chamber and 6×10^4 BMMCs were set in upper chamber, in triplicate, of a 24-well transwell apparatus (BD Biosciences, San Jose, CA), and incubated at 37°C for 6 h. Here we used the un-coated filter, not the filter coated with fibronectin or other ECM proteins as commonly used, in the apparatus to avoid the possibility that some tumor-derived factors might affect mast cell migration by influencing the interaction of mast cells with fibronectin or other ECM proteins. Thus the efficiency of mast cell migration might be not high, but the result was significant and could be used to well evaluate the chemotactic effect of SCF on mast cells. BMMCs migrating into the lower chamber were enumerated under microscopy, and the migrated cell percentage was calculated. SCF (PeproTech) was used as positive control. Anti-SCF neutralizing antibody, anti-c-Kit antibody, and isotype controls (10
Analysis of gene expression by conventional RT-PCR and real-time RT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA) or from tissues homogenized in TRIzol according to the manufacturer’s instructions. The relative quantity of mRNA was determined by RT-PCR (twenty-eight cycles, One-step RT-PCR kit, Qiagen, Valencia, CA). The mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer sequences were shown in Supplementary Table 1.

For real time RT-PCR assays, the cDNA sequences of all detected genes were retrieved from NCBI database. The primers were designed with the Oligo Primer Analysis 4.0 software and the sequences were blasted (http://www.ncbi.nlm.nih.gov/BLAST/). The primer sequences were shown in Supplementary Table 2. 100 ng of total RNA was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 µl. Then 2 µl of cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA) in duplicate. For sample analysis, the threshold was set based on the exponential phase of products, and CT value for samples was determined. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against house keeping gene GAPDH.

Western blot analysis

Cell lysates or tumor tissue homogenates (30 µg of total protein) and prestained molecular weight markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% of Triton X-100) containing 5% nonfat milk, and probed with anti mouse or human SCF antibodies (R&D
Systems). After incubation with the secondary antibody conjugated with horseradish peroxidase, membranes were extensively washed, and the immunoreactivity was visualized by enhanced chemiluminescence according to the manufacturer’s protocol (ECL kit, Santa Cruz, Santa Cruz, CA). All antibodies were purchased from Cell Signaling (Santa Cruz).

**Assay of soluble SCF and IFN-γ by ELISA**

For the assay of SCF, tumor cells were cultured with or without BMMCs for 24 h, or 400 mg of tumor tissue was cut into small pieces and cultured in 24-well plate in 1 ml of RPMI 1640 medium for 24 h. The soluble SCF in the supernatants was assessed by Mouse SCF Quantikine ELISA Kit (R &D systems) according to the manufacturer’s protocol.

For the analysis of the effect of MMP-9 inhibitor on the production of soluble SCF, MMP-9 inhibitor 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid (Calbiochem, La Jolla, CA) was injected into mice (1 mg/kg, i.p.) 30 min before mast cell injection. The control mice received an equal amount of vehicle (1% DMSO). In the *in vitro* experiments, the inhibitor was added to the culture medium to a final concentration of 0.2 mM.

For the assay of IFN-γ, NK cells were cultured in the presence of IL-2 (50 U/ml) for 24 h, and IFN-γ in the supernatant was measured by mouse IFN-γ ELISA Kit (R&D Systems).

**Construction of H22 tumor cell line expressing SCF-siRNA**

SCF sense and antisense siRNAs were generated using Silencer™ siRNA construction kit according to the manufacturer’s instruction (Ambion, Austin, TX). After hybridization and purification, the different double-stranded SCF-siRNAs and control siRNA were transiently transfected into H22 tumor cells using GeneSilencer siRNA transfection reagent (Gene Therapy Systems, San Diego, CA). The most efficient SCF-siRNA sequence (cagtcaagtttcaaggg) and
its control siRNA (ctggtcaagtctacaagag) were verified by RT-PCR detection of SCF mRNA 24 h after transfection, and inserted into RNAi-Ready pSIREN-RetroQ expressing vector with U6 promoter (BD Biosciences, Clontech, Palo Alto, CA). The recombinant SCF siRNA-expressing plasmids and control plasmids were transfected into H22 tumor cell using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) for stable expression after selection.

