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IL-6 Cooperates with G-CSF To Induce Protumor Function of Neutrophils in Bone Marrow by Enhancing STAT3 Activation

Bin Yan,1 Jing-Jing Wei,1 Ye Yuan, Rui Sun, Dong Li, Jing Luo, Sheng-Jun Liao, Yuan-Hong Zhou, Yu Shu, Qi Wang, Gui-Mei Zhang, and Zuo-Hua Feng

Neutrophils are known to have antitumor potential. However, in recent years the tumor-promoting effect of neutrophils has been well demonstrated. So far, it remains unclear what causes the conversion of neutrophil function from tumor suppressive to tumor promoting. In this article, we report that the conversion of murine neutrophil function occurs in bone marrow, and that IL-6 cooperation with G-CSF is required for this conversion. IL-6 cooperated with G-CSF to modulate neutrophils in bone marrow, altering the activation potential of signaling pathways in neutrophils, especially that of STAT3. Costimulation with G-CSF and IL-6 induced a higher level of phospho-STAT3 in neutrophils, which was further increased by upregulation of STAT3 expression in neutrophils owing to downregulation of IFN-β expression in bone marrow macrophages by IL-6. Augmented STAT3 activation was crucial for upregulating the expression of Mmp9 and Bv8 genes and downregulating the expression of Trail and Rab27a genes in neutrophils. Moreover, G-CSF/IL-6–modulated neutrophils could not efficiently release azurophilic granules because of downregulation of Rab27a and inefficient activation of PI3K and p38 MAPK pathways. Because of premodulation by G-CSF and IL-6, neutrophils in response to complex stimuli in tumor released much less myeloperoxidase, neutrophil elastase, and TRAIL, but showed much higher expression of Mmp9 and Bv8 genes. Taken together, these results demonstrate that G-CSF and IL-6, despite their well-known physiological functions, could modulate the activation potential of signaling pathways in neutrophils, resulting in the production or release of the above-mentioned factors in a way that favors tumor angiogenesis and tumor growth. The Journal of Immunology, 2013, 190: 5882–5893.

Poly morphonuclear leukocytes (PMNs or neutrophils) make up a significant portion of the inflammatory cell infiltrate found in a wide variety of human cancers and murine models (1). Although neutrophils have the potential to suppress tumor growth through their cytotoxicity against tumor cells, and cause damage to vascular endothelium through oxidants and proteolytic enzymes (2), substantial evidence now shows significant protumor actions of neutrophils, in particular their role in promoting tumor angiogenesis (3). Moreover, increased peripheral blood neutrophil counts and neutrophil-to-lymphocyte ratios have been associated with poor clinical outcomes and short overall survival (4–8). However, it remains unclear what causes the conversion of neutrophil function from tumor suppressive to tumor promoting.

In response to suitable stimuli, neutrophils could release TRAIL, myeloperoxidase (MPO), and neutrophil elastase (NE). TRAIL not only induces apoptosis in tumor cells (9) but also has the potential to inhibit tumor angiogenesis (10, 11). MPO catalyzes the formation of HOCl (12). HOCl and NE at high concentration inhibit tumor growth (4). They are also involved in neutrophil-mediated damage of vascular endothelium. In contrast, however, neutrophils in tumors could produce proangiogenic factors such as MMP-9 and Bv8 (13–16). The proangiogenic function of neutrophils has been demonstrated, suggesting that neutrophils in tumors may produce more proangiogenic factors but fewer antiangiogenic factors. Nevertheless, so far it is unclear how neutrophils are modulated to produce these factors in a way that favors tumor angiogenesis and tumor growth.

The functions of neutrophils can be modulated by a variety of cytokines and chemokines (17). Among them, G-CSF and IL-6, which could be produced by tumor cells and/or stromal cells in malignant tumors (18), are potential candidates for modulating neutrophils to induce their protumor function. G-CSF and IL-6 are the cytokines that promote neutrophil production (19, 20). However, in recent years some studies showed that G-CSF could promote tumor angiogenesis (15, 21) and that increased levels of serum IL-6 have been associated with poor prognosis in different types of cancers (22–26). These findings have the important implication that G-CSF and IL-6, despite their well-known physiological functions, might be able to cause the conversion of neutrophil function. IL-6 is known to promote neutrophil production and may exert its effects in conjunction with G-CSF (20). However, so far it is unclear whether, and how, IL-6 cooperation with G-CSF is involved in inducing protumor function of neutrophils. In this study, we focused on the cooperative effect of G-CSF and IL-6 on neutrophils before they entered the tumor milieu,

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Abbreviations used in this article: BM, bone marrow; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; N-BM, bone marrow of naive mice; NE, neutrophil elastase; N-PC, peritoneal cavity of naive mice; PC, peritoneal cavity; pG/P6-PC, peritoneal cavity of pG/P6-PC mice; PMN, polymorphonuclear leukocyte or neutrophil; T-BM, bone marrow of tumor-bearing mice; TIMP, tissue inhibitor of metalloproteinase; T-PC, peritoneal cavity of tumor-bearing mice; T-SM, soluble molecule from tumor; VEGF, vascular endothelial growth factor.

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and investigated the mechanisms underlying the modulatory effect of G-CSF and IL-6. Our data showed that IL-6 cooperated with G-CSF to modulate neutrophils in bone marrow (BM). Modulating the activation of signaling pathways, especially those of STAT3, G-CSF, and IL-6, increased the expression of Mmp9 and Bv8 genes, decreased Trail expression in neutrophils, and attenuated the exocytosis of neutrophil azurophilic granules, thus favoring tumor growth and tumor angiogenesis.

