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The IL-6–STAT3 axis mediates a reciprocal crosstalk between cancer-derived mesenchymal stem cells and neutrophils to synergistically prompt gastric cancer progression

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Emerging evidence indicate that mesenchymal stem cells (MSCs) affect tumor progression by reshaping the tumor microenvironment. Neutrophils are essential component of the tumor microenvironment and are critically involved in cancer progression. Whether the phenotype and function of neutrophils is influenced by MSCs is not well understood. Herein, we investigated the interaction between neutrophils and gastric cancer-derived MSCs (GC-MSCs) and explored the biological role of this interaction. We found that GC-MSCs induced the chemotaxis of neutrophils and protected them from spontaneous apoptosis. Neutrophils were activated by the conditioned medium from GC-MSCs with increased expression of IL-8, TNFα, CCL2, and oncostatin M (OSM). GC-MSCs-primed neutrophils augmented the migration of gastric cancer cells in a cell contact-dependent manner but had minimal effect on gastric cancer cell proliferation. In addition, GC-MSCs-primed neutrophils prompted endothelial cells to form tube-like structure *in vitro*. We demonstrated that GC-MSCs stimulated the activation of STAT3 and ERK1/2 pathways in neutrophils, which was essential for the functions of activated neutrophils. We further revealed that GC-MSCs-derived IL-6 was responsible for the protection and activation of neutrophils. In turn, GC-MSCs-primed neutrophils induced the differentiation of normal MSCs into cancer-associated fibroblasts (CAFs). Collectively, our results suggest that GC-MSCs regulate the chemotaxis, survival, activation, and function of neutrophils in gastric cancer via an IL-6–STAT3–ERK1/2 signaling cascade. The reciprocal interaction between GC-MSCs and neutrophils presents a novel mechanism for the role of MSCs in remodeling cancer niche and provides a potential target for gastric cancer therapy.
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Accumulating evidence suggest that neutrophils are critical for cancer initiation and progression.[1,2] The increased presence of intratumoral neutrophils has been linked to a poorer prognosis for patients with renal cancer, hepatocellular carcinoma (HCC), melanoma, head and neck squamous cell carcinoma (HNSCC), pancreatic cancer, colorectal carcinoma, and gastric adenocarcinoma.[3] Recent studies using murine tumor models or involving cancer patients have suggested an important functional role of neutrophils during tumor progression.[4–7] Neutrophils-derived factors promote genetic mutations leading to tumorigenesis or promote tumor cell proliferation,[8] migration, and invasion.[9,10] Neutrophils have also been demonstrated to induce tumor vascularization by the production of pro-angiogenic factors[11,12]

The infiltration of neutrophils into tumors has been shown to be mediated by factors produced by both tumor and stromal cells. Recent reports suggest that tumor cells actively modulate the functions of neutrophils. Tumor-derived CXCL5 modulates the chemotaxis of neutrophils, which in turn enhances the migration and invasion of human HCC cells.[13] HNSCC cells-derived MIF induces the recruitment and activation of neutrophils through a p38-dependent manner.[14,15] Neutrophils respond to hyaluronan fragments in tumor supernatants via PI3K/Akt signaling, leading to prolonged survival and stimulating effect on HCC cell motility.[16] Kuang *et al.*[17] suggest that IL-17 promotes the migration of neutrophils into HCC through epithelial cell-derived CXC chemokines, resulting in increased MMP-9 production and angiogenesis at invading tumor edge However, much less is known about the role of stromal cells in modulating the phenotype and function of neutrophils in cancer thus far.

Cancer-associated fibroblasts (CAFs) have a key role in cancer mainly through secretion of soluble factors, as growth

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Abbreviations: MSCs, mesenchymal stem cells; CAFs, cancer-associated fibroblasts; CM, conditioned medium; IL-6, interleukin-6; IL-8, interleukin-8; CCL2, monocyte chemotactic protein 1; TNF-α, tumor necrosis factor-α; OSM, oncostatin M; MCL-1, myeloid cell leukemia-1; Bcl-2, B-cell lymphoma 2; STAT3, signal transducers and activators of transcription 3; ERK, extracellular signal-regulated kinase; α-SMA, α-smooth muscle actin; FAP, fibroblast activation protein; IL-6R, interleuki-6 receptor; TAN, tumor-associated neutrophils; MDSCs, myeloid-derived suppressor cells; MMP-9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor; IFN-β, interferon-β; TGF-β, transforming growth factor-β; HUVECs, human umbilical vein endothelial cells
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factors or inflammatory mediators, as well as production of extracellular matrix proteins and their proteases. These activated fibroblasts are involved in creating a niche for cancer cells, promoting their proliferation, motility and chemoresistance. Activated fibroblasts express several mesenchymal markers such as α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and vimentin. CAFs actively participate in reciprocal interaction with tumor cells and with other cell types in the microenvironment, contributing to a tumor-permissive niche and promoting tumor progression.

Mesenchymal stem cells (MSCs) are adult stromal cells with self-renewal and pluripotent differentiation abilities. MSCs can be mobilized from bone marrow to the site of damage, respond to the local microenvironment, and exert wound repair and tissue regeneration functions upon injury and inflammation conditions.[18] MSCs have been considered as one of the major components of the tumor stroma and are believed to be the precursors of CAFs.[19,20] We have previously demonstrated that human bone marrow MSCs prompt tumor growth *in vivo*.[21] In addition, we have recently isolated MSCs-like cells from the gastric cancer tissues (GC) and the adjacent normal tissues (GCN) and shown that the gastric cancer-derived MSCs (GC-MSCs) possess the properties of CAFs.[22,23] As tumor-derived MSCs are often exposed to distinct inflammatory cells and factors in the tumor microenvironment, they may acquire novel functions that are not present in normal MSCs, and these unique functions may have a role in reshaping the tumor microenvironment and ultimately affect tumor progression. As neutrophils are key mediators of tumor progression and tumor angiogenesis, it is likely that an intense interaction may exist between the tumor-derived MSCs and tumor-infiltrating neutrophils.

The emerging roles of CAFs in cancer immunoeditting led us to investigate whether GC-MSCs are able to regulate the phenotype and function of neutrophils in gastric cancer. We have shown that there is a reciprocal interaction between GC-MSCs and neutrophils. GC-MSCs enhanced the chemotaxis of peripheral blood-derived neutrophils and protected them from spontaneous apoptosis. GC-MSCs induced the activation of neutrophils to highly express IL-8, CCL2, TNFα, and oncostatin M (OSM), leading to the increase of gastric cancer cell migration and angiogenesis *in vitro*. GC-MSCs exerted this effect through the IL-6–STAT3–ERK1/2 signaling axis, and blockade of the IL-6–IL-6R interaction or pharmacological inhibition of STAT3 and ERK1/2 activation abrogated this role. In turn, GC-MSCs-activated neutrophils could trigger the CAF differentiation of normal MSCs. Therefore, these results establish a bi-directional interaction between GC-MSCs and neutrophils that may be critically involved in the progression of gastric cancer.