**MMP-9 assay by gelatin zymography**

Active MMP-9 mediates the shedding of membrane-associated SCF. To evaluate the influence of mast cell-derived MMP-9 on the release of SCF, the production of MMP-9 by mast cells was analyzed by gelatin zymography as described previously. Briefly, proteins prepared from mast cells were separated by 7.5% SDS-PAGE containing 1% gelatin. The gels were incubated in MMP activation buffer containing 50 mM Tris (pH8.0) and 10 mM CaCl₂ at 37°C overnight, and then stained with 1% Coomassie Brilliant Blue R-250 for 3 h and destained in 10% (V/V) methanol and 5% (V/V) acetic acid.

**Clinical tumor specimens and murine tumor specimens**

Clinical tumor specimens were acquired by surgery from untreated cancer patients, which was approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained according to the Declaration of Helsinki from all subjects. Seven types of tumor specimens were collected, including breast cancer, gastric cancer, colon cancer, lung cancer, ovarian cancer, liver cancer, and esophageal cancer. For each type of tumor, specimens were acquired from four to five patients.

To obtain different murine tumor specimens, mice were inoculated with tumor cells by subcutaneous injection of 1×10⁵ cells to the left flank. BALB/c mice were inoculated with H22
cells, colon-26 cells, or 4T1 cells, respectively. C57BL/6 mice were inoculated with RM1 cells, B16 cells, or LLC1 cells, respectively. Tumors were surgically dissected for the experiments when tumor size reached ~5×5 mm².

**Flow cytometric analysis**

Mouse tumor cells or human tumor cells were incubated with biotin-conjugated goat-anti-mouse SCF or goat-anti-human SCF and the corresponding isotype control IgG for 30 min at 4°C. After washing, the cells were further incubated with PE-conjugated streptavidin for flow cytometric analysis. All antibodies were purchased from R&D Systems. Parameters were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

For IL-17 intracellular staining, tumor-infiltrating immune cells were isolated from tumor tissue, and then fixed and permeabilized with Fix/Perm solution (BD Biosciences). The cells were then resuspended in Perm buffer and incubated with FITC-labeled anti-mouse IL-17 antibody or isotype control (eBioscience) at room temperature in the dark for 20 min.

For intracellular staining of Foxp3, tumor-infiltrating immune cells were stained with PE-cy3-conjugated anti-mouse CD3. After cellular surface staining, the cells were treated as above, restained with FITC-labeled anti-mouse Foxp3 antibody or isotype control (eBioscience).

**Assay of the activities of NF-κB and AP-1**

5×10⁶ BMMCs, with or without anti-c-Kit antibody as indicated, were injected via tail vein into mice (n=5 per group) bearing WT H22 tumor or SCF-knockdown H22 tumor. The mice were sacrificed 72 h later and tumor cells were isolated from peripheral tumor tissues. The nuclear extract was prepared with Nuclear Extraction Kit (Millipore, Billerica, MA). The
activities of NF-κB and AP-1 in nuclear extract were determined by NF-κB Assay kit and AP-1 Assay kit (Millipore), respectively, according to the manufacturer’s protocol.

**Isolation of T cells and NK cells**

Tumor tissues from different groups were digested with collagenase and hyaluronidase for 1 hour at 37°C, and minced. After lysis of red blood cells, the dissociated cells were underlaid with 5 ml of Lymphocyte-M solution, and centrifuged at 2,200 rpm for 20 min. Tumor-infiltrating lymphocytes were harvested from the interface. T cells were isolated on a T cell enrichment column (R&D Systems), and NK cells were purified by magnetic sorting with biotinylated-DX5 antibody. T cells and NK cells were also isolated from spleen of normal mice, and used in the related experiments.

**T cell proliferation assay**

The isolated T cells (1×10⁴) were cultured in the presence or absence of anti-CD3 and anti-CD28 antibodies (1 µg/ml each) in 96-well culture plates. [³H]-thymidine was added during the last 10 hours of 72-h culture, and then the incorporation of [³H]-thymidine was measured to determine T cell proliferation. The results were expressed as stimulation index (cpm of stimulated cells/cpm of un-stimulated cells).

**Assay of adenosine**

The peripheral tumor tissues were frozen and powdered in liquid nitrogen. The tissue powder (1 mg) was mixed with 1 ml of 0.6 M perchloric acid by vigorous vortexing and sonicated with a microsonicator for 10 seconds at 45 W. Then, the mixture was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was neutralized with 5 M K₂CO₃ to pH 7.2, centrifuged at 3000 rpm to remove precipitates, and stored at -80°C until HPLC analysis. HPLC analysis of adenosine
was performed as previously described.\textsuperscript{35}

For the assay of adenosine in the culture supernatant, 1 ml of supernatant was mixed with 30 µl of perchloric acid (4.4 M), and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was neutralized to pH7.2 and analyzed by HPLC.