Materials and Methods

Animals and cell lines
BALB/c mice and C57BL/6 mice, 6 to 8 wk old, were purchased from the China National Laboratory Animal Center of Wuhan University (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. B6.FVB(Bg)-Mmp9tm1wrj mice (MMP-9−/−) were kindly provided by Professor Hong-Liang Li (Cardiovascular Research Institute of Wuhan University, Wuhan, People’s Republic of China). The BALB/c background H22 hepatocarcinoma cell line and C57BL/6 background melanoma B16F1 cell line were purchased from the China Center for Type Culture Collection (Wuhan, China) and cultured according to the center’s guidelines.

Reagents and plasmids
STAT3 inhibitor VIII, PD98059, and wortmannin were purchased from Merck4Biosciences (Calbiochem). All inhibitors were dissolved in DMSO as a stock solution and diluted with culture medium to the desired concentration without toxicity to neutrophils. Murine G-CSF and IL-6 were purchased from PeproTech (Rocky Hill, NJ). Plasmids pG, pGR, pGCaCL1, and pIFN-β are expression vectors carrying the cDNA encoding murine G-CSF, IL-6, pGCaCL1, and IFN-β, respectively. Plasmids pGR, psGR, and ps130 are expression vectors carrying the cDNA encoding the extracellular domain of murine G-CSF, IL-6, and gp130, respectively. These plasmids were constructed by the insertion of cDNA into plasmid pCORNAs.13 (Invitrogen, Carlsbad, CA) in our laboratory. All of the vectors were identified by in vivo expression (Supplemental Fig. 1).

In vivo gene transfection
Plasmids were prepared and analyzed as described previously (27). Mice received the injection of plasmid DNA (in 100 μl saline) into the muscle tissue (i.m. injection) at the inoculation site or the injection of plasmid DNA via the tail vein (i.v. injection) using the hydrodynamics-based gene delivery technique (27).

Recruitment of neutrophils to the peritoneal cavity
To acquire CXCL1-expressing hepatocytes, mice received an i.v. injection of pCXCL1 plasmid (200 µg per mouse). Then, 12 h later, hepatocytes were prepared from peritoneal cells harvested 12 h after i.v. injection for the isolation of neutrophils (Supplemental Fig. 2).

Isolation of neutrophils
Murine neutrophils were isolated from BM cells or peritoneal cells, as described previously (29). Briefly, the cells were washed once in HBSS, layered over a Percoll gradient (54%/64%/72% for BM cells and 54%/64%/70% for peritoneal cells), and centrifuged at 1060 × g for 30 min. The dense band at the 64%/72% or 64%/70% interface was collected as the neutrophil fraction. The isolated cells were >90% neutrophils, as assessed by flow cytometric analysis and Giemsa–Wright stain (Supplemental Fig. 3). To isolate neutrophils from tissues, single-cell suspensions were prepared by digesting the tissues with collagenase, hyaluronidase, and DNase. Neutrophils were isolated using PE-anti-Ly6G Ab (eBioscience), magnetic microbeads, and MiniMACS columns (Miltenyi Biotec) according to the manufacturer’s protocol.

Animal experiments and treatment protocols
To acquire mice with in vivo expression of G-CSF and/or IL-6, mice received an i.m. injection of pG6 or pG6 plasmids (100 µg of each per injection) in the left hind thigh, once every 2 d, four times. The p3.1 (pCORNAs.13) plasmid was used as control. The mice were used for further experimentation on day 10 after the first injection of plasmid.

Immunohistochemistry
Tissue sections were prepared and subjected to immunohistochemical analysis, as described earlier (27). Anti-mouse Ly6G Ab and anti-mouse CD34 Ab (Santa Cruz Biotechnology) were used as primary Abs for detecting neutrophils and microvessels, respectively. HRP-conjugated secondary Ab was used for detecting neutrophils and microvessels in the H22 tumor. Cy3-conjugated secondary Ab was used for detecting microvessels in the H22 tumor. Immunohistochemistry was performed using an Nicon microscope at 40 × 10 magnification. The neutrophils were counted using Image-Pro Plus 6.0 software. The neutrophil density was defined as the number of neutrophils per microscopical field. Criteria for positive-staining microvessels and microvessel counting followed the method described by Weidner (30). Microvessel density was defined as the number of microvessels per 0.2 mm².

Assay of gene expression by real-time RT-PCR
Total RNA was extracted from cells with TRIzol reagent (Invitrogen). The relative quantity of mRNA was determined by real-time RT-PCR according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (31). Gapdh, Hprt, and Ppiα were chosen as reference genes. The relative expression of genes was calculated using GeNorm software. The primer sequences were as follows: MMP-9, sense 5′-GCTTCTGACCAGCAGAC-3′, antisense 5′-GACACCAGTAGACTCCAGACA TA-3′; GACACCAGTAGACTCCAGACA TA-3′; Hprt, sense 5′-GGACATT-3′, antisense 5′-GAAGGCGTTGGTGGTGTAG-3′; Ppiα, sense 5′-GGTGGTGAGAAAATGGAACAC-3′, antisense 5′-TGCTACTTCTGCTGCTACC-3′; Gapdh, sense 5′-ATTGCCCAAGGCTCTGCAAG-3′, antisense 5′-GGCAATGTCCATCCAGGT-3′; T-βR, sense 5′-TACTGGAGATCCTGGAAGAG-3′, antisense 5′-ACGTCGGTTGGAAGAATGACGCG-3′, antisense 5′-CTGTATTGAGACGGTATG-5′-CTACCCCTGACCGAGATTT-3′; SOCS3, sense 5′-ATTCACCAGGATGCAGCTAC-3′, antisense 5′-G-CCAATGTCCTCCAGGT-3′; IFN-β, sense 5′-TGGCTTTCTGGTCTTTCTGTC-3′, antisense 5′-CGCCCTTGAAGTGAGTTGCAGTA-3′; STAT3, sense 5′-ACCTCCAGGACGACCGTATG-3′, antisense 5′-TGCTTCTGTCG-AGTACCTTCA-3′; Rac2, sense 5′-GGGCTTTTCTCCAGGTATG-3′, antisense 5′-GAAGGCGTTGAGTGTGATG-3′; Rab27a, sense 5′-GCCTGGGATI-CACCAGGT-3′; TGFβ-3, sense 5′-TGGGATTGTCCTCTGTTC-3′, antisense 5′-TGCTTTTCTCCAGGTT-3′; Rab1b, sense 5′-TGGTCTTCTGG-3′, antisense 5′-GGTTGTCTGACCTTCA-3′; GADPH, sense 5′-ATGGTCCATGATGCACCTAC-3′, antisense 5′-G-ACACCTAGATCCACGACATA-3′; Hprt, sense 5′-GGCTTAAAGTCTTCTTCTAC-3′, antisense 5′-CCGTTGAGTTGTTACATCA-3′; PPIα, sense 5′-CTAAAAGGCTTCCCTCTCTAC-3′, antisense 5′-CAGGACCTGTAAGCCTTACG-3′.