Results

GC-MSCs recruit neutrophils and protect neutrophils from spontaneous apoptosis. GC-MSCs were isolated from gastric cancer tissues and characterized by phenotypic analyses. In consistent with our previous studies,[22,23] the isolated GC-MSCs were positive for CD29, CD44, and CD90 but negative for CD14, CD34, CD45, CD71, and HLA-DR (Supplementary Figure 1). To determine the effect of GC-MSCs on neutrophil survival, human peripheral blood-derived neutrophils were cultured in the presence or absence of conditioned medium (CM) from GC-MSCs for 18 h. The viability of neutrophils was determined by using MTT assay. As shown in Figure 1a, incubation with GC-MSCs CM obviously improved the survival of neutrophils. To demonstrate whether GC-MSCs CM enhance the viability of neutrophils through the inhibition of spontaneous apoptosis, neutrophils incubated with or without GC-MSCs CM were harvested and stained with Annexin V/PI to evaluate the apoptotic rate. The results of flow cytometry showed that GC-MSCs CM significantly decreased the rate of apoptotic neutrophils (Figure 1b). To further confirm this result, neutrophils treated with GC-MSCs CM were collected and cell lysates were prepared for immunoblotting assay. The results of western blot showed that the level of Bax decreased, whereas that of Bcl-2 increased in neutrophils treated with GC-MSCs CM (Figure 1c). We also demonstrated that incubation with GC-MSCs CM inhibited the activation of caspase-3 in neutrophils (Supplementary Figure 2). Quantitative PCR analyses showed that GC-MSCs treatment increased the expression of Mcl-1 but reduced that of Fas in neutrophils (Figure 1d), supporting the notion that GC-MSCs CM has protective effect on neutrophils. We next wanted to know whether GC-MSCs CM has any effect on neutrophil chemotaxis. Neutrophils were seeded on the upper chamber of transwell plate (core size 3.0 μm) and GC-MSCs CM or RPMI 1640 medium were put in the lower chamber as the chemoattractant. As shown in Figure 1e, GC-MSCs CM remarkably induced the chemotaxis of neutrophils compared with medium control. To demonstrate whether GC-MSCs CM could activate neutrophils, we determined the expression of IL-8, CCL2, TNFα, and OSM in neutrophils incubated with or without GC-MSCs CM. The results of quantitative PCR analyses showed that treatment with GC-MSCs CM significantly upregulated the mRNA levels of IL-8, CCL2, TNFα, and OSM in neutrophils (Figure 1f). We further confirmed the upregulation of IL-8, CCL2, TNFα, and OSM in neutrophils by using another GC-MSCs-derived CM, suggesting that the activation of neutrophils by GC-MSCs CM is not source-specific. In summary, these results indicate that GC-MSCs recruit neutrophils, protect them from spontaneous apoptosis, and skew them towards an activated state in a cell contact-independent manner.

GC-MSCs-primed neutrophils prompt gastric cancer cell migration and angiogenesis. We next wanted to know the biological role of neutrophils stimulated with GC-MSCs CM in gastric cancer. To this end, neutrophils were primed with GC-MSCs CM for 12 h and the primed neutrophils were harvested to coculture with human gastric cancer cells directly or indirectly. For direct coculture, GC-MSCs CM-primed neutrophils were mixed with SGC-7901 in a 5 : 1 ratio and seeded in the upper chamber of a transwell system. The migrated SGC-7901 cells were determined by using crystal violet staining. For indirect coculture, GC-MSCs CM-primed neutrophils were seeded in the lower chamber, whereas SGC-7901 cells in the upper chamber of a transwell system. The results of cell migration assay showed that

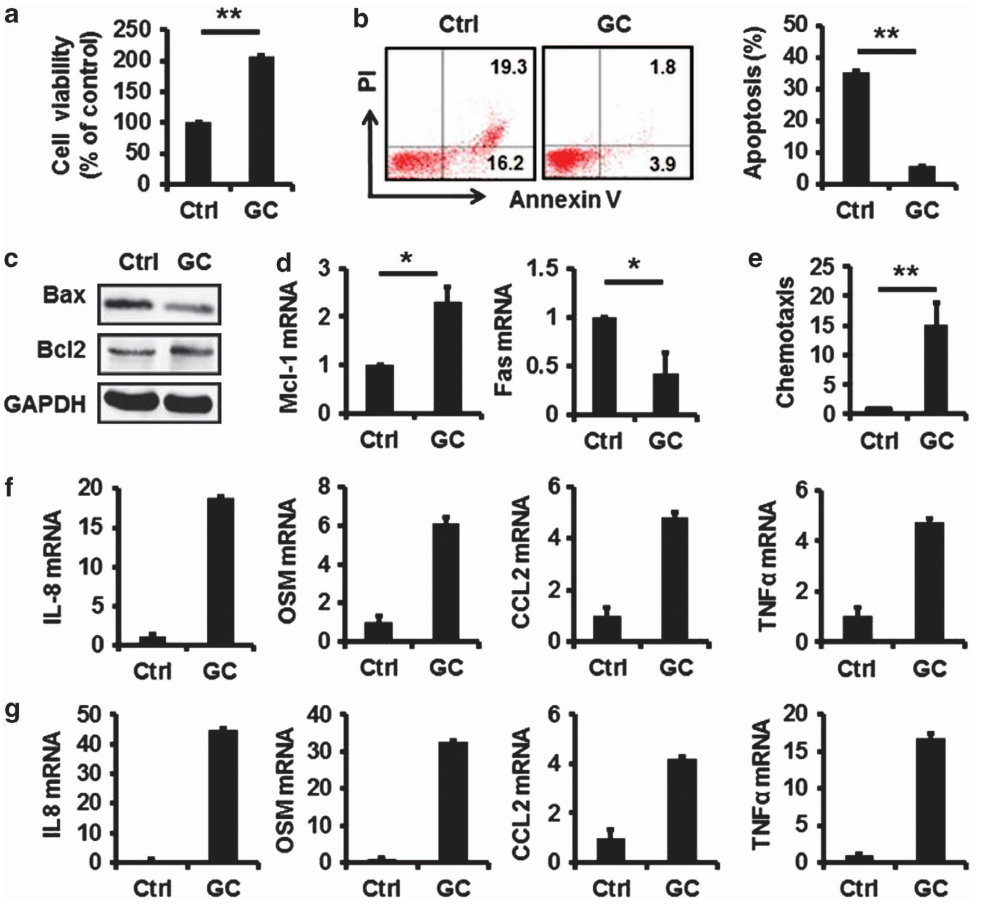

Figure 1 Gastric cancer-derived MSCs recruit, protect, and activate neutrophils. Neutrophils were incubated in the presence or absence of GC-MSCs conditioned medium for 18 h, the viability of neutrophils was determined by using MTT assay (**a**); The apoptosis of neutrophils was quantified by using an Annexin V/PI apoptosis detection kit (**b**); Total protein was extracted from neutrophils and the levels of Bax and Bcl-2 were detected by using western blot. GAPDH was used as loading control (**c**); Total RNA was extracted from neutrophils and real-time PCR was performed to determine the mRNA levels of Mcl-1 and FAS (**d**), IL-8, CCL2, TNFα, and OSM (**f**, **g**); Neutrophils were seeded on the upper chamber of a transwell system with 3 μm pore inserts. The bottom chambers were loaded with conditioned medium from GC-MSCs. After 3 h of culture, migrated cells were counted by using the Beckman Coulter HMX (**e**). $^{*}P<0.05$, $^{**}P<0.01$

direct coculture with GC-MSCs CM-primed neutrophils significantly improved the number of migrated SGC-7901 cells, whereas indirect coculture with GC-MSCs CM-primed neutrophils had less effect on the migratory ability of SGC-7901 cells (Figure 2a). In addition, the proliferation of SGC-7901 cells did not seem to be affected by the supernatants from GC-MSCs CM-primed neutrophils (data not shown). As factors released by neutrophils have been implicated in mediating tumor angiogenesis[24] we then analyzed the neutrophil supernatants for pro-angiogenic activity by using an *in vitro* endothelial cell tube formation assay. Human umbilical vein endothelial cells (HUVECs) were seeded on matrigel in the presence or absence of supernatants from GC-MSCs CM-primed neutrophils. As shown in Figure 2b, incubation with supernatants from GC-MSCs CM-primed neutrophils enhanced the formation of tube-like structure compared with control neutrophils. These results, when taken together, suggest that GC-MSCs regulate the function of neutrophils, which, in turn, modulate the functions of gastric cancer cells and endothelial cells.