\textit{Statistics}

Results were expressed as mean value ± SD and interpreted by ANOVA-repeated measure test. Differences were considered to be statistically significant when $P < 0.05$.

\textbf{Results}

\textit{Mast cells promote tumor growth on the basis of SCF/c-Kit-mediated migration into tumor}
To verify whether the chemotactic migration of mast cells into tumor is the prerequisite for their tumor-promoting activity, we first investigated whether SCF is the main chemokine mediating the migration of mast cells into tumor. The result from transwell assay showed that the migration of bone marrow-derived mast cells (BMMCs) was induced by the cultured tumor tissues, and the migration was significantly inhibited by SCF neutralizing antibody and c-Kit-blocking antibody (Fig. 1A). When CFSE-labeled BMMCs were injected into mice bearing H22 hepatocarcinoma via tail vein, both anti-SCF and anti-c-Kit antibodies effectively impaired the infiltration of BMMCs into tumor (Fig. 1B), indicating that the migration is mainly mediated by SCF released from tumor tissues. We then injected BMMCs into tumor-bearing mice to analyze the effect of mast cells on tumor growth. The i.v. injection of BMMCs markedly accelerated the growth of tumor (Fig. 1C) and shortened the survival of mice (Fig. 1D), both anti-SCF and anti-c-Kit antibodies abolished the tumor-promoting effect of the i.v. injected BMMCs (Fig. 1E). These data suggest that SCF/c-Kit axis-triggered mast cell migration into tumor is prerequisite for their tumor-promoting activity.

**Tumor cell-derived SCF is responsible for the infiltration of mast cells into tumor**

Next, we tested whether SCF is mainly produced by tumor cells. By detecting the expression of SCF, we found that the cultured H22 cells expressed both mRNA and protein of SCF, whereas SCF was not detectable in the culture supernatants (Fig. 2A), suggesting that SCF is a membrane-associated protein on tumor cells and might be released in tumor tissue. To confirm this, we additionally detected SCF expression in another five murine tumor cell lines, seven human tumor cell lines, and the corresponding mouse and human tumor tissues. SCF expression was very high in tumor cells and tumor tissues, but very low in the adjacent tissues around tumor
(Fig. 2B). All of these tumor cells expressed membrane-associated SCF (Fig. 2C), whereas SCF was only detectable in the supernatants of the cultured tumor tissues, but not in the supernatants of either tumor cells or adjacent tissues around tumor (Fig. 2D). Thus, we constructed a H22 tumor cell line stably expressing SCF siRNA (Fig. 2E). Both SCF expression and SCF release were strikingly decreased in siRNA-expressing tumor (Fig. 2E), indicating that the soluble SCF in tumor tissue is mainly released from tumor cells. More importantly, SCF-knockdown tumor could not efficiently attract BMMCs either in vitro or in vivo (Fig. 2F), suggesting that mast cell infiltration into tumor is mainly induced by tumor cell-produced SCF.

**Activation of mast cells by SCF is necessary for their tumor-promoting effect**

We then investigated the role of SCF in mast cell-mediated tumor promotion. Compared with WT tumor, the inoculation of SCF-knockdown tumor cells generated a much retarded tumor growth. The SCF-knockdown tumor on d40 after inoculation reached similar size as that of WT tumor on d25 (Fig. 3A), indicating that SCF and mast cells are crucial for tumor development. As expected, the i.v. injection of BMMCs had no effect on the growth of SCF-knockdown tumor (Fig. 3B). Unexpectedly, the intra-tumor injection of BMMCs, untreated or pretreated with low concentration of SCF (0.2 ng/ml or 10 ng/ml), to SCF-knockdown tumor did not promote tumor growth either (Fig. 3B), whereas the injection of BMMCs pretreated with higher concentration of SCF (200 ng/ml) promoted the growth of tumor (Fig. 3C). This finding implies that SCF not only mediates the chemotactic migration of mast cells into tumor, but also activates, at higher concentration, mast cells to generate the tumor-promoting effect.