Preparation of soluble molecules from tissues
Palpable tumors were dissected. The mixture of soluble molecules from tumor (T-Ms) was prepared by digesting the tissues with collagenase and removing debris by centrifugation. The concentration of T-Ms was defined by the concentration of protein, which was determined using Coomassie Brilliant Blue G250 and Bio-Rad protein assay according to the manufacturer’s instructions. For the detection of TRAIL, MPO, and NE in tissues, soluble interstitial molecules in the tissues at inoculation sites were prepared on day 4 after tumor inoculation.

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To detect intracellular MPO and NE, 5 × 10⁶ (for MPO) or 2.5 × 10⁵ (for NE) neutrophils were lysed with TBS containing 0.2% Triton X-100 (50 μl). To detect MPO and NE in tissues, soluble interstitial molecules prepared from tissues were diluted to 0.5 mg/ml (for MPO) and 2.5 mg/ml (for NE) of proteins. The activities of MPO and NE were measured as described previously (32, 33) and expressed as the values of OD (OD450 for MPO and OD405 for NE). To induce the release of azurophilic granules, neutrophils were stimulated for 30 min with TMs (0.5 mg/ml). Neutrophil degranulation was determined by detecting the release of MPO. Percentage of MPO release was calculated as described previously (32).

Western blot assay
Western blot assay was accomplished as described previously (34). Abs were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA).

Assay of MPO and NE
To detect intracellular MPO and NE, 5 × 10⁶ (for MPO) or 2.5 × 10⁵ (for NE) neutrophils were lysed with TBS containing 0.2% Triton X-100 (50 μl). To detect MPO and NE in tissues, soluble interstitial molecules prepared from tissues were diluted to 0.5 mg/ml (for MPO) and 2.5 mg/ml (for NE) of proteins. The activities of MPO and NE were measured as described previously (32, 33) and expressed as the values of OD (OD450 for MPO and OD405 for NE). To induce the release of azurophilic granules, neutrophils were stimulated for 30 min with TMs (0.5 mg/ml). Neutrophil degranulation was determined by detecting the release of MPO. Percentage of MPO release was calculated as described previously (32).

Matrix metalloproteinase-9 assay
Neutrophils were incubated at a concentration of 5 × 10⁵ per milliliter at 37°C for 4 h in RPMI 1640 medium. Matrix metalloproteinase-9 (MMP-9) in supernatants was detected by gelatin zymography, and the relative activity of MMP-9 was calculated as described previously (35). To detect MMP-9 in tumor, 1 × 10⁶ neutrophils or an equal volume of PBS (50 μl) was carefully injected into palpable tumor, once a day, three times. The soluble interstitial molecules in the tissues were prepared 1 d after the last injection. MMP-9 in soluble interstitial molecules was detected using the Biotrak Activity Assay System (Amersham Biosciences, Piscataway, NJ) per the protocol of the manufacturer.

Cytotoxicity assay
The cytotoxicity of neutrophils to tumor cells was determined as described earlier (9). Briefly, H22 target cells were labeled with CFSE and incubated with neutrophils at different E:T ratios (10:1, 20:1) in triplicate at 37°C for 8 h. Tumor cells incubated without neutrophils were used as control. The cells were then stained with allophycocyanin-Annexin V (BD Biosciences, San Diego, CA) and analyzed by flow cytometry. The cytotoxicity of neutrophils to tumor cells was evaluated by the percentage of Annexin V− cells in CFSE− cells, which was calculated by the following equation: Annexin V− cell % = (Annexin−V− /CFSE− cells /CFSE− cells in the mixture of tumor cells and neutrophils) − (Annexin−V− /CFSE− cells /CFSE− cells in control tumor cells).

Isolation of BM F4/80+ cells
Murine BM cells were washed once in HBSS. F4/80+ cells were isolated from BM cells using PE-anti-F4/80 Ab (eBioscience), magnetic microbeads, and MiniMACS columns (Miltenyi Biotec).

ELISA assay
Cell-free supernatants from untreated or treated cells were harvested after 48-h culture. IFN-γ and IL-6 in the supernatants was quantified using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Statistical analysis
Results were expressed as mean value ± SD and interpreted by one-way ANOVA. Differences were considered statistically significant when p < 0.05.

Results

G-CSF and IL-6 modulate neutrophils in BM to induce their protumor function
To explore whether the function of neutrophils could be modulated before they entered the tumor milieu, we first tested the effect of BM neutrophils on tumor growth in the coinoculation test. The neutrophils from bone marrow of naive mice (N-BM) suppressed tumor growth, whereas the neutrophils from bone marrow of tumor-bearing mice (T-BM) promoted tumor growth (Fig. 1A), suggesting that the function of neutrophils could be modulated in BM. To clarify the roles of G-CSF and IL-6, we evaluated their effect on the function of BM neutrophils by expressing soluble extracellular domains of G-CSFR, IL-6R, and gp130 in local tissues to block tumor-derived G-CSF and IL-6 (Fig. 1A), or expressing G-CSF and/or IL-6 in vivo in the absence of tumor (Fig. 1B). The results showed that G-CSF and IL-6 cooperated to modulate BM neutrophils, converting their function from tumor suppressive to tumor promoting.

