Clinical Cancer Research

Interleukin-17–Producing Neutrophils Link Inflammatory Stimuli to Disease Progression by Promoting Angiogenesis in Gastric Cancer

Tuan-Jie Li[1,2], Yu-Ming Jiang[1], Yan-Feng Hu[1], Lei Huang[3], Jiang Yu[1], Li-Ying Zhao[1], Hai-Jun Deng[1], Ting-Yu Mou[1], Hao Liu[1], Yang Yang[2,4], Qi Zhang[2,5], and Guo-Xin Li[1]

Abstract

Purpose: Elevated levels of neutrophils have been associated with poor survival in various cancers, but direct evidence supporting a role for neutrophils in the immunopathogenesis of human cancers is lacking.

Experimental Design: A total of 573 patients with gastric cancer were enrolled in this study. Immunohistochemistry and real-time PCR were performed to analyze the distribution and clinical relevance of neutrophils in different microanatomic regions. The regulation and function of neutrophils were assessed both *in vitro* and *in vivo*.

Results: Increased neutrophil counts in the peripheral blood were associated with poor prognosis in gastric cancer patients. In gastric cancer tissues, neutrophils were enriched predominantly in the invasive margin, and neutrophil levels were a powerful predictor of poor survival in patients with gastric cancer. IL17$^+$ neutrophils constitute a large portion of IL17-producing cells in human gastric cancer. Proinflammatory IL17 is a critical mediator of the recruitment of neutrophils into the invasive margin by CXC chemokines. Moreover, neutrophils at the invasive margin were a major source of matrix metalloproteinase-9, a secreted protein that stimulates proangiogenic activity in gastric cancer cells. Accordingly, high levels of infiltrated neutrophils at the invasive margin were positively correlated with angiogenesis progression in patients with gastric cancer.

Conclusions: These data provide direct evidence supporting the pivotal role of neutrophils in gastric cancer progression and reveal a novel immune escape mechanism involving fine-tuned collaborative action between cancer cells and immune cells in the distinct tumor microenvironment. *Clin Cancer Res; 23(6); 1575–85. ©2016 AACR.*

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

Introduction

Tumor progression is the product of evolving crosstalk between malignant cells and various stromal and immune cell subsets of the surrounding microenvironment (1). Inflammatory cells and mediators are key components of the tumor microenvironment. Proinflammatory IL17 cytokines were initially noted for their pathogenic role in chronic inflammatory diseases, and subsequent accumulating evidence has indicated the involvement of neutrophils in carcinogenesis and angiogenesis. Clinical studies have suggested that elevated numbers of IL17-producing cells infiltrating in tumors are positively correlated with microvessel density (MVD) in tissues and are also an independent marker of adverse prognosis in patients with cancer (2–4). However, the molecular mechanisms by which IL17 fosters angiogenesis and promotes tumor progression in gastric cancer are poorly understood.

There is substantial evidence that infiltrating immune cells in the tumor are polarized toward a tumor-promoting phenotype by stimulating angiogenesis and tissue remodeling (5–7). Neutrophils are the predominant circulating granulocyte in humans and comprise 50% to 75% of circulating leukocytes. Increased levels of neutrophils have been observed in several types of human tumors. Studies of mouse and zebrafish cancer models have suggested that neutrophils in cancer biology can be pro- or antitumorigenic, depending on the tumor microenvironment (8–10). The basic mechanisms by which neutrophils promote the development of cancer are diverse. In addition to contact-dependent mechanisms, neutrophils secrete soluble factors that can activate endothelial and parenchymal cells, enhancing tumor cell adhesion in distant sites (11–14). Circulating tumor cells are trapped in neutrophil extracellular traps (NET) *in vitro*, and NET trapping is associated with increased formation of hepatic micrometastases and compromised organ function in murine models

[1]Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China. [2]Guangdong Provincial Key Laboratory of Liver Disease Research, Guangzhou, China. [3]German Cancer Research Center (DKFZ), Heidelberg, Germany. [4]Department of Hepatic Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. [5]Cell-gene Therapy Translational Medicine Research Center, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, China.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

T.-J. Li, Y.-M. Jiang, and Y.-F. Hu contributed equally to this article.