**SCF-stimulated mast cells augment the release of SCF from H22 tumor cells**

Active MMP-9 can release membrane-bound SCF. Coincidently, mast cells have been
found to produce MMP-9 for their migration.\textsuperscript{36} Therefore, we wondered whether SCF/c-Kit signal can regulate the expression of MMP-9 in mast cells, therefore promoting the release of SCF from tumor cells. The cultured BMMCs expressed a low level of latent form of MMP-9. However, as low as 2 ng/ml SCF could stimulate BMMCs to produce more latent MMP-9 and active MMP-9 (Fig. 4A). SCF-promoted production of MMP-9 by mast cells could be inhibited by anti-c-Kit antibody (Fig. 4A), suggesting that SCF/c-Kit signal promotes the production and activation of MMP-9 in mast cells. Additionally, SCF-treated BMMCs significantly increased the concentration of SCF in the supernatant of H22 cells, whereas this effect was inhibited by MMP-9 inhibitor. Moreover, the release of SCF was not increased if BMMCs were pretreated with SCF in the presence of anti-c-Kit (Fig. 4B). Under each of the above conditions, SCF mRNA levels were not significantly influenced (Fig. 4B). Consistent with the \textit{in vitro} data, much higher level of SCF was detected in tumor tissue after intra-tumor injection or i.v. injection of mast cells (Fig. 4C), but SCF mRNA levels were not changed (Fig. 4C). In these situations, the increased release of SCF was also suppressed by MMP-9 inhibitor and anti-c-Kit (Fig. 4C). Taken together, these data suggest that SCF/c-Kit signal can stimulate mast cells to produce active MMP-9, and the latter then increases the level of soluble SCF in tumor by promoting the release of SCF from tumor cells, thereby favoring the activation of mast cells in tumor.

\textbf{SCF-activated mast cells remodel tumor inflammatory microenvironment}

The activated mast cells can release a large number of proinflammatory factors. Here we found that the expressions of proinflammatory genes encoding IL-6, TNF-α, VEGF, Cox-2, iNOS, and CCL2 were upregulated in BMMCs in the presence of SCF at high concentration (200 ng/ml), but not low concentration (5 ng/ml) (Fig. 5A). This upregulation could be abolished
by anti-c-Kit antibody (Fig. 5A), indicating that SCF/c-Kit signal activates mast cells. Consistent with in vitro data, the i.v. injection of BMMCs resulted in the significant increase of mRNA levels of the above proinflammatory genes in WT tumor, which was abolished by anti-c-Kit antibody (Fig. 5B). The i.v. injection of BMMCs into SCF-knockdown tumor did not significantly change the mRNA levels of these genes in tumor (Figure 5B). More importantly, the i.v. injection of BMMCs also increased the transcription of IL-17 gene and the amount of IL-17-producing cells in WT tumor, but not in SCF-knockdown tumor (Fig. 5C and 5D). These data suggest that SCF-activated mast cells remodel tumor inflammatory microenvironment. Since the activities of NF-κB and AP-1, two key transcription factors, in tumor cells can be increased by the proinflammatory factors such as TNF-α and others, we then further analyzed the influence of mast cell-remodeled environment on tumor cells by detecting the activities of NF-κB and AP-1 in tumor cells. The result showed that the activities of p65/p50 and c-Jun/c-Fos were significantly increased in the tumor cells isolated from the peripheral tumor tissue after mast cell injection (Fig. 5E). Anti-c-Kit antibody hindered the increase of such activities, and the injection of BMMCs into SCF-knockdown tumor did not significantly alter the activities of NF-κB and AP-1 (Fig. 5E).

**SCF-activated mast cells remodel tumor microenvironment by intensifying immunosuppression**

To investigate whether SCF-activated mast cells contribute to tumor immune suppression, we analyzed the expressions of immune-associated genes in tumor. Among them, IL-2 is related to immune activation, IL-10, TGF-β, and Foxp3 are related to immune suppression. After i.v. injection of mast cells into mice, IL-2 mRNA in tumor was decreased, and the levels of IL-10,
TGF-β, and Foxp3 mRNAs were increased (Fig. 6A). Consistent with the increased expression of TGF-β and Foxp3, the percentage of Treg cells in total T cells in tumor was also increased (Fig. 6B). In line with this, the i.v. injection of mast cells intensified the suppression of T lymphocyte and NK cell, two major types of cytotoxic effector cells, in tumor microenvironment, evaluated by the further decreased response of T cells to the stimulation of anti-CD3 and anti-CD28 antibodies (Fig. 6C), and the reduced production of IFN-γ by NK cells (Fig. 6D). The administration of anti-c-Kit antibody suppressed the effect of mast cell injection on gene expression pattern and the activities of T cells and NK cells in tumor microenvironment (Fig. 6A, 6C and 6D). On the other hand, the injection of mast cells into SCF-knockdown tumor produced a much less effect on gene expression pattern and the activities of T cells and NK cells (Fig. 6A, 6C and 6D). Taken together, these data indicate that SCF-activated mast cells can exacerbate the immunosuppression in tumor microenvironment.