To investigate whether the function of neutrophils could be maintained in blood and in the process of chemotaxis, we recruited neutrophils to the tissues at the inoculation site by expressing CXCL1 in an H22 hepatocarcinoma model (Fig. 1C, 1D). The recruitment of neutrophils suppressed the growth of tumor in control mice but promoted tumor growth in pG/pi6 mice (Fig. 1E), suggesting that the function of neutrophils was maintained in blood and in the process of chemotaxis. To further prove this, we recruited neutrophils to the PC of mice, using CXCL1-expressing hepatocytes, and isolated the neutrophils from the PC. In the coinoculation test, neutrophils from the peritoneal cavity of naive mice (N-PC) suppressed tumor growth, whereas neutrophils from that of pG/pi6 mice (pG/pi6-PC) and tumor-bearing mice (T-PC) promoted tumor growth (Fig. 1F).

We then tested whether G-CSF/IL-6–modulated neutrophils could promote tumor angiogenesis. Neutrophils isolated from the PC were injected into palpable tumors. The results showed that the neutrophils from N-PC reduced the density of microvessels in tumor, whereas the neutrophils from pG/pi6-PC and T-PC increased the density of microvessels in tumor (Fig. 1G), indicating that premodulation of neutrophils by G-CSF and IL-6 enabled the cells to promote tumor angiogenesis.

The above results showed that G-CSF and IL-6 modulated the function of neutrophils in BM, and that the function of neutrophils was maintained in blood and in the process of chemotaxis. The neutrophils isolated from the PC were the cells that had finished the process of chemotaxis but had not been stimulated by the stimuli in the tumor milieu. Therefore, in the following experiments, the neutrophils from BM were used to investigate the stimulating effects of G-CSF and IL-6 and the related mechanisms, whereas the neutrophils from the PC were used to test the function of neutrophils and their response to the stimuli in the tumor milieu.

G-CSF and IL-6 influence production or release of functional molecules by neutrophils
To further prove that premodulation by G-CSF and IL-6 altered the function of neutrophils, we analyzed the neutrophils recruited to the tissues at inoculation sites. The function of these neutrophils might represent the final function of the neutrophils in the tumor milieu, because these neutrophils not only finished the process of chemotaxis but also were stimulated by complex stimuli in the tissues. Compared with neutrophils from control mice, those from pG/pi6 mice expressed higher levels of Mmp9 and Bov mRNAs, but lower levels of Trail mRNA (Fig. 2A). The mRNA of Mpo or NE was undetectable or unchanged, respectively, in neutrophils isolated from the tissues (data not shown). The neutrophils from pG/pi6 mice contained more intracellular MPO and NE (Fig. 2B), but much less intracellular Trail (Fig. 2C). We then assessed the extracellular Trail (soluble Trail), MPO, and NE in the tissues. Extracellular Trail was detectable in the tissues of control mice, but not pG/pi6 mice (Fig. 2D). The activities of MPO and NE in local tissues of pG/pi6 mice were significantly lower than those in control mice (Fig. 2E). To compare the effect of G-CSF/IL-6 with that of tumor on neutrophils, we used another model in which the mice received secondary inoculation with tumor cells after the removal of primary tumor. The effect of primary tumor on neutrophils was analyzed by recruiting neutrophils to the tissues at the sites of secondary inoculation. The results showed that the modulatory
The effect of G-CSF and IL-6 on neutrophils was similar to that of primary tumor (Fig. 2A–E). Taken together, these data suggested that G-CSF and IL-6 could alter the expression of \textit{Mmp9}, \textit{Bv8}, and \textit{Trail} genes in neutrophils, as well as attenuate neutrophil degranulation to decrease the release of MPO and NE.

We then isolated neutrophils from the PC of mice to test their function. The neutrophils from N-PC had cytotoxicity to tumor cells in the absence or presence of the mixture of T-sMs, which might represent complex stimuli in the tumor milieu. The cytotoxicity of the neutrophils from pG/pI6 mice, pI6 mice, or pG/pI6 mice, pI6 mice, or tumor-bearing mice after recruitment by CXCL1. The neutrophils were used for coinoculation with H22 cells. (G) Neutrophils were isolated from the PC of naive mice, p3.1 mice, pI6 mice, or tumor-bearing mice after recruitment by CXCL1. The neutrophils were injected into palatable tumor. Microvessels in H22 tumor (Hematoxylin staining) and B16 melanoma were detected by immunohistochemical analysis (left; scale bar, 50 μm). Microvessel density in tumor tissues was determined as described in Materials and Methods (right). Tumors (n = 8 per group) were dissected and weighed on day 10 after tumor inoculation (A, B, E, F). Data are representative of three independent experiments (C), or pooled from four independent experiments with a total of eight samples in each group (D, G). *p < 0.05, **p < 0.01.

\textbf{G-CSF attenuates the activation of PI3K and p38 MAPK pathways in neutrophils}

To explore the mechanism involved in the attenuation of neutrophil degranulation, we next analyzed the activation of PI3K and p38 MAPK pathways in neutrophils.
p38 MAPK pathways in neutrophils, because the activation of these pathways is required for the induced degranulation of neutrophils (37). For this purpose, we stimulated the neutrophils from the PC with T-sMs. T-sMs could activate PI3K and p38 MAPK pathways in neutrophils (Fig. 3A, 3B). Intriguingly, the phosphorylation levels of Akt and p38 MAPK in neutrophils from pG/pI6-PC and T-PC were significantly lower than those in neutrophils from N-PC (Fig. 3A, 3B). In vivo expression of G-CSF, but not IL-6, resulted in the inefficient activation of PI3K and p38 MAPK pathways (Fig. 3C). Consistently, in vivo expression of G-CSF attenuated T-sM–induced degranulation of neutrophils, evaluated by the release of MPO (Fig. 3D). However, both