GC-MSCs activate STAT3 and ERK1/2 pathways in neutrophils. To demonstrate the signaling pathways that are responsible for the activation of neutrophils by GC-MSCs CM, human promyelocytic leukemia HL-60 cells were induced to differentiate towards neutrophils by DMSO for 5 days and then incubated with CM from GC-MSCs for different times. As shown in Figures 3a, 1.5% DMSO efficiently induced the differentiation of HL-60 cells into neutrophil-like cells. To test the response of neutrophilic-differentiated HL-60 cells to GC-MSCs CM stimulation, we incubated these cells with GC-MSCs CM for 15 min, 30 min, 1 h, and 2 h. Western blot analyses demonstrated that STAT3 (T705) was strongly activated already at 15 min after stimulation and that it remained constantly activated until 1 h poststimulation (Figure 3b). ERK1/2 was also strongly activated at 30 min after stimulation and decreased afterward (Figure 3b). To confirm that the induction of STAT3 and ERK1/2 phosphorylation by GC-MSCs CM is not source-specific, we stimulated neutrophilic-differentiated HL-60 cells with different GC-MSCs CM for 30 min. The results of western blot indicated that neutrophilic-differentiated HL-60

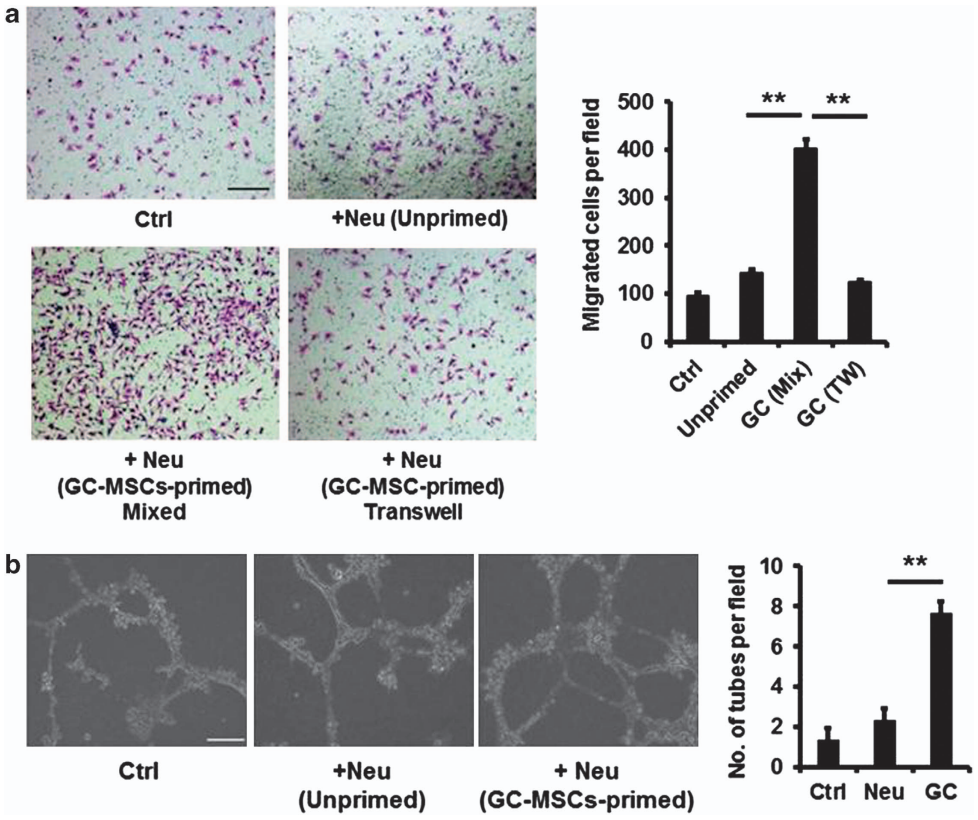

Figure 2 GC-MSCs-primed neutrophils prompt gastric cancer cell migration and endothelial cell tube formation. Neutrophils were pretreated with GC-MSCs conditioned medium for 12 h, and then cocultured with 2×10^4 SGC-7901 cells in the upper compartment of the chamber. After incubation for 12 h, the migrated cells on the lower surface of the membrane were stained with crystal violet after fixation and then counted under a light microscope. For the transwell coculture, SGC-7901 cells were seeded in the upper chamber and neutrophils in the lower chamber (**a**); HUVECs were seeded at 2×10^4 cells/well and incubated with conditioned media from GC-MSCs-primed neutrophils for 10 h. The formation of tube-like structure by HUVECs was observed under a phase-contrast microscope and photographed at 100 × magnification (**b**). Scale bar = 200 μm. $^{**}P < 0.01$

cells responded with STAT3 and ERK1/2 phosphorylation upon different GC-MSCs CM stimulation (Figure 3c). We then wanted to elucidate whether ERK1/2 is a downstream target of STAT3. To this end, primary neutrophils were stimulated with GC-MSCs CM for 30 min in the presence or absence of the STAT3 inhibitor WP1066 (10 μM) or ERK1/2-specific inhibitor U0126 (10 μM). Western blot analyses revealed that primary neutrophils responded with ERK1/2 and STAT3 phosphorylation in a manner comparable to neutrophilic-differentiated HL-60 cells. However, inhibition of STAT3 by WP1066 reduced the levels of ERK1/2 phosphorylation, whereas inhibition of ERK1/2 had no effect on STAT3 phosphorylation (Figure 3d), suggesting that GC-MSCs CM-trigged signaling cascade in neutrophils involves ERK1/2 as downstream target of STAT3. We next tested whether the activation of neutrophils is modulated by GC-MSCs CM through the STAT3–ERK1/2 signaling cascade. To this end, we stimulated neutrophils with GC-MSCs CM in the presence or absence of the STAT3 inhibitor WP1066 (10 μM) or ERK1/2-specific inhibitor U0126 (10 μM) and then evaluated the percentage of apoptotic neutrophils. The results of flow cytometry showed that both WP1066 and U0126 significantly increased the rate of apoptotic neutrophils (Figure 3e), suggesting that GC-MSCs CM protects neutrophils from apoptosis through the STAT3–ERK1/2 signaling cascade. The results of quantitative PCR analyses showed that treatment with GC-MSCs CM in the presence or absence of U0126 or WP1066 reversed the enhancement of IL-8, TNFα, and CCL2 mRNA levels in neutrophils (Figures 3g and h), suggesting that the activation of neutrophils by GC-MSCs CM is dependent on STAT3 and ERK1/2. In summary, these results indicate that STAT3–ERK1/2 signaling cascade is responsible for GC-MSCs-induced neutrophil activation.

Blocking STAT3–ERK1/2 signaling cascade inhibits GC-MSCs-induced pro-migratory and pro-angiogenic roles in neutrophils. To verify that STAT3–ERK1/2 signaling cascade is involved in the regulation of neutrophil functions in gastric cancer, we stimulated neutrophils with GC-MSCs CM in the presence or absence of 10 μM U0126 or WP1066. As shown in Figure 4a, pre-incubation with U0126 resulted in strong inhibition of the pro-migratory role of GC-MSCs CM-primed neutrophils. The presence of U0126 also inhibited the pro-angiogenic role of GC-MSCs CM-primed neutrophils (Figure 4b). In addition, pre-incubation with WP1066 resulted in strong inhibition of the pro-migratory as well as the pro-angiogenic roles of GC-MSCs CM-primed neutrophils (Figures 4c and d). Taken together, these data indicate that STAT3–ERK1/2 signaling cascade is an