**SCF-activated mast cells release adenosine in tumor microenvironment**

The cultured BMMCs may release adenosine after stimulation.35 Recently, adenosine has been reported to inhibit the production of IL-2 and IFN-γ by T cells.38,39 Thus, SCF-activated mast cells might release adenosine to directly suppress T cells and NK cells. To test this, we first detected the release of adenosine by BMMCs in the presence of SCF. SCF could stimulate BMMCs to release adenosine in a dose-dependent manner (Fig. 7A). On the other hand, the i.v. injection of mast cells into mice bearing WT tumor increased the level of adenosine in tumor, which was suppressed by anti-c-Kit antibody, whereas the intra-tumor injection of mast cells did not increase the level of adenosine in SCF-knockdown tumor (Fig. 7B). We next investigated the effect of mast cell-released adenosine on T cell proliferation driven by anti-CD3 and anti-CD28
antibodies and the production of IFN-γ by NK cells in response to IL-2 stimulation. Both T cell proliferation and the production of IFN-γ by NK cells were inhibited in the presence of the supernatants of mast cells stimulated with 100 ng/ml of SCF (Fig. 7C). The inhibitory effect was significantly relieved by adenosine receptor A2A antagonist SCH-58261 (Sigma) (Fig. 7C). We then used SCH-58261 to interfere with adenosine signaling pathway in vivo. The results showed that SCH-58261 not only relieved the suppression of T cells and NK cells after the injection of mast cells, but also augmented the activities of T cells and NK cells in control tumor (Fig. 7D).

Discussion

Our present findings strongly suggest that mast cells infiltrating into tumor produce a protumor effect by remodeling tumor microenvironment. Given that the expression of SCF was found in all the tumor cell lines or tumor tissues of different tumor types tested in this study, such remodeling process may be general in most types of tumors.
During the development of tumor, circulating mast cells migrate into tumor and form one of the major stromal cell populations. Although various factors may induce the chemotactic migration of mast cells, our data in this report suggest that SCF/c-Kit signal is mainly responsible for the migration of mast cells into tumor, since mast cells failed to migrate into SCF-knockdown tumor, and anti-c-Kit antibody abolished the migration of mast cells into WT tumor. Except for mast cell migration and adhesion to extracellular matrix components, SCF/c-Kit signal is also critical for the survival and functional activation of mast cells and the secretion of mediators from mast cells. Importantly, our data reveal that SCF acts on mast cells in a dose-dependent manner. Low dose of SCF induces both the chemotactic migration of mast cells and the production of active MMP-9 by mast cells, whereas higher concentration of SCF is required for the activation and mediator-secretion of mast cells. The accumulation and activation of mast cells in tumor can be intensified by a vicious feedback circle between mast cells and SCF. SCF-stimulated mast cells produce MMP-9 which not only facilitates the migration of mast cells into tumor, but also augments the release of SCF from tumor cells. The increased release of SCF is in favor of both the accumulation and further activation of mast cells.

Inflammation is a fundamental character of tumor microenvironment. Numerous proinflammatory factors existent in tumor microenvironment influence the growth and metastasis of tumor. Many of them are produced by mast cells. Mast cells were thought to be detrimental to tumor cells by releasing IL-4, TNF-α, and others to induce the apoptosis of tumor cells. However, many evidences obtained in recent years indicate that these inflammatory cytokines are beneficial to tumors. In addition, other proinflammatory factors and inflammation-related enzymes also benefit tumor development. Our previous studies have
demonstrated that IL-6, iNOS, and CCL2 have the protumor effects through different mechanisms.\textsuperscript{44,45} TNF-\(\alpha\), VEGF, and Cox-2 also play important roles in tumor initiation and progression.\textsuperscript{18} In this study, we found that SCF/c-Kit axis is crucial for the production of these factors and enzymes in tumor, since the transcription activities of these genes in SCF-knockdown tumor were very low. On the other hand, mast cells, relying on SCF/c-Kit signal, make main contribution to the production of these factors and enzymes in tumor. Mast cells remodeled the tumor inflammatory microenvironment by not only producing the above factors but also increasing the production of IL-17, a critical proinflammatory cytokine.\textsuperscript{46,47} The increased expression of these genes is obviously beneficial to tumor development. SCF-stimulated production of VEGF by mast cells is consistent with the previous report that mast cells can promote tumor angiogenesis.\textsuperscript{48,49} Moreover, the proinflammatory factors such as TNF-\(\alpha\) and others can increase the activities of NK-\(\kappa\)B and AP-1 in tumor cells.\textsuperscript{37} Our data show that the activities of NK-\(\kappa\)B and AP-1 were indeed increased in the tumor cells in the tumor microenvironment remodeled by mast cells. The activated NF-\(\kappa\)B and AP-1 may favor the proliferation of tumor cells by inducing the expression of cyclins, the survival of tumor cells by blockade of apoptosis, and the invasiveness of tumor cells by inducing the production of MIF and EMMPRIN.\textsuperscript{50,51}