FIGURE 2. Gene expression or release of factors by neutrophils in tissues. (A–C) Neutrophils were isolated from the tissues with CXCL1 expression on day 4 after inoculation of H22 cells. The expression of Mmp9, Bv8, and Trail genes was detected at the mRNA level by real-time RT-PCR (A). Intracellular MPO and NE were detected by activity assay (B). Intracellular TRAIL was detected by Western blot (C). (D and E) Soluble interstitial molecules were prepared from the tissues with CXCL1 expression on day 4 after inoculation of H22 cells. TRAIL was detected by Western blot (D). MPO and NE were detected by activity assay (E). (F and G) Neutrophils were isolated from the PC of naive mice, p3.1 mice, pG/pI6 mice, or tumor-bearing mice after recruitment by CXCL1. The neutrophils were used for analysis of cytotoxicity to tumor cells (F) and release of MMP-9 (G), as described in Materials and Methods. (H) Neutrophils isolated from the PC of naive mice, p3.1 mice, pG/pI6 mice, or tumor-bearing mice were injected into palpable tumor (H22). MMP-9 in tumor was then detected as described in Materials and Methods. (I) Neutrophils isolated from the PC of pG/pI6-MMP-9−/− (MMP9KO) mice and pG/pI6-WT mice were injected into palpable tumor (B16). MMP-9 and microvessel density in tumor were determined as described in Materials and Methods. Tumors were dissected and weighed on day 7 after the last injection of neutrophils. Data are representative of three independent experiments (C, D), or pooled from four independent experiments with a total of eight samples in each group (A, B, E–I). *p < 0.05, **p < 0.01. Sec, Secondary inoculation, described in Materials and Methods.
spontaneous degranulation and induced degranulation of neutrophils from pG/pI6 mice were significantly decreased, suggesting that the attenuated degranulation was partially attributed to the inefficient activation of PI3K and p38 MAPK pathways.

**G-CSF and IL-6 upregulate Mmp9 and Bv8 expression but downregulate Trail expression in neutrophils**

Next, we investigated whether G-CSF and IL-6 could modulate the expression of *Mmp9*, *Bv8*, and *Trail* genes in BM neutrophils. The result showed that in vivo expression of G-CSF increased the expression of *Mmp9* and *Bv8*, and that IL-6 increased the expression of *Mmp9*. However, none of them alone influenced the expression of *Trail* (Fig. 4A). Intriguingly, coexpression of G-CSF and IL-6 in vivo reduced *Trail* mRNA level and further increased mRNA levels of *Mmp9* and *Bv8* in neutrophils, similar to the effects of tumor (Fig. 4A). The cooperative effect of G-CSF and IL-6 was also demonstrated by stimulating the neutrophils from N-BM with G-CSF and/or IL-6 in vitro (Fig. 4B). To test whether premodulation by G-CSF and IL-6 might influence the response of neutrophils in tumors, we stimulated neutrophils from the PC with T-sMs. In the presence of T-sMs, the expression of *Mmp9* and *Bv8* genes was further increased, whereas the expression of *Trail* was further reduced in neutrophils from pG/pI6-PC and T-PC (Fig. 4C). Compared with neutrophils from N-PC, those from pG/pI6-PC and T-PC showed much higher expression of *Mmp9* and *Bv8*, and much lower expression of *Trail*, suggesting that the response of neutrophils to T-sMs was augmented by G-CSF and IL-6.

**STAT3 is crucial for the modulatory effect of G-CSF and IL-6 on neutrophils**

G-CSF could activate the STAT3, PI3K, and ERK pathways in neutrophils (19, 38). IL-6 is known to activate STAT3 (39). To ascertain the crucial signaling pathway(s) involved in the effect of G-CSF/IL-6 on the expression of *Mmp9*, *Bv8*, and *Trail* genes, we stimulated neutrophils from N-BM with G-CSF and IL-6 in the presence of STAT3 inhibitor VIII, PD98059 (inhibitor of ERK pathway), or wortmannin (PI3K inhibitor). Inhibiting STAT3 abrogated the effect of G-CSF/IL-6 on the expression of *Mmp9* and *Trail*, and decreased the expression of *Bv8* (Fig. 5A, Supplemental Fig. 4A). Inhibiting the ERK pathway suppressed the expression of *Bv8* (Fig. 5A, Supplemental Fig. 4B). However, inhibiting PI3K increased the effect of G-CSF/IL-6, especially on the expression of *Trail* (Fig. 5A, Supplemental Fig. 4C). Moreover, G-CSF alone decreased the expression of *Trail* in the presence of PI3K inhibitor, whereas the effect of G-CSF was abrogated by STAT3 inhibitor (Fig. 5B), indicating that G-CSF–activated STAT3 could suppress the expression of *Trail* if PI3K was inhibited.

When neutrophils from the PC were stimulated with T-sMs, the modulatory effect of T-sMs on the expression of *Mmp9*, *Bv8*, and *Trail* was also suppressed by STAT3 inhibitor (Fig. 5C), further indicating that STAT3 was crucial for upregulating the expression of *Mmp9* and *Bv8* and downregulating the expression of *Trail* in neutrophils. In line with this, phosphorylated STAT3 was increased in neutrophils from pG-BM and pI6-BM, and further increased in neutrophils from pGpI6-BM and T-BM, compared with that in neutrophils from N-BM (Fig. 5D). Moreover, the expression of STAT3 was increased in neutrophils from pI6-BM, pG/pI6-BM, and T-BM (Fig. 5D, 5E). Similar levels of STAT3 were maintained after neutrophils were recruited to the PC by CXCL1 (Fig. 5E).