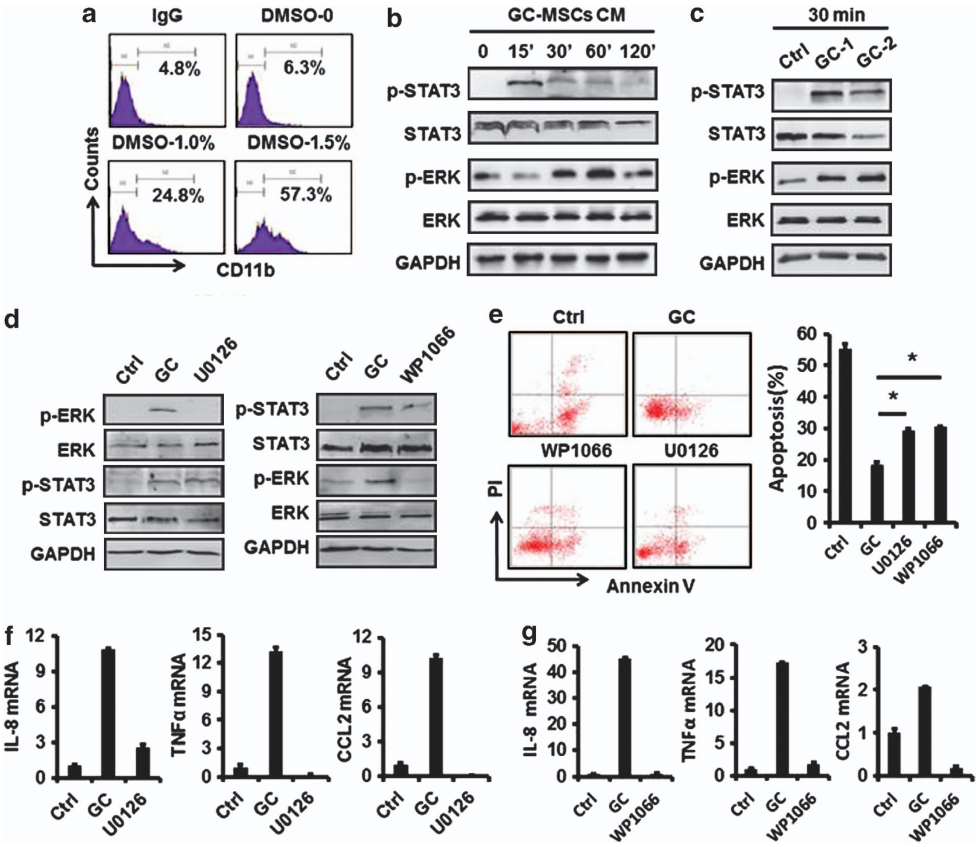

Figure 3 GC-MSCs CM activates the STAT3 and ERK1/2 pathways in neutrophils. (**a**) Human promyelocytic leukemia HL-60 cells were induced to differentiate towards neutrophils by DMSO for 5 days. The percentage of CD11b-positive population was determined by using flow cytometry. (**b**) Neutrophilic-differentiated HL-60 cells were incubated with conditioned medium from GC-MSCs for different times. The levels of p-ERK1/2, p-STAT3, ERK1/2 and STAT3 were examined by using western blot. (**c**) Western blot analyses of ERK1/2 and STAT3 (T705) phosphorylation in neutrophilic-differentiated HL-60 cells that were stimulated with different GC-MSCs CM for 30 min. (**d**) Neutrophils were stimulated with GC-MSCs CM in the presence or absence of the ERK1/2-specific inhibitor U0126 (10 μM) or STAT3 inhibitor WP1066 (10 μM) for 30 min. The levels of p-ERK1/2 and ERK1/2 were examined by using western blot. (**e**) Neutrophils were harvested and stained with Annexin V/PI to evaluate the rate of apoptotic cells by using flow cytometry. $^*P<0.05$. (**f**) Neutrophils stimulated with GC-MSCs CM in the presence or absence of the ERK1/2-specific inhibitor U0126 (10 μM) (**g**) or STAT3 inhibitor WP1066 (10 μM) (**h**) for 18 h. Total RNA was extracted from neutrophils and real-time PCR was performed using human-specific primers for the expression of IL-8, TNFα, and CCL2 mRNA

important regulator of neutrophil functions upon stimulation by GC-MSCs CM.

GC-MSCs-derived IL-6 is crucial for the recruitment, protection, and activation of neutrophils. We performed multiplex screening arrays to detect the soluble factors present in GC-MSCs CM that might be responsible for neutrophil activation. Among the 10 cytokines/chemokines tested, IL-6, IL-8, and MCP-1 were the most abundant (Figure 5a). Although the role of IL-6 in MSCs-mediated improvement of neutrophil survival has been partially clarified,[25] almost nothing is known about the role of CAFs-derived IL-6 in the activation and function of neutrophils in gastric cancer. Therefore, we next evaluated IL-6 expression in GC-MSCs and the matched GCN-MSCs. IL-6 was found to be strongly expressed in GC-MSCs compared with GCN-MSCs, as indicated by ELISA (Figure 5b) and quantitative RT-PCR analyses (Figure 5c). Furthermore, we demonstrate that GC-MSCs CM strongly activates neutrophils, whereas GCN-MSCs have no or weak effects (Figure 5d), suggesting that IL-6 in GC-MSCs CM is the major regulator of neutrophil activation. To investigate whether the *in vitro* effects of GC-MSCs CM on neutrophils were mediated by IL-6, we stimulated neutrophils with GC-MSCs CM in the presence of IL-6R antagonist (IL-6RA) or IgG control. As shown in Supplementary Figure 3, pre-incubation with IL-6RA inhibited the activation of STAT3 and ERK1/2 signaling by GC-MSCs CM in neutrophils. The results of real-time PCR analyses showed that the inhibition of IL-6–IL-6R interaction in neutrophils significantly inhibited the upregulation of IL-8, CCL2, and TNFα induced by GC-MSCs CM (Figure 5e). To confirm the role of IL-6–IL-6R interaction in neutrophil function, we stimulated neutrophils with GC-MSCs CM containing IL-6RA or IgG control. The neutrophils and supernatants from stimulated neutrophils were collected and used for cell migration and tube formation assays, respectively. The results showed that blockade of IL-6–IL-6R interaction significantly reduced the pro-migratory and pro-angiogenic roles of stimulated neutrophils (Figures 5f and g). These data strongly support that IL-6 produced by GC-MSCs