Immunosuppression is another basic feature of tumor microenvironment. Although immune surveillance works at early stage of tumorigenesis, the established tumors primarily induce immune tolerance,\textsuperscript{52} resulting in the shift of the immune balance from activation to tolerance induction. An absolute immune suppression is usually generated in tumor microenvironment at the later stage of tumor. Our data in this report indicate that mast cells play important roles in
such immune balance shifting. The infiltration of mast cells into tumor significantly increased the expressions of TGF-β, IL-10, and Foxp3 in tumor. Consistently, Treg cells in tumor microenvironment was also increased, since CD4⁺CD25⁻ T cells can be converted into CD4⁺CD25⁺ regulatory T cells by TGF-β-induced expression of transcription factor Foxp3.⁵³ Both the infiltration of mast cells and the increase of Treg cells can explain the increased expression of IL-10, a cytokine mediating complex immunosuppressive effect. Given that Treg cells release IL-9 to activate mast cells for immune suppression,²⁵ here we propose a novel partnership between mast cells and Treg cells in tumor microenvironment: they activate each other and cooperate to suppress immune responses.

Adenosine release from the stimulated mast cells was found 23 years ago.³⁵ However, its connection with tumor progression has not yet been elucidated. Our data show that mast cells augment the release of SCF by tumor cells, and then the increased SCF in turn stimulates the release of adenosine by mast cells. Adenosine not only inhibits the production of IFN-γ and IL-2 by CD4⁺ T cells⁴⁸,⁴⁹ but also suppresses the migration of mast cells.⁵⁴ The latter may partly explain the accumulation of mast cells around the vasculature in peripheral region of tumor tissue. Thus, mast cells localizing around tumor vasculature may form the first layer of barrier, where the tumor-infiltrating T cells initially accept the immunosuppressive signal, adenosine, leading to the attenuation of immune attack on tumor cells.

In summary, the findings in this report disclose the complex relationship between mast cells, tumor cells and other immune cells in tumor microenvironment. The remodeling of tumor microenvironment can actually be initiated by tumor cell-released SCF. The following events include the mutual influence between mast cells and tumor cells, mast cells and other immune
cells. Therefore, SCF/c-Kit pathway is not only very important for the remodeling of tumor microenvironment, but also a very important target for tumor therapy.

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Authorship

Contribution: B.H. designed and conducted research, analyzed data, and wrote the paper; Z.L., G.M.Z., D.L., S.C., B.L., Y.L. and Y.Y. conducted research and analyzed the data. J.U., H.X. and Z.H.F. designed the research, analyzed the data, and wrote the paper. All authors have reviewed and contributed to the manuscript.

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**Figure legends**

**Figure 1.** Mast cells promote tumor growth with SCF/c-Kit-mediated chemotactic migration as the prerequisite. (A) SCF induces the migration of mast cells (MCs). The migration of MCs in transwell assay was determined in the presence of tumor tissues, antibodies, or SCF. *, P<0.05, compared with tumor tissue group. (B) Infiltration of circulating MCs into tumor. CFSE-labeled BMMC with or without antibodies were injected into tumor-bearing mice
via tail vein. The peripheral tumor tissues were surgically excised from mice 24 h after the injection, and frozen sections were prepared and analyzed by fluorescence microscopy. (C) Mast cells promote tumor growth. BMMCs were injected into tumor-bearing mice by i.v. injection. Bone marrow cells were used as control. The growth of tumor was monitored. (D) Survival rate follow-up after the i.v. injection of BMMCs. The survival period of tumor-bearing mice in BMMC injection group was significantly shortened, compared with that in control groups (n=12, \( P<0.05 \); Kaplan-Meier analysis). The data were the representative of two independent experiments in which the similar results were obtained. (E) Dependence of tumor-promoting effect of MCs on SCF/c-Kit axis. BMMCs, with or without antibodies, were injected into tumor-bearing mice via tail vein. Both of two antibodies abolished the tumor-promoting effect of MCs. *, \( P<0.05 \), compared with control group.