Corresponding Authors: Guo-Xin Li, Department of General Surgery, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510-515, China. Phone: 86-20-61641681; Fax: 86-20-61641683; E-mail: gzliguoxin@163.com; Qi Zhang, Cell-gene Therapy Translational Medicine Research Center, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. Phone: 86-20-85253106; Fax: 86-20-5252276; E-mail: keekee77@126.com; and Yang Yang, Department of Hepatic Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. Phone: 86-20-85252702; Fax: 86-20-85252276; E-mail: yysysu@163.com

doi: 10.1158/1078-0432.CCR-16-0617

©2016 American Association for Cancer Research.

Translational Relevance

Tumor-associated neutrophils contribute to tumor progression, invasion, and angiogenesis in many types of cancers. In this research, we found that accumulation of neutrophils in the peripheral blood and tumor invasive margin predicted a poor survival in patients with gastric cancer and that neutrophils secreted matrix metalloproteinase-9 to stimulate proangiogenic activity of gastric cancer cells. We also found that IL17 protein was primarily expressed by neutrophils in gastric cancer samples. IL17 stimulated gastric cancer cells to express CXC chemokines, and $IL17^+$ neutrophil polarization might be promoted by IL6 and IL23 *in vitro*. These data provide direct evidence supporting the pivotal role of $IL17^+$ neutrophils in promoting angiogenesis during gastric cancer progression and reveal that selective modulation of the functional activities of $IL17^+$ neutrophils may provide a novel strategy for anticancer therapy.

(15, 16). These novel aspects of neutrophil biology may contribute to cancer progression and metastasis. However, direct evidence supporting a role of neutrophils in the immunopathogenesis of human cancers is lacking.

Neutrophils are a source of IL17 in human psoriatic lesions and in several mouse models of infectious and autoimmune inflammation (17, 18). Elevated IL17 expression has also been observed in patients with corneal ulcers caused by filamentous fungi; neutrophils are the predominant infiltrating cells in these ulcers (19–21). Although IL17 has been detected in several human tumors (22), its cellular sources remain unclear. Gastric cancer is the fourth most common human malignant disease and the second leading cause of cancer-related death worldwide (23). In the present study, gastric cancer was used as a model system. Both clinical sample analysis and experimental studies suggested that IL17 is predominantly expressed by neutrophils and that IL17 recruits neutrophils into the invasive margin by inducing CXC chemokine production in gastric cancer cells. Furthermore, tumor-associated macrophages (TAM) may promote $IL17^+$ neutrophils via the secretion of IL6 and IL23. Accumulated neutrophils in the invasive margin are the major source of matrix metalloproteinase (MMP)-9, which may in turn stimulate the proangiogenic activity of gastric cancer cells at the adjacent invading edge. Our research provides direct evidence for the important role of neutrophils in the immunopathogenesis of human cancers by rerouting inflammation in a protumoral direction.

Materials and Methods

Patients and specimens

A total of 573 patients at Nanfang Hospital of Southern Medical University (Guangzhou, China) with pathologically confirmed gastric cancer were enrolled in this research. None of the patients received anticancer therapy before sampling. Individuals with concurrent autoimmune disease, HIV, or syphilis were excluded. Blood and paired intratumoral and nontumoral (at least 3 cm from the tumor site) tissues from patients (Group 1) who received therapy in 2014 were processed for the preparation of tumor and nontumor tissue conditioned medium ($n = 3$), and samples from another 16 patients were used for real-time PCR or immunofluorescence. Paraffin-embedded and formalin-fixed samples were obtained from 245 patients (Group 2) who underwent curative resection between 2005 and 2007 and had complete follow-up data, and these patients were further enrolled for analyses of progression-free survival (PFS) and overall survival (OS). We defined PFS as the time from the date of diagnosis to the date of confirmed tumor progression. OS was defined as the interval between the date of diagnosis and the date of death from any cause. Regular blood cell counts were obtained from 554 patients (Group 3) who underwent curative resection between 2005 and 2009 and had complete follow-up data. Clinical stages were classified according to the guidelines of the International Union against Cancer (AJCC, 7th edition). The clinical characteristics of all patients are summarized in Supplementary Table S1. All samples were coded for anonymity in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki). Written informed consent was obtained from all patients, and the protocol was approved by the Review Board of Southern Medical University.