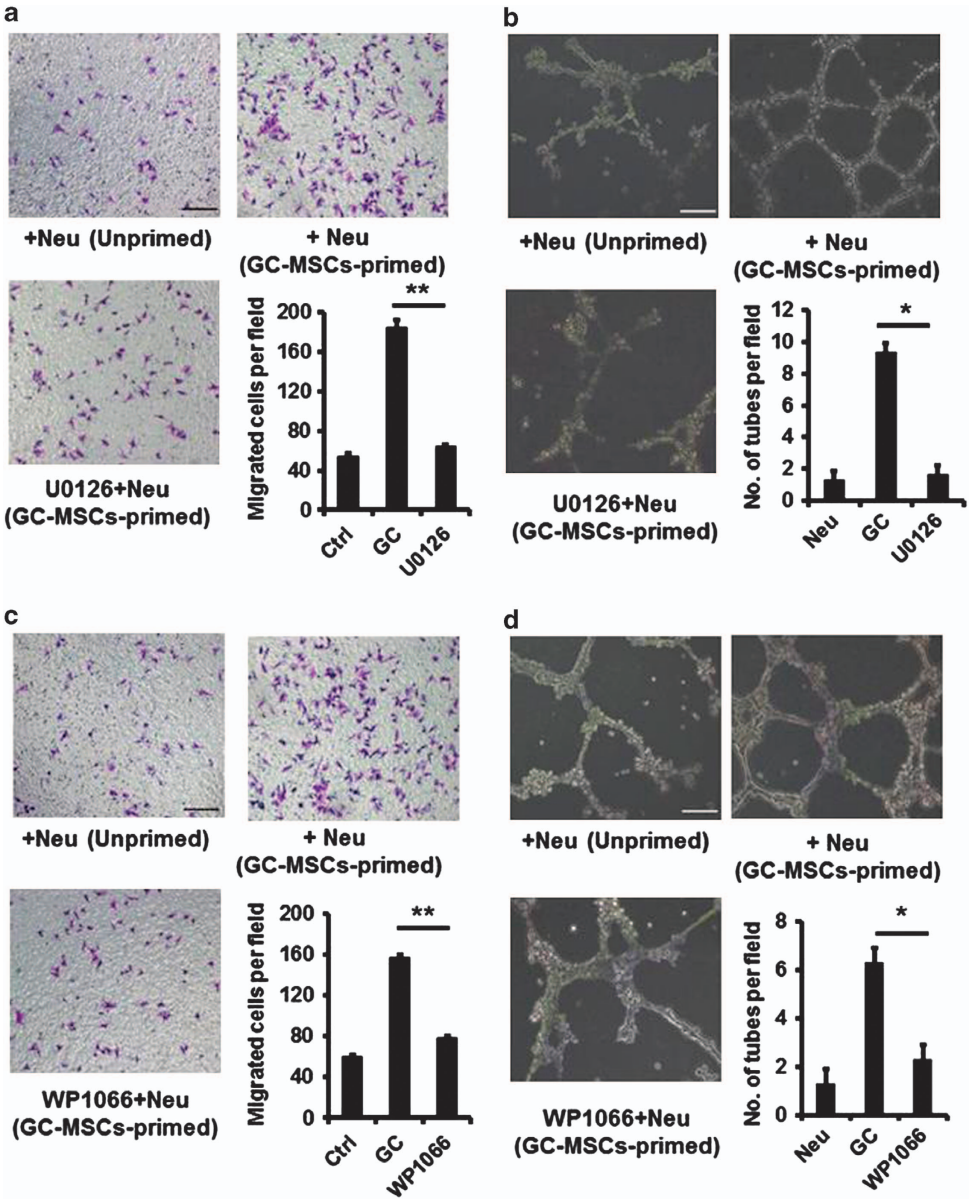

Figure 4 Blocking STAT3–ERK1/2 signaling cascade inhibits GC-MSCs-induced pro-migratory and pro-angiogenic roles in neutrophils. Neutrophils were stimulated with GC-MSCs CM in the presence or absence of U0126 (10 μM) or WP1066 (10 μM), and then the cells were collected. Neutrophils were seeded with 2×10^4 SGC-7901 cells in the upper compartment of the chamber for coculture. After incubation for 12 h, the migrated cells on the lower surface of the membrane were stained with crystal violet after fixation and then counted (**a**, **c**); HUVECs were seeded at 2×10^4 cells/well with conditioned media from neutrophils for 10 h. The formation of tube-like structure by HUVECs were observed under a phase-contrast microscope and photographed at 100 $\times$ magnification (**b**, **d**). Scale bar = 200 μm. $^*P<0.05$, $^{**}P<0.01$

is responsible for the modulation of neutrophil activation and functions.

GC-MSCs-primed neutrophils induce the CAF differentiation of normal MSCs. To demonstrate the crosstalk between CAFs and neutrophils, we next investigated the role of GC-MSCs CM-primed neutrophils in the modulation of a reactive state of normal MSCs. GCN-MSCs were treated with the supernatants from GC-MSCs CM-primed neutrophils, and α-SMA and vimentin were determined as markers for fibroblast activation. Western blot results demonstrated that supernatants from GC-MSCs CM-primed neutrophils upregulated the levels of α-SMA and vimentin in GCN-MSCs at both 24 and 48 h poststimulation (Figure 6a). To further confirm this result, GCN-MSCs were treated with the supernatants from GC-MSCs CM-primed neutrophils for 48 h, the expression of α-SMA and vimentin were determined by using immunofluorescence (Figure 6b). In addition to α-SMA, we also demonstrated that GC-MSCs CM-primed neutrophils induced the expression of FAP, an important marker for CAFs, in GCN-MSCs. In addition, GC-MSCs CM-primed neutrophils improved IL-6 mRNA levels in GCN-MSCs (Figure 6c). To demonstrate the functional role of activated neutrophils-induced CAF differentiation of