Figure 2. Tumor cell-derived SCF is responsible for the infiltration of mast cells into tumor.

(A) Assay of SCF expression in H22 tumor or tumor cells. SCF expression was detected by RT-PCR and Western blot respectively. SCF in the supernatants of the cultured tumor tissue or tumor cells was assayed by ELISA. Mouse monocyte system cell line RAW246.7 was used as negative control. (B) Assay of SCF expression in tumor cells and tumor tissues. SCF expression in murine tumor cell lines and human tumor cell lines, corresponding murine tumor and specimens from human tumor, and normal tissue adjacent to tumor was detected by RT-PCR and Western blot respectively. (C) SCF on the surface of different tumor cells was analyzed by flow cytometry. (D) Assay of soluble SCF produced by different tumors. Tumor cell lines, the corresponding tumor tissues, and the adjacent tissues around tumor were cultured in vitro. SCF
in the supernatants was detected by ELISA. (E) Silence of SCF expression in H22 tumor cells by SCF siRNA. SCF expression was detected by RT-PCR and Western blot respectively. The soluble SCF released from tumor tissue was assayed by ELISA. (F) SCF-knockdown tumor can not efficiently induce the migration of mast cells (MCs). SCF-knockdown or control tumor tissues were used for transwell assay of MC migration (left). The infiltration of circulating MCs into SCF-knockdown or control tumor tissue (right) was analyzed using the same protocol as that in Figure 1B. *, \( P<0.05 \), compared with control tumor.

**Figure 3. Activation of mast cells by SCF is necessary for their tumor-promoting effect.** (A) SCF-knockdown retards tumor growth. Mice (n=8 per group) were inoculated with SCF-knockdown H22 cells and control WT H22 cells respectively. The growth of tumor was monitored. (B and C) Mice (n=8 per group) were inoculated with SCF-knockdown H22 tumor cells. When tumor size reached ~5×5 mm\(^2\), the mice received BMMCs either by i.v. injection or by intra-tumor (i.t.) injection (B), or received the intra-tumor injection of BMMCs pretreated with different concentrations of SCF and anti-c-kit antibody (20 µg/ml) as indicated (C). The growth of tumor was promoted only by the intra-tumor injection of mast cells (MCs) pretreated with higher concentration of SCF (200 ng/ml), which was abolished by anti-c-kit antibody.

**Figure 4. SCF-stimulated mast cells augment the release of SCF from tumor cells.** (A) SCF stimulates the production of active MMP-9 by mast cells (MCs). BMMCs were cultured for 24 h in the presence of different concentrations of SCF and anti-c-Kit (10 µg/ml). The production of MMP-9 was detected by RT-PCR and gelatin zymography. (B) MC-derived MMP-9 increased
the release of SCF from H22 tumor cells. BMMCs were treated with 5 ng/ml of SCF for 4 h in
the absence or presence of 10 µg/ml anti-c-Kit antibody. H22 cells and SCF-treated BMMCs
were cultured alone or in two chambers separated by semipermeable membrane. SCF in the
supernatants was detected by ELISA (*left*). SCF mRNA was detected by real-time RT-PCR
(*right*). *, \( P<0.05 \), compared with H22/MC group. (C) Assay of SCF in tumor tissues.
Tumor-bearing mice received the i.p. injection of MMP-9 inhibitor and the intra-tumor (i.t.)
injection of MCs, or received the i.v. injection of MCs with anti-c-Kit antibody. The tumor
tissues were excised 48 h after MC injection and cultured *in vitro*. SCF in the supernatants was
detected by ELISA (*left*). The mRNA level of SCF in tumor tissues was detected by real-time
RT-PCR (*right*). *, \( P<0.05 \), compared with MC injection groups.