Immunohistochemistry and immunofluorescence

Paraffin-embedded and formalin-fixed samples were cut into 4-μm sections, which were then processed for immunohistochemistry as previously described. Following incubation with an antibody against human CD66b (BD, Pharmingen), IL17 (R&D Systems), MMP-9 (Thermo Fisher), or CD34 (Abcam), the sections were stained using an EnVision System (Dako Cytomation). At low power (200×), the tissue sections were screened using an inverted research microscope (Leica DM IRB), and the 5 most representative fields were selected. This analysis was performed by 2 independent observers who were blinded to the clinical outcome. For immunofluorescence analysis, tissues were stained with antibodies against CD66b and MMP9 or CD66b and IL17, followed by Alexa Fluor 555–conjugated anti-mouse IgG and Alexa Fluor 488–conjugated anti-rabbit IgG (Molecular Probes). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were viewed and assessed using a fluorescence microscope (Leica DMI 4000B) and analyzed using Leica Application Suite software (version 4.0).

Blood sample analyses

Peripheral blood was obtained at the time of diagnosis prior to surgery. The numbers of neutrophils were determined with a hemocytometer. The percentages of specific types of cells were determined using a Hematology Analyzer (Sysmex). Absolute counts of specific cells were calculated by multiplying the percentage of specific cells by the total number of white blood cells.

Tumor cell lines and preparation of tumor cell line culture supernatants

The human gastric cancer cell lines SGC7901 and BGC-823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These 2 cell lines have not been tested and authenticated. The AGS cell line was obtained from the ATCC (CRL-1739) and was tested and authenticated in November 10, 2015. AGS cells gDNA was extracted with Axygen extraction kit, after amplification with 20-STR amplification protocols. STR gene loci and gender Amelogenin were tested using

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

ABI 3730XL Genetic Analyzer. All cell types were tested for mycoplasma contamination using a single-step PCR method and maintained in complete medium composed of DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (Gibco). Tumor culture supernatants were prepared by plating 5×10^6 tumor cells in 10 mL of complete medium in 100-mm dishes, and after 48 hours, the supernatants were harvested, centrifuged, and stored in aliquots at −80°C.

Neutrophil isolation and culture

Human neutrophils were isolated from the peripheral blood of healthy donors. Density-gradient separation on Polymorphprep was performed at 500 × *g* for 35 minutes at room temperature. The pale-red granulocyte layer was washed, and the contaminated erythrocytes were lysed by brief hypotonic lysis. Neutrophils with more than 96% purity were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were then cultured for the indicated times in medium alone or with 20% culture supernatants from gastric cancer cells. The culture supernatants were collected, and apoptosis of the cells was assessed by staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (Life Technologies). In some experiments, the neutrophils were cultured for 24 hours in serum-free medium, and the culture supernatants were collected for tube formation assays.

In vitro activation of human neutrophils

Human neutrophils were suspended at a density of 2.5×10^6 cells per mL and were incubated at 37°C in 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated FCS or recombinant IL6 and IL23 as the indicated concentration.

Real-time PCR

Gastric cancer cells were left untreated or were exposed for the indicated times to 40 ng/mL IL17 or to 10% neutrophil culture supernatant in triplicate. The total RNA of gastric cancer cells and total RNA of nontumoral, invasive margin, and tumor center tissues from patients with gastric cancer were isolated using TRIzol reagent. Aliquots containing 2 μg of total RNA were reverse-transcribed using MMLV reverse transcriptase. The specific primers used to amplify the genes are listed in Supplementary Table S2. PCR was performed in triplicate using SYBR Green real-time PCR master mix (Roche) in a Roche LightCycler 480 System. All results are presented as arbitrary units relative to GAPDH rRNA expression.

Tissue culture and preparation of tumor tissue and nontumor tissue culture supernatants

Gastric tissues were obtained from 3 independent cases of pathologically confirmed gastric cancer by gastroscopy. The tumor tissues were selected from minimally necrotic regions of the tumor mass. Tumor tissue culture supernatants (TTCS) or nontumor tissue culture supernatants (NTCS) were prepared as previously described (4, 24). Briefly, the tissues were cut into small pieces and digested with collagenase type IV (1 mg/mL; Sigma) and hyaluronidase (125 units/mL; Sigma) at 37°C with agitation for 1 hour in DMEM with 10% heat-inactivated FBS. The dissociated tissues were centrifuged, resuspended in DMEM with 10% FBS, and cultured in one 25-cm^2 flask per 1 g of tissue. After a 48-hour culture period, the supernatants were harvested, centrifuged, and stored.