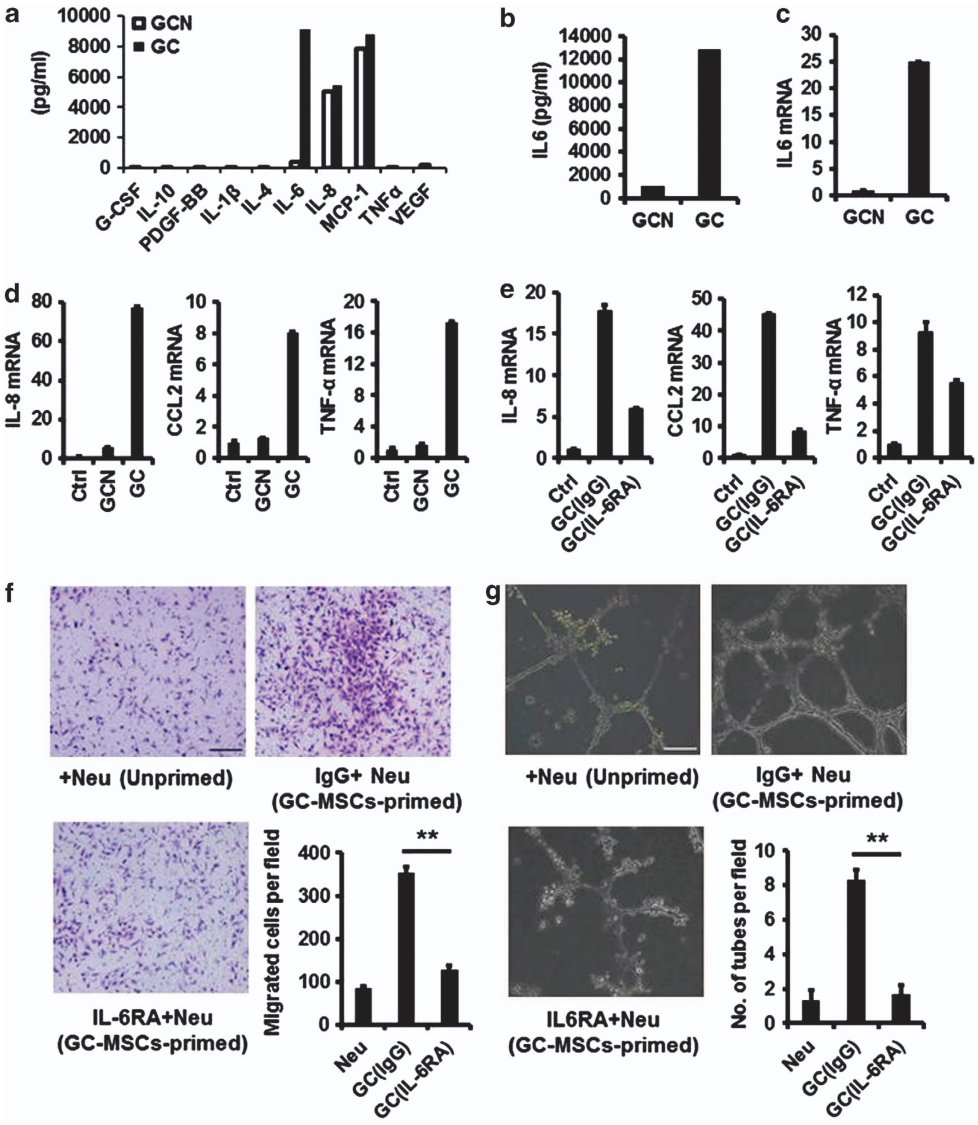

Figure 5 GC-MSCs induce the activation of neutrophils through IL-6–IL-6R interaction. (**a**) Multiplex screening assays for cytokine/chemokine levels in GC-MSCs supernatants by using Human Cytokine/Chemokine Panel 1. (**b**) IL-6 levels in GC-MSCs and GCN-MSCs supernatants were determined by using ELISA. (**c**) Total RNA was extracted from GC-MSCs and GCN-MSCs, and real-time PCR was performed to determine the expression of IL-6 mRNA. (**d**) Neutrophils were incubated with GC-MSCs or GCN-MSCs-derived conditioned medium. Total RNA was extracted from neutrophils and real-time PCR was performed by using human-specific primers for the quantification of IL-8, CCL2, and TNFα. (**e**) Neutrophils were cultured with GC-MSCs-derived conditioned medium or control medium in the presence or absence of IL-6RA (10 μg/ml) for 18 h. Total RNA was extracted from neutrophils and real-time PCR was performed by using human-specific primers for the quantification of IL-8, CCL2 and TNFα. (**f**) After coculture with GC-MSCs CM for 12 h in the presence or absence of IL-6RA (10 μg/ml), neutrophils were harvested and seeded with 2×10^4 SGC-7901 cells in the upper compartment of the chamber. After incubation for 12 h, the migrated cells on the lower surface of the membrane were stained with crystal violet after fixation and then counted (**g**) HUVECs were seeded at 2×10^4 cells/well with conditioned media from GC-MSCs-primed neutrophils in the presence or absence of IL-6RA (10 μg/ml) for 10 h. The formation of tube-like structure by HUVECs were observed under a phase-contrast microscope and photographed at 100 $\times$ magnification. Scale bar = 200 μm. $^{**}P < 0.01$

normal MSCs, the supernatants from activated neutrophils-primed MSCs were collected and used for cell migration assay. The results showed that the supernatants from the CAF-differentiated MSCs significantly prompted gastric cancer cell migration (Figure 6d). In brief, these results indicate that GC-MSCs CM-primed neutrophils are able to elicit activation of GCN-MSCs to CAFs, suggesting a bi-directional role between fibroblast activation and neutrophils.

Discussion

In this study, we demonstrated that GC-MSCs induced the chemotaxis of neutrophils and protected them from spontaneous apoptosis. Neutrophils primed with GC-MSCs supernatants displayed an activated state and prompted the migration of gastric cancer cells and the tube formation of endothelial cells *in vitro*. GC-MSCs exerted the activation of neutrophils via the IL-6–STAT3 axis. In turn, the activated neutrophils induced the differentiation of normal MSCs

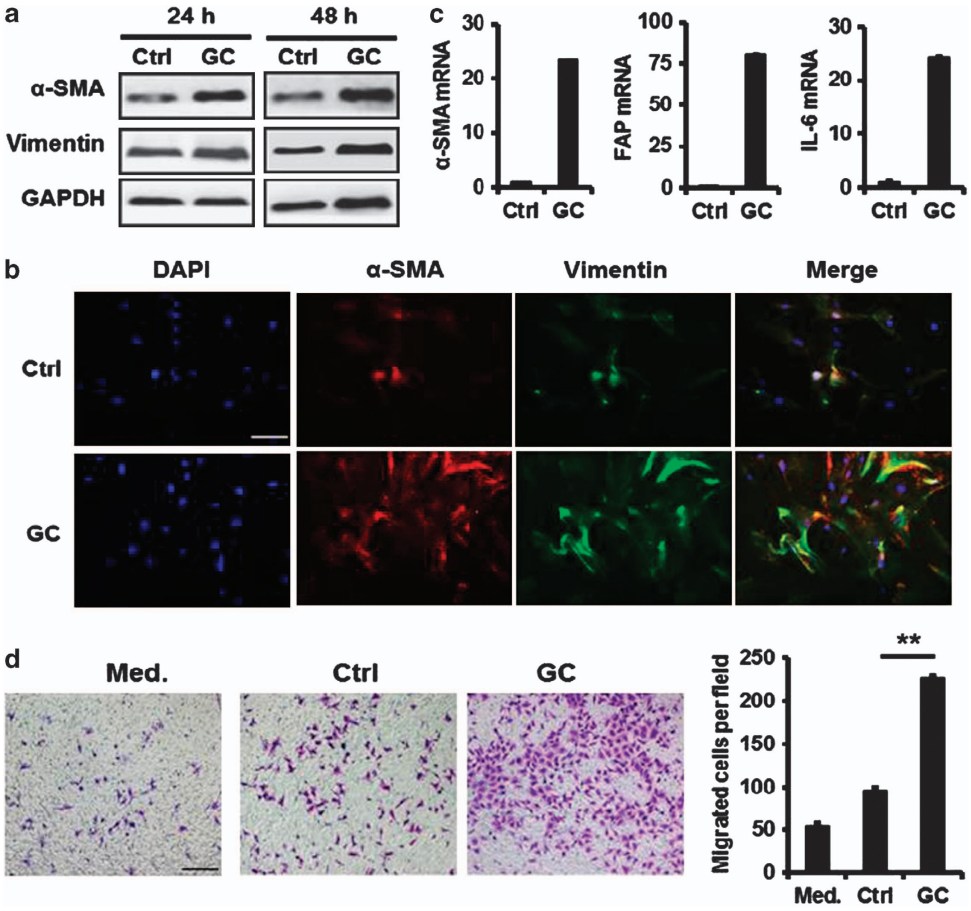

Figure 6 GC-MSCs-primed neutrophils induce the CAF differentiation of normal MSCs. (**a**) GCN-MSCs were incubation with conditioned media from GC-MSCs-primed neutrophils for 24 or 48 h. Total protein was extracted and the levels of α-SMA and vimentin were detected by using western blot. (**b**) GCN-MSCs were incubated with conditioned medium from GC-MSCs-primed neutrophils for 48 h. Then the cells were incubated with anti-α-SMA or anti-vimentin primary antibodies followed by fluorescence-conjugated secondary antibodies. Nuclei were visualized by DAPI staining. The images were acquired by using a Nikon eclipse Ti-S microscope. Scale bar = 100 μm. (**c**)Total RNA was extracted from GCN-MSCs and real-time PCR was performed using human-specific primers for the quantification of α-SMA, FAP, and IL-6. (**d**) Cell migration assay for SGC-7901 cells stimulated with the conditioned medium from CAF-differentiated MSCs that were induced by GC-MSCs-primed neutrophils. Scale bar = 200 μm. $^{**}P < 0.01$

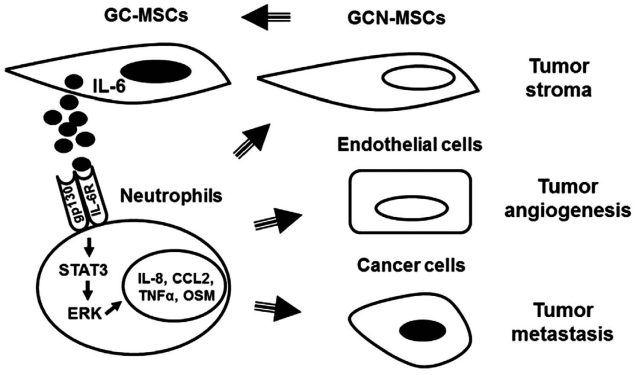

Figure 7 Proposed model for reciprocal crosstalk between GC-MSCs and neutrophils via IL-6–STAT3 axis. GC-MSCs-derived IL-6 activates the STAT3–ERK1/2 signaling cascade in neutrophils, resulting in the prolonged survival and the functional activation of neutrophils. In turn, GC-MSCs-activated neutrophils prompt the metastatic potential of gastric cancer cells and the angiogenic ability of endothelial cells, as well as the transition of normal MSCs to CAFs in tumor stroma

towards CAFs. These results establish a bi-directional interaction between GC-MSCs and neutrophils and elaborate the mechanistic link between GC-MSCs and neutrophils in synergistically driving tumor progression (Figure 7). Therefore, our findings provide important insights into the role of MSCs in directing the reshape of the tumor microenvironment.