**Figure 5. SCF/c-Kit signal induces the mast cell-mediated remodeling of tumor
inflammatory microenvironment.** (A) Expression of proinflammatory genes in mast cells.
Mast cells were cultured in the presence or absence of SCF and anti-c-Kit antibody. The levels of
IL-6, TNF-\( \alpha \), VEGF, Cox-2, iNOS, and CCL2 mRNAs were detected by real-time PCR. (B–E)
Expression of proinflammatory genes in tumor and the activities of NF-\( \kappa \)B and AP-1 in tumor
cells. The mice bearing WT H22 tumor received the i.v. injection of mast cells and anti-c-Kit
antibody as indicated. The mice bearing SCF-knockdown H22 tumor received the intra-tumor
injection of mast cells. The levels of IL-6, TNF-\( \alpha \), VEGF, Cox-2, iNOS, CCL2, and IL-17
mRNAs in tumor tissues were detected by real-time PCR (B and C). IL-17-expression (IL-17\( ^{+} \))
cells in immune cells from tumor were analyzed by flow cytometry (D). Tumor cells were
isolated from tumor tissue, and the activities of NF-\( \kappa \)B and AP-1 were assayed as described in
Materials and Methods (E). *, P<0.05, compared with control tumor cells or WT tumor control.

Figure 6. SCF/c-Kit signal activates mast cells to exacerbate the immunosuppression in tumor microenvironment. When tumor size reached ~5×5 mm², the mice bearing WT H22 tumor received the i.v. injection of mast cells (MCs) and anti-c-Kit antibody as indicated, and the mice bearing SCF-knockdown tumor received the intra-tumor injection of MCs. (A) The expression of Foxp3 and cytokine genes in tumor. The levels of Foxp3, IL-10, TGF-β, and IL-2 mRNAs in tumor tissues were detected by real-time PCR 72 h after the injection of mast cells. (B) Treg cells (Foxp3⁺) in T cells (gated CD3⁺ cells) from tumor were analyzed by flow cytometry. (C and D) Mast cells intensify the suppression of T cells and NK cells in tumor. 72 h after the injection of mast cells, T cells and NK cells were isolated from tumor. The proliferation of T cells (C) and the production of IFN-γ by NK cells (D) were determined as described in Materials and Methods. T cells and NK cells isolated from normal spleen were used as control. *, P<0.05, compared with WT tumor control.

Figure 7. SCF-activated mast cells release adenosine to suppress the immune response. (A) Assay of adenosine released by BMMCs after the stimulation with SCF. BMMCs were cultured in the absence or presence of SCF for 48 h. The adenosine in the supernatant was assayed as described in Materials and Methods. (B) Assay of adenosine in tumor tissues. The mice bearing WT H22 tumor received the i.v. injection of mast cells and anti-c-Kit antibody as indicated. The mice bearing SCF-knockdown H22 tumor received the intra-tumor injection of mast cells. 72 h later, the adenosine in tumor tissues was assayed as described in Materials and Methods. *,
$P<0.05$, compared with 0 ng/ml SCF group or WT tumor group. (C and D) Mast cell-produced adenosine inhibits T cells and NK cells. Splenic T cells and NK cells were cultured with the culture supernatant of SCF-stimulated mast cells or control SCF medium in the presence or absence of adenosine receptor A$_{2A}$ antagonist SCH-58261 (C). The T cells and NK cells from tumor were isolated from the mice bearing WT H22 tumor 72 h after the intra-tumor injection of mast cells or control bone marrow cells with or without SCH-58261 (D). The proliferation of T cells (left) and the production of IFN-$\gamma$ by NK cells (right) were determined as described in Materials and Methods. *, $P<0.05$, compared with 0 ng/ml SCF, control, or WT tumor group; #, $P<0.05$, compared with no-SCH-58261 group.
Figure 2

A

B

C

D

E

F

37
Figure 3

A

B

C

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Figure 5

A. 1. control 2. SCF (50ng/ml) 3. SCF (200 ng/ml) 4. SCF (200 ng/ml) + anti-c-kit

B. Relative to GAPDH

C. IL-17

D. Comparison of IL-17 among different groups

E. ELISA analysis of cytokines and proteins

1. WT tumor
2. WT tumor/MC
3. WT tumor/MC+anti-c-kit
4. SCF-knockdown tumor
5. SCF-knockdown tumor/MC
Figure 6

A and B:
1. WT tumx
2. WT tumor/MC
3. WT tumor/MC+anti c-kit
4. SCF-knockdown tumor
5. SCF-knockdown tumor/MC

C and D:
1. control
2. WT tumx
3. WT tumx/MC
4. WT tumor/MC+anti c-kit
5. SCF-knockdown tumor
6. SCF-knockdown tumor/MC
SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment

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