**Augmented STAT3 activation results in downregulation of Rab27a in neutrophils**

On the basis of the above results, we further investigated whether the augmented STAT3 activation might influence neutrophil degranulation. For this purpose, we analyzed the expression of Rac2 and Rab27a, which are crucial for exocytosis of neutrophil azurophilic granules (32, 40). In pG/pI6 mice and tumor-bearing mice, the expression of *Rab27a* gene was decreased in BM neutrophils and the neutrophils were recruited to the PC (Fig. 6A). Consistently, the expression of *Rab27a* gene in neutrophils from N-BM could be downregulated by costimulation with G-CSF and IL-6 in vitro (Fig. 6B). The effect of G-CSF and IL-6
was abolished by inhibiting STAT3, but enhanced by inhibiting PI3K (Fig. 6C). These data suggested that augmented STAT3 activation in neutrophils was another reason for attenuated neutrophil degranulation.

Both G-CSF and IL-6 can activate STAT3. Of interest, coexpression of G-CSF and IL-6 in vivo resulted in a higher level of phospho-STAT3 in neutrophils. Moreover, IL-6 cooperation with G-CSF was

FIGURE 4. IL-6 and G-CSF regulate the expression of Mmp9, Bv8, and Trail genes in neutrophils. The expression of Mmp9, Bv8, and Trail genes in neutrophils was detected at the mRNA level by real-time RT-PCR. Neutrophils were isolated and treated as follows. (A) Neutrophils in BM were isolated from naive mice, p3.1 mice, p6 mice, pG mice, pG/p6 mice, or tumor-bearing mice. The cells were directly used for analysis of gene expression. (B) Neutrophils were isolated from BM of naive mice and stimulated with G-CSF and/or IL-6 (50 ng/ml of each) for 12 h. (C) Neutrophils were isolated from the PC of naive mice, pG/p6 mice, or tumor-bearing mice after recruitment by CXCL1. The cells were stimulated with T-sMs (0.5 mg/ml) for 12 h. Data are pooled from three independent experiments with a total of six samples in each group. *p < 0.05, **p < 0.01.

FIGURE 5. Effect of signaling pathways on G-CSF/IL-6–modulated gene expression in neutrophils. (A) Neutrophils were isolated from BM of naive mice. The cells (2 × 10⁶/ml) were untreated or pretreated for 2 h with STAT3 inhibitor VIII (SI; 50 μM), PD98059 (PD; 20 μM), or wortmannin (WT 10 nM), and then stimulated for 12 h with G-CSF and IL-6 (50 ng/ml of each) in the absence or presence of the same inhibitor. The expression of Mmp9, Bv8, and Trail genes was detected at the mRNA level by real-time RT-PCR. (B) Neutrophils from BM of naive mice were stimulated for 12 h with G-CSF (50 ng/ml) in the absence or presence of wortmannin and/or STAT3 inhibitor VIII. The expression of Trail was detected by real-time RT-PCR. (C) Neutrophils were isolated from the PC of naive mice, pG/p6 mice, or tumor-bearing mice after recruitment by CXCL1. The cells were stimulated for 12 h with T-sMs (0.5 mg/ml) in the absence or presence of STAT3 inhibitor VIII. The expression of Mmp9, Bv8, and Trail genes was detected by real-time RT-PCR. (D) and (E) Neutrophils in BM or the PC were isolated from naive mice, pG mice, p6 mice, pG/p6 mice, or tumor-bearing mice. The phospho-STAT3 (p-STAT3) and total STAT3 in BM neutrophils were detected by Western blot (D). The relative levels of total STAT3 protein to β-actin protein (STAT3/β-actin) in BM PMNs and PC PMNs were calculated after densitometric analysis of Western blots (E). Data are representative of three independent experiments (D), or pooled from three independent experiments with a total of six samples in each group (A, B, C, E). *p < 0.05, **p < 0.01.
expression of real-time RT-PCR and Western blot. (Neutrophils were isolated from BM (BM-PMN) or the PC (PC-PMN) of six samples in each group. * by real-time RT-PCR. Data are representative of three independent experiments. **p < 0.01, ***p < 0.001.

Motivated by these results, we further investigated STAT3 activation in neutrophils. To test this, we stimulated the neutrophils from BM neutrophils (Fig. 5E), which might also favor the activation of STAT3 in BM neutrophils (Fig. 7C). However, IL-6 alone was inefficient in promotingSTAT3 expression in vitro. However, in vivo expression of IL-6 alone increased the expression of STAT3 in BM neutrophils, similar to the effect of coexpression of G-CSF and IL-6 (Fig. 5E). To explore the mechanism underlying the promoting effect of IL-6 on STAT3 expression in vivo, we investigated whether IL-6 might suppress IFN-β expression in BM, because IFN-β has been found to maintain lower expression of STAT3 in neutrophils (14). In pG/p6 mice and tumor-bearing mice, the expression of IFN-β in BM cells was significantly reduced (Fig. 8B). In vivo expression of IL-6, but not G-CSF, resulted in downregulation of IFN-β expression (Fig. 8B). F4/80+ monocytes/macrophages have been found to be the main source of Ifnb1 mRNA in BM cells (42). We then isolated F4/80+ cells from N-BM and stimulated the cells with IL-6. The result showed that IFN-β expression in F4/80+ cells was decreased by IL-6 in the absence, but not the presence, of STAT3 inhibitor (Fig. 8C), suggesting that IL-6 suppressed the expression of the Ifnb1 gene in F4/80+ cells through STAT3.

To further clarify whether downregulation of IFN-β expression was crucial for the increase of STAT3 expression and STAT3 activation in neutrophils in pG/p6 mice and tumor-bearing mice, we expressed IFN-β in the mice by in vivo transfection of the IFN-β expression vector. In vivo expression of IFN-β not only hindered the upregulation of STAT3 expression but also significantly reduced the phosphorylation level of STAT3 in BM neutrophils in pG/p6 mice and tumor-bearing mice (Fig. 5D).