Tumors have long been regarded as wounds that do not heal.[26] During this process, the immune cells and stromal cells are 'educated' by the tumor to acquire tumor-promoting activities. Several lines of evidence have shown that the stromal cells influence the growth, progression, and outcome of cancer cells. Studies have confirmed that MSCs could differentiate into CAFs under the tumor microenvironment conditions. We have previously demonstrated that MSCs promote gastric cancer growth *in vivo*. The mechanism responsible for this effect is not well understood but is linked to the pro-angiogenic role of MSCs.[27] Comito *et al.*[28] recently reported that CAFs and M2-polarized macrophages synergized to prompt prostate carcinoma progression. Based on

our findings, we propose that the reciprocal crosstalk between cancer-derived MSCs and neutrophils synergistically prompt gastric cancer progression. Neutrophils-derived TNFα and IL-6 may have a major role in the induction of MSCs differentiation towards CAF as both TNFα and IL-6 are upregulated in activated neutrophils (data not shown) and have been previously suggested to participate in CAF transition.[29]

Recent studies suggest that in murine lung cancer model, infiltrating neutrophils are driven by TGF-β to acquire a polarized N2 protumor phenotype.[30] After TGF-β inhibition, a shift to N1 occurs with acquisition of antitumor activity *in vitro* and *in vivo*. The N1–N2 polarization proposed by Fridlender *et al.*[31] mirrors the M1–M2 polarization of macrophages. Transcriptomic analysis of tumor-associated neutrophils (TAN), naive bone marrow neutrophils (NN) and granulocytic MDSCs (G-MDSC) suggests that TANs differ from NN and G-MDSC in their genetic profile, indicating that TANs are a distinct population of neutrophils. In contrary, IFN-β-deficient mice injected with melanoma or fibrosarcoma cells develop faster-growing tumors with rich blood vessels than the wild-type counterparts.[4] These tumors are infiltrated with increased number of neutrophils expressing high level of MMP-9 and VEGF. These findings suggest that endogenous IFN-β maintains neutrophils at antitumoral N1 type and the lack of IFN-β convert the neutrophils to a pro-tumoral N2 type. Considering the notable differences between mice and human, the existence and properties of N1–N2 in humans is still being investigated. The activation of neutrophils by tumor or stromal cells raises a question regarding if the activated neutrophils are N2-like TAN. A careful comparison of *in vitro* activated neutrophils and *in vivo* N2 TAN may provide further information for understanding the polarization of neutrophils in human cancer.

Spicer *et al.*[9] suggest that neutrophils promote liver metastasis via interactions with circulating tumor cells through Mac-1–ICAM-1 interaction, indicating that neutrophils may act as a bridge to facilitate interactions between cancer cells and the liver parenchyma. Queen *et al.*[32] suggest that breast cancer bells stimulate neutrophils to produce OSM, which in turn induces vascular endothelial growth factor from breast cancer cells in coculture and increases breast cancer cell detachment and invasive capacity. In our study, we demonstrated that the activated neutrophils had increased expression of Mac-1 (data not shown) and OSM, and could enhance the migration of gastric cancer cells and angiogenesis *in vitro*, supporting the notion that neutrophils are critically involved in cancer progression by modulating cancer metastasis and angiogenesis.

The molecular mechanisms mediating neutrophil activation and function in the tumor microenvironment are poorly understood. In a melanoma-neutrophils coculture experiment, Peng *et al.*[33] demonstrate that NFκB pathway is activated in neutrophils and is responsible for CXCL8 production by neutrophils upon melanoma cell stimulation. Tumor-derived hyaluronan fragments activate the PI3K/Akt pathway in neutrophils to prolong their survival and production of inflammatory factors.[16] Dumitru *et al.*[15] suggest that HNSCC supernatants strongly activate p38 and its downstream kinases CREB and p27 in neutrophils to induce release of CCL4, IL-8, and MMP-9. They have shown that HNSCC prolongs the survival of neutrophils via p38 activation, whereas the PI3K signaling pathway seems not to be involved in this process. These findings indicate that the molecular mechanisms through which tumor and its microenvironment modulate the functions of neutrophils are very complex and may be cancer type-specific. In this study, we demonstrated that GC-MSCs supernatants activated neutrophils through STAT3 and ERK1/2 signaling cascade. Further studies using inhibitors validated that GC-MSCs signals via STAT3 and ERK1/2 to prolong the survival of neutrophils and induce release of pro-inflammatory and pro-angiogenic mediators. In addition, we found that Akt and NFκB signaling pathways in neutrophils were not affected by GC-MSCs (data not shown). Understanding the exact molecular mechanisms used by cancer-derived MSCs to skew the functions of neutrophils may ultimately provide a novel strategy for anticancer therapy.

Raffaghello *et al.*[25] suggest that human bone marrow MSCs (BM-MSCs)-derived IL-6 rescues neutrophils from apoptosis by activating STAT3 transcription factor. In addition, human MSCs activated by poly(I:C) or LPS significantly prolong the survival and function of neutrophils, indicating a role of MSCs in recruiting peripheral blood neutrophils and enhancing their inflammatory activity to respond to microbial challenge.[34] In this study, we have further established a link between MSCs and neutrophils in cancer. Hsu *et al.*[35] demonstrate that BM-MSCs instruct anti-CD3/anti-CD28 antibody-activated $CD4^+CD45RO^+$ memory T cells to produce IL-17. Whether MSCs may modulate neutrophils biology and function in gastric cancer through IL-17-producing cells is an interesting question to be answered. Ren *et al.*[36] recently reported that MSCs isolated from spontaneous lymphoma (L-MSCs) enhanced tumor growth in comparison with BM-MSCs. L-MSCs recruited both macrophages and neutrophils to the tumor, but the depletion of macrophages while not neutrophils abolished tumor promotion of L-MSCs, suggesting that the recruitment of neutrophils by MSCs may have little effect on primary tumor growth but may influence the other aspects of tumor progression such as metastasis. Our findings that GC-MSCs-activated neutrophils prompt gastric cancer cell, migration while not affect cell proliferation, is in support of this notion. Further study using animal model to determine the role of MSCs-neutrophils interaction in gastric cancer metastasis will be actively performed.

In conclusion, our data suggest the establishment of a feedback loop between MSCs and neutrophils, resulting in the setup of a pro-inflammatory microenvironment due to the enrichment in CAF-differentiated MSCs and activated neutrophils in gastric cancer. These stromal components synergistically contribute to enhance invasiveness of gastric cancer cells and angiogenesis, ultimately fostering the metastatic spread of cancer cells. Strategies that target MSC-neutrophils interaction should provide a novel avenue of therapy for inflammation-related cancer.

Materials and Methods

Neutrophil isolation. Peripheral blood was collected from healthy volunteers after obtaining written informed consent. Carefully layer 5.0 ml of anti-coagulated whole blood over 5.0 ml of PolymorphPrep (Axis-Shield PoC AS, Oslo, Norway) in a 15 ml centrifuge tube. Centrifuge the samples layered over Polymorphprep at

800 *g* for 30 min in a swing-out rotor at room temperature. After centrifugation, two leukocyte bands should be visible. The top band at the sample/medium interface will consist of mononuclear cells and the lower band of polymorphonuclear cells (PMN). The cell bands may be harvested using a Pasteur pipette. The remnant RBC was lysed using hypotonic lysing procedure to obtain the pure PMN population. The morphological examination and cell count were performed to determine the number and purity of PMN. Neutrophils were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (fetal bovine serum; Invitrogen) and 1% penicillin/streptomycin. Purity of neutrophils was 98% after this procedure.