Detection of MMP-9 activity by gelatin zymography

MMP-9 activity was detected by gelatin zymography as previously described (3). The conditioned medium from neutrophils, tumor cells, normal liver cells, T cells, or macrophages was collected, centrifuged at 10,000 × *g* for 10 minutes to remove cell debris, and stored in aliquots at −80°C. To assay gelatinolytic activity, aliquots of medium were mixed with 4× nonreducing sample buffer and loaded in a 10% acrylamide gel containing 1 mg/mL gelatin. After electrophoresis, the gel was soaked in 2.5% Triton X-100 to remove SDS, incubated at 37°C overnight in development buffer, and stained with Coomassie brilliant blue R-250.

ELISA

The concentrations of IL6 and IL23 (Enzo Life Sciences) in the monocyte medium were determined using ELISA kits according to the manufacturer's instructions.

Angiogenic tube formation assays

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in serum-free medium for endothelial cells (SFM; Invitrogen). The tube formation assay was performed using HUVECs at passages 2 to 7, as described (25), in the presence of serum-free conditioned medium from neutrophils, AGS cells, or AGS cells exposed to neutrophil-conditioned medium.

Chick embryo chorioallantoic membrane assay

White leghorn chicken eggs were purchased from Guangdong Research Institute of Animal Science on the fifth day after fertilization. The air chambers of these eggs were windowed after disinfection, and 100 μL of conditioned medium for each egg was pipetted onto the chorioallantoic membrane (CAM) after the shell membranes were removed. The windows were sealed with sterile ventilated tapes, and the eggs were incubated at 80% relative humidity and 37.5°C in the incubator. After 2 days, the tapes were removed, and the results were observed under a stereomicroscope (ZEISS Lumar.V12).

External validation cohort survival analysis

An online database was used to assess the relevance of IL17A, VEGF, and CD34 mRNA expression to OS and PFS. Kaplan–Meier curves were generated with the KM plot software using a database of public microarray datasets (http://kmplot.com/analysis). The database was established using gene expression data and survival information of 882 patients with gastric cancer downloaded from the Gene Expression Omnibus (GEO). Kaplan–Meier plots were generated after averaging the probes. Patients were divided according to the median expression value. The number at risk is indicated below the main plot, and HR (and 95% CIs) and log-rank *P* values were calculated and are displayed on the webpage.

Statistical analysis

The results are expressed as the mean ± SEM. The statistical significance of differences between groups was determined using the Student *t* test. Correlations between parameters were assessed using Pearson correlation analysis and linear regression analysis as appropriate. Cumulative survival time was calculated using the

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

Kaplan–Meier method, and survival was measured in months from resection to recurrence or the last review. The log-rank test was applied to compare groups. Multivariate analyses of prognostic factors for OS and PFS were performed using the Cox proportional hazards model. SPSS statistical software (version 21.0) was used for all statistical analyses. All data were analyzed using 2-tailed tests unless otherwise specified, and $P < 0.05$ was considered statistically significant.

Results

Accumulation of neutrophils in the peripheral blood and the invasive margin promotes disease progression and predicts poor survival in patients with gastric cancer

To determine the functional significance of neutrophils in tumor immunopathology, we first investigated the prognostic role of increasing neutrophil counts in the peripheral blood. A total of 554 patients with gastric cancer, who had received curative resection with complete follow-up data, were divided into 2 groups according to the median neutrophil count in peripheral blood. As shown in Fig. 1A, patients with high neutrophil counts exhibited worse OS and PFS than patients with low neutrophil levels at diagnosis. This result suggests that elevated neutrophil counts are positively correlated with gastric cancer progression. Next, we investigated neutrophil infiltration and distribution in human gastric cancer tissues. Neutrophils were visualized by immunohistochemical staining of CD66b in paraffin-embedded tissues from 245 untreated patients with gastric cancer. As shown in Fig. 1B, neutrophils were present throughout the tissue but predominated in the invasive margin rather than in the tumor center (101.1 ± 5.3 and 66.7 ± 4.6 cells/field, respectively; $n = 245$ for both; $P < 0.0001$; Fig. 1C). The number of $CD66b^+$ cells in the invasive margin was significantly increased and correlated with disease progression in patients with gastric cancer [stage I–II ($n = 79$) vs. stages III–IV ($n = 166$); $P = 0.0002$; Fig. 1C].

On the basis of the above observations, we predicted that the presence of invasive margin $CD66b^+$ cells would have an adverse effect on survival. To test this assumption, we divided the 245 patients into 2 groups according to the median CD66b density in different areas. There was a striking inverse association between $CD66b^+$ cell density in the invasive margin and both OS and PFS ($P < 0.001$ for both; Fig. 1D). The invasive margin $CD66b^+$ cell density was also associated with lymph node metastasis ($P = 0.037$) and tumor–node–metastasis (TNM) stage ($P = 0.001$; Supplementary Table S3). Univariate and multivariate analyses indicated that the number of $CD66b^+$ cells in the invasive margin was an independent prognostic factor for both OS and PFS (Supplementary Table S4).

To further evaluate the prognostic role of invasive margin $CD66b^+$ cells in different subgroups, patients were stratified according to tumor TNM stage. As expected, a high density of invasive margin $CD66b^+$ cells remained predictive of worse survival in different TNM stages (Supplementary Fig. S1 and Supplementary Table S3) and thus represents a powerful prognostic factor for patients with gastric cancer in different risk groups.

IL17 protein is primarily expressed by neutrophils in gastric cancer

Neutrophils at the site of inflammation not only exert microbicidal effects but also contribute to angiogenesis and tissue remodeling (5). The role of IL17 in gastric cancer remains controversial. Kaplan–Meier analyses indicated that high IL17 expression in patients with gastric cancer is correlated with poor prognosis, PFS, and OS (Supplementary Fig. S1), indicating that IL17 in gastric cancer may promote cancer progression. Although IL17 production by T cells has been widely studied, it is increasingly clear that diverse types of innate immune cells also produce IL17 (26). $IL17^+$ neutrophils have been observed in many types of cancers, but the correlation between $IL17^+$ cells and neutrophils in gastric cancer is unknown. Therefore, we assessed the association between $CD66b^+$ cells and $IL17^+$ cells in human gastric cancer, with a specific focus on the tissue microlocalization of the cells. Both $CD66b^+$ cells and $IL17^+$ cells accumulated in the peritumoral stroma (Fig. 2A), and a significant correlation between levels of $CD66b^+$ cells and $IL17^+$ cells was observed in the same area ($R^2 = 0.555$, $P < 0.001$; Fig. 2B). Notably, using confocal microscopy, we confirmed that most ($63.4 \pm 9.1\%$; $n = 10$) of the IL17 protein was expressed by $CD66b^+$ neutrophils in the tumor stroma (Fig. 2C). Taken together, these observations suggest that IL17 is primarily expressed by neutrophils in gastric cancer.

CXC chemokines that bind to and activate CXCR1 and/or CXCR2 generally mediate the migration of neutrophils across the tumor vasculature (27). We hypothesized that IL17 signals are involved in the accumulation of neutrophils in gastric cancer, and we examined the chemokine profiles of IL17-treated gastric cancer cells by real-time PCR. As shown in Fig. 2D, exposure of gastric cancer cells to IL17 markedly upregulated the expression of several CXC chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, and CXCL11. In contrast, IL17 did not stimulate CC chemokine expression in gastric cancer cells (Fig. 2D). Consistent with these *in vitro* data, significantly increased expression of several of these chemokines, including CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8, was detected in the invasive margin compared with the tumor center in patients with gastric cancer (Fig. 2E). These data suggest that IL17 may promote the migration of neutrophils into gastric cancer tumors via gastric cancer cell–derived CXC chemokines.

$IL17^+$ neutrophil polarization may be promoted by TAM-derived IL6 and IL23

TAMs promote IL17 expression in lymphoid cells via IL6 and IL23. To determine whether these cytokines operate in the polarization of $IL17^+$ neutrophils in gastric cancer, we first quantified the cytokines in TAMs culture medium by ELISA. IL6 and IL23 were significantly upregulated compared with the medium of monocytes isolated from autologous nontumor tissues (Fig. 3A). Similar results were obtained for TTCS- and NTCS-conditioned blood monocyte culture supernatants (Fig. 3B). To further ascertain the role of these cytokines in the expression of IL17 by neutrophils, we isolated neutrophils and incubated them alone or with recombinant human IL6 and IL23 for different times and analyzed *il17a* expression by quantitative PCR. We detected Il17a in neutrophils stimulated with recombinant human IL6 and IL23. Stimulation with IL6 or IL23 alone was not sufficient to induce expression of *il17a* mRNA in human neutrophils, and IL6 and IL23 were necessary and sufficient for IL17a expression in those cells. Because RORγt mediates the production of IL17 by lymphoid cells, we further investigated whether the combination of recombinant human IL6 and IL23 induces expression of RORγt in neutrophils. As shown in Fig. 3E, we detected RORC expression by