In vivo expression of IFN-β in pG/p6 mice and tumor-bearing mice increased Rab27a expression in BM neutrophils (Fig. 8E) and neutrophils recruited to the PC (data not shown). When neutrophils from the PC were stimulated with T-sMs, both spontaneous degranulation and induced degranulation of neutrophils from pG/p6 mice and tumor-bearing mice were significantly decreased (Fig. 8F). In vivo expression of IFN-β recovered spontaneous degranulation of neutrophils, but failed to fully recover the induced degranulation (Fig. 8F) because the activation of PI3K and p38 MAPK pathways were still inefficient (data not shown). Moreover, IFN-β attenuated or abrogated the modulatory effect of G-CSF/IL-6 and tumor on the expression of Mmp9, Bv8, and Trail genes (Fig. 8G), as well as abrogating the tumor-promoting potential of BM neutrophils (Fig. 8H).

Discussion
Our data in this study showed that IL-6 cooperated with G-CSF to induce protumor function of neutrophils before they entered the tumor milieu. Although the function of neutrophils could be modulated by a variety of cytokines and chemokines, G-CSF and IL-6 might play key roles in inducing the protumor function of neutrophils in the presence of tumor. The modulation of neutrophils by G-CSF and IL-6 not only altered the expression of genes related to antitumor and protumor functions of neutrophils but also attenuated neutrophil degranulation, enabling neutrophils to promote tumor growth and tumor angiogenesis.
Angiogenesis is a key event in tumor growth and progression. Neutrophils have been found to exert a dual influence on blood vessel formation by producing proangiogenic or antiangiogenic factors. Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis (43). However, VEGF secreted from cells is sequestered to the extracellular matrix (3). Neutrophil-derived MMP-9, which is essential for blood vessel formation (4), promotes angiogenesis by remodeling extracellular matrix and releasing sequestered VEGF, and has been functionally implicated in VEGF activation (3, 13). Depletion of neutrophils could significantly suppress VEGF:VEGF receptor association (13). Neutrophils, in contrast to other cell types, do not express tissue inhibitors of metalloproteinases (TIMPs), and therefore release TIMP-free MMP-9 (3, 36). Thus, neutrophil-derived TIMP-free MMP-9 is rapidly and freely available to immediately release sequestered VEGF and remodel the ECM to promote angiogenesis (3). In contrast, Bv8 produced by neutrophils also promotes tumor angiogenesis, which is independent of the effect of VEGF (18). On the antiangiogenic side, neutrophils could adhere to the vascular endothelium, causing endothelial damage by releasing proteolytic enzymes and producing oxidants (2), the representatives of which are NE and HOCl (4). Neutrophils may also suppress angiogenesis by producing TRAIL, which could induce vascular disruption and inhibit the production of VEGF from tumor cells (10, 11). Therefore, the attenuated release of NE, MPO, and TRAIL is in favor of a proangiogenic effect of neutrophils. Our data showed that G-CSF and IL-6 could alter the function of neutrophils by modulating the production or release of these factors. Naïve neutrophils could suppress tumor angiogenesis, whereas G-CSF/IL-6–modulated neutrophils had the opposite effect. In line with this, IL-6 cooperated with G-CSF to increase the expression of Mmp9 and Bv8 genes, decrease the expression of Trail gene in neutrophils, and attenuate the release of MPO and NE from neutrophils. The cooperation of G-CSF with IL-6 was required for modulating neutrophils to promote tumor angiogenesis. The requirement for such cooperation is demonstrated via the coexpression of G-CSF and IL-6 in vivo in the absence of tumor. Therefore, the finding in this study does not contradict the previously reported proangiogenic effect of G-CSF in the presence of tumor, because the presence of tumor results in the production of IL-6 in vivo.

Efficient release of TRAIL, MPO, NE, and defensins is important for the antitumor effect of neutrophils. Inefficient release of these factors may result in the conversion of neutrophil function. At high concentrations, these factors are cytotoxic to tumor cells (2, 4, 44). Reducing their release may attenuate or even abrogate the antitumor function of neutrophils. More importantly, NE and defensins at low concentration could promote the proliferation of tumor cells (4, 44). As complex stimuli in the tumor milieu, T-sMs could stimulate naïve neutrophils to release azurophilic granules, which contain TRAIL, MPO, NE, and defensins (2, 37, 45). However, G-CSF and IL-6 could downregulate Trail gene expression and decrease the exocytosis of neutrophil azurophilic granules. The inefficient degradation was partially attributed to the inefficient activation of PI3K and p38 MAPK pathways. More importantly, the augmented STAT3 activation resulted in downregulation of Rab27a in neutrophils, which even decreased the spontaneous release of azurophilic granules from neutrophils. Therefore, enhancing STAT3 activation and attenuating activation of PI3K and p38 MAPK pathways are important mechanisms through which G-CSF and IL-6 suppress the release of TRAIL, MPO, NE, and defensins from neutrophils.

The activation of STAT3 is important for the modulation of neutrophil function (23, 38). Although both G-CSF and IL-6 could activate STAT3 in neutrophils, our data showed that G-CSF was a stronger inducer for STAT3 activation in BM neutrophils, whereas IL-6 was a relatively weaker one. However, IL-6 could enhance the activation of STAT3 in neutrophils by functioning as a...
costimulator and increasing STAT3 expression. SOCS3 is a feedback inhibitor that is induced by STAT3 and in turn attenuates IL-6R– and G-CSFR–mediated STAT3 activation (46, 47). Co-stimulation with G-CSF and IL-6 resulted in higher levels of phosphorylated STAT3 and Socs3 mRNA in neutrophils, suggesting that simultaneous activation of G-CSFR and IL-6R was more efficient in activating STAT3, resulting in the equilibrium of STAT3 activation and Socs3 expression at higher levels at steady state.