MSCs isolation. The tumor specimens were obtained from patients who underwent radical gastrectomy for gastric cancer at the department of Surgery, the Affiliated Hospital of Jiangsu University. Written informed consent was obtained from all patients and the study was approved by the ethical committee of Jiangsu University. The fresh tumor specimens were washed in phosphate-buffered saline (PBS) with 10% penicillin and streptomycin. The tissues were cut into 1-mm^3-sized pieces and cultured in low glucose (L-DMEM) supplemented with 15% FBS and 1% penicillin and streptomycin. The medium was replaced every 3 days after the initial plating. When adherent fibroblast-like cells appeared after 10 days culture, the cells were trypsinized and passaged into a new flask for further expansion. The cells at passage 3 were used for further study. The phenotype of isolated MSCs was determined by using flow cytometry. MSCs were collected and stained with monoclonal antibodies against CD29, CD44, CD45, and CD90 (PE-conjugated); CD14, CD34, CD71, and HLA-DR (FITC-conjugated) (Becton-Dickinson, San Jose, CA, USA). PE-IgG1 and FITC-IgG1 were used as the isotype control.

CM from MSCs. GC-MSCs were seeded on 35-mm culture dishes with L-DMEM supplemented with 15% FBS at a density of 1×10^5/ml for 48 h. The confluent cells were washed once with PBS and cultured in fresh L-DMEM supplemented with 15% FBS for another 48 h. The medium was collected and cell debris were removed by rinsing the supernatant through a 0.22 μm filter and designated as CM.

Cell culture. Human promyelocytic leukemia HL-60 cell line and gastric cancer SGC-7901 cell line were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 containing 10% FBS.

Cell viability assay. The viability of neutrophils was determined by using the MTT assay. Briefly, 5×10^4 neutrophils were incubated in the presence or absence of GC-MSCs CM in 96-well plate. At scheduled times, 20 μl MTT was added to each well and cells were further incubated at 37 °C in a humidified, 5% CO_2 for 4 h. Then the media were discarded. 150 μl DMSO was added to each well. Soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan formed is directly proportional to the number of viable cells in the culture and was determined by a microplate reader at 490 nm.

Cell apoptosis assay. Neutrophil apoptosis was quantified using an Annexin V apoptosis detection kit according to the manufacturer's instructions (Invitrogen). The binding of Annexin V-FITC and PI to the cells was measured by FACS Calibur (BD Biosciences, NJ, USA) using Cell Quest software.

Neutrophil chemotaxis assay. Neutrophils were seeded on the upper chamber of a 24-well transwell system with 3 μm pore inserts (5×10^5 cells in 0.2 ml medium). The bottom chambers were loaded with control medium or with CM from GC-MSCs. After 3 h of culture at 37 °C in a humidified atmosphere of 5% CO_2, the inserts were removed, and migrated cells were counted by using Beckman Coulter HMX. The number of cells that migrated toward control medium was set as 1.

Cell migration assay. Cell migration assay was performed in a 24-well Boyden chamber with an 8-μm pore size polycarbonate membrane (Corning, Union City, CA, USA). Cancer cells (2×10^4 in 100 μl of serum-free medium) were added to the upper compartment of the chamber whereas the lower compartment was filled with 600 μl of DMEM containing 10% FBS. After incubation for 12 h, cancer cells remaining on the upper surface of the membrane were removed. The migrated cells on the lower surface of the membrane were rinsed with PBS for 5 min to remove residual neutrophils and subsequently stained with crystal violet after fixation and then counted under a light microscope. For coculture studies, the neutrophils (1.0×10^5) were pre-seeded in the upper or lower compartment.

Endothelial tube formation assay. Neutrophils were cultured in the presence or absence of GC-MSCs CM for 12 h, followed by washing with PBS once and culturing in fresh medium for additional 6 h. The CM from activated neutrophils were collected and filtered through a 0.22 μm filter. 96-well slide chambers were coated with 50 μl of Matrigel (BD Biosciences) and allowed to gel at 37 °C for 30 min. HUVECs were seeded at 2×10^4 cells/well and incubated with or without conditioned media from neutrophils at 37 °C for 10 h. The formation of tube-like structure by HUVECs were observed under a phase-contrast microscope and photographed at 100 × magnification. The number of tubules from five random fields in each well was counted. The experiments were repeated for three times.

Quantitative real-time PCR. Total RNA was extracted from neutrophils using Trizol reagent (Invitrogen). The cDNAs were synthesized by using a reverse transcription kit according to the manufacturer's instruction (Vazyme, Shanghai, China). Quantitative RT-PCR was performed using human-specific primers for the quantification of IL-8, CCL2, TNFα, and OSM (Table 1). β-actin was used as an internal control. Reactions were performed using SYBR-Green PCR mix (Applied Biosystems, Shanghai, China) in the Bio-Rad CFX96 Real-Time System.

Western blot. Total protein was extracted from differentiated HL-60 cells and neutrophils that were stimulated with GC-MSCs CM for different times. Equal amounts of proteins were separated by 12% SDS-PAGE and immunoblotted with antibodies against Bax, p-ERK1/2, ERK1/2 (Bioworld, Nanjing, China), cleaved Caspase-3, Bcl-2, p-STAT3, STAT3 (Cell signaling technology, Shanghai, China), and GAPDH (CWBIO, Beijing, China), followed by the secondary goat anti-rabbit or goat anti-mouse antibodies (Bioworld). Detection was performed using the ECL kit (Applygen Gene Technology, Beijing, China).

Immunofluorescence. Cells were seeded in 24-well chamber slides and led to adhere overnight. After incubation with conditioned media from GC-MSCs-primed neutrophils for 48 h, cells were washed with cold PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. After blocking with 5% BSA, cells were incubated with mouse anti-α-SMA or rabbit anti-vimentin primary antibodies at 4 °C overnight (1 : 100, Bioworld) and followed by Cy3-conjugated anti-mouse or FITC-conjugated anti-rabbit secondary antibodies (1 : 100, Invitrogen) at 37 °C for 2 h. Nuclei were visualized by DAPI staining, and the images were acquired by using a Nikon eclipse Ti-S microscope.

Multiplex bead-based immunoassay and ELISA. Multiplex screening assays for GC-MSCs supernatants were performed using Human Cytokine/Chemokine Panel 1 according to the protocol provided by the manufacturer

Table 1 Primer sequences of target genes

Genes	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp (°C)
Mcl-1	For: ACGGCCTTCCAAGGCAT Rev: TTGTTACGCCGTCGCTGA	103	63
Fas	For: TCGGAGGATTGCTCAACAAC Rev: ATGATGCAGGCCTTCCAAGT	183	63
IL-8	For: GCTCTGTGTGAAGGTGCAGTTT Rev: TTCTGTGTTGGCGCAGTGT	144	62
OSM	For: CACAGAGGACGCTGCTCAGT Rev: GCTGGTGTCCTGCATGAGA	143	60
CCL2	For: GAACCGAGAGGCTGAGACTA Rev: GCCTCTGCACTGAGATCTTC	151	52
TNFα	For: CCGAGTGACAAGCCTGTAGC Rev: AGGAGGTTGACCTTGGTCTG	493	57
IL-6	For: TACATCCTCGACGGCATCTC Rev: AGCTCTGGCTTGTTCCTCAC	252	61
α-SMA	For: CTGACTGAGCGTGGCTATTC Rev: CCACCGATCCAGACAGAGTA	452	58
FAP	For: ATAGCAGTGGCTCCAGTCTC Rev: GATAAGCCGTGGTTCTGGTC	278	59
β-actin	For: CACGAAACTACCTTCAACTCC Rev: CATACTCCTGCTTGCTGATC	265	56

(Millipore, Billerica, MA, USA). Data were analyzed using the Luminex 200 system (Millipore). IL-6 levels in GC-MSCs supernatants were determined using ELISA kit according to the manufacturer's instructions (Dakewe, Beijing, China).

Statistical analyses. Results were expressed as mean ± S.D. Statistical analyses were performed using student's *t* test with Prism Version 5.0 software (Graphpad Software). $P < 0.05$ was considered as statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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