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

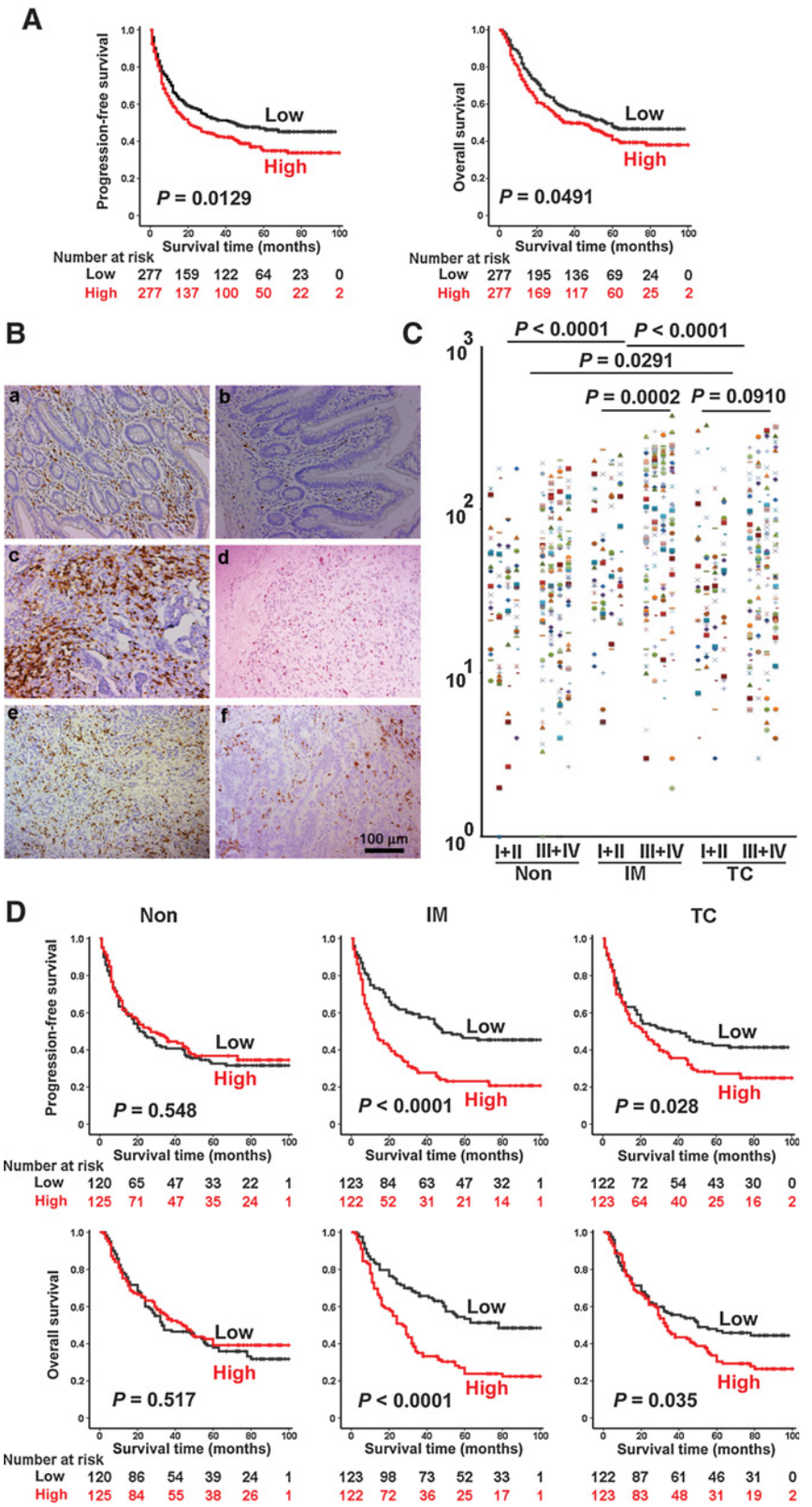

Figure 1.

Neutrophils promote disease progression and predict poor survival in patients with gastric cancer. **A,** Cumulative OS and PFS curves of patients. The patients were divided into 2 groups according to the median neutrophil count in peripheral blood. Paraffin-embedded gastric cancer samples ($n = 245$) were stained with an anti-CD66b antibody. **B** and **C,** Distribution of $CD66b^+$ cells in nontumoral (Non; a, b), invasive margin (IM; c, d), and tumor center (TC; e, f) tissues of gastric cancer samples. Left (