FIGURE 8. IL-6 increases STAT3 expression in neutrophils by downregulating IFN-β expression in BM. (A) Neutrophils isolated from BM of naive mice were stimulated with G-CSF and/or IL-6 for 12 h. The expression of Stat3 gene in neutrophils was detected by real-time RT-PCR and Western blot. The relative levels of total STAT3 protein to β-actin protein (STAT3/β-actin) were calculated after densitometric analysis of Western blots. (B) BM cells from naive mice, pG6 mice, pG mice, pG/pI6 mice, or tumor-bearing mice were incubated at 37°C for 48 h, and IFN-β in supernatants was detected by ELISA. Alternatively, the cells were incubated at 37°C for 12 h. The expression of Ifnb1 was detected by real-time RT-PCR. Alternatively, the cells were further cultured for 48 h under the same conditions, after replacement of the culture medium with fresh medium. Then, IFN-β in supernatants was detected by ELISA. (D) The pG/pI6 mice and tumor-bearing mice were untreated or treated by i.v. injection of pIFN-β or p3.1 plasmid, as described in Materials and Methods. Neutrophils were isolated from BM of the mice or BM of naive mice. The expression of the Stat3 gene in neutrophils was detected by real-time RT-PCR (left) and Western blot (middle). The phosphorylation of STAT3 was detected by Western blot (right). The relative levels of Rab27α in neutrophils was detected by real-time RT-PCR (left) and Western blot (right). The relative level of Rab27α to β-actin was calculated after densitometric analysis of Western blots. (F) Mice were treated as described in (D). Neutrophils were isolated from the PC of the mice after recruitment by CXCL1, or from N-PC. The release of MPO from neutrophils was detected after stimulation with T-sMs, as described in Materials and Methods. (H) Neutrophils were isolated from BM of mice, as described in (D), and used in the coinoculation test as outlined in Materials and Methods. The mice without neutrophil injection (none) were also used as control. Tumors (n = 8 per group) were dissected and weighed on day 10 after tumor inoculation. Data are pooled from three independent experiments with a total of six samples in each group (A–G). *p < 0.05, **p < 0.01.
of others (14). Lower expression of STAT3 in neutrophils could be maintained by IFN-β produced in BM in which F4/80+ monocytes/macrophages are the main source of Ifnb1 mRNA (14, 42). Our data in this study showed that IL-6 could suppress the expression of Ifnb1 in BM F4/80+ cells by activating STAT3. Consistent with this result, our previous study showed that the phosphorylation level of STAT3 in BM F4/80+ cells was increased in tumor-bearing mice (48). Our result is also supported by a recent report that IL-6 could inhibit the expression of Ifnb1 in DENV-ADE–infected macrophages (49). Downregulation of IFN-β expression was crucial for the increase of STAT3 expression in BM neutrophils in the presence of tumor or after in vivo expression of G-CSF and IL-6. Therefore, a higher phosphorylation level of STAT3 in neutrophils could be induced by G-CSF and IL-6 owing to the stronger activating effect of G-CSF/IL-6 and the enhancing effect of IL-6.

It has been demonstrated that STAT3 has a negative effect on the cytotoxicity of neutrophils to tumor cells (50), and that increased expression and activation of STAT3 are involved in the proangiogenic potential of neutrophils (14). Our data in this present study further showed that the augmented activation of STAT3 could modulate the expression and/or release of the factors related to the antitumor and protumor function of neutrophils. STAT3 has been found to positively or negatively regulate the expression of different genes (14, 51). Consistently, our data showed that G-CSF and IL-6–activated STAT3 to upregulate the expression of Mmp9 and Bv8 genes and downregulate the expression of Trail and Rab27a genes in neutrophils. Intriguingly, none of them alone could downregulate the expression of Trail and Rab27a genes, indicating that a higher activity of STAT3 was necessary for downregulating the expression of Trail and Rab27a. As a relatively weaker inducer, IL-6 alone could not efficiently induce STAT3 activation to influence the expression of Trail and Rab27a. G-CSF was more efficient in inducing STAT3 activation.

However, the effect of STAT3 activated by G-CSF alone was antagonized by the PI3K pathway, because G-CSF could reduce the expression of Trail if the PI3K pathway was inhibited. In addition to phosphorylation at Tyr705 by JAK, STAT3 can act as a scaffold for the kinases TAK1 and NLK following binding to gp130, and promote its phosphorylation at Ser727, which is also important for the transcriptional activity of STAT3 (52). The PI3K pathway has a negative effect on the serine phosphorylation of STAT3 (53). When neutrophils were stimulated with G-CSF and IL-6, the effect of the PI3K pathway might be overwhelmed by the augmented activation of STAT3. Moreover, when neutrophils were stimulated by T-sMs, inefficient activation of the PI3K pathway owing to modulation by G-CSF might also increase the regulatory effect of STAT3 on gene expression in neutrophils. Moreover, it has also been found that IFN-β could downregulate the expression of c-myc in BM neutrophils, and that c-Myc could cooperate with STAT3 to promote the proangiogenic function of neutrophils (14). Downregulation of IFN-β by IL-6 may also promote c-Myc expression in neutrophils, favoring the proangiogenic function of neutrophils.

In summary, in this study we demonstrated that G-CSF and IL-6 cooperated to augment the protumor function of neutrophils. The cooperation of G-CSF and IL-6 involved not only their simultaneous stimulation but also their different influence on neutrophils, such as increasing STAT3 expression and attenuating the activation of the PI3K and p38 MAPK pathways. Because of the altered activation of these signaling pathways, the complex stimuli in the tumor milieu could stimulate neutrophils to promote tumor growth. Given that increased serum levels of G-CSF and IL-6 have been found in cancer patients, antagonizing their modulatory effect on neutrophils might be very important for tumor therapy. Our data showed that IFN-β could antagonize the effect of G-CSF and IL-6 by downregulating STAT3 expression. Further identifying the factor(s) that could augment the activation of the PI3K and p38 MAPK pathways in G-CSF/IL-6–modulated neutrophils might lead to a new strategy of tumor therapy by fully recovering the antitumor function of neutrophils.

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Disclosures

The authors have no financial conflicts of interest.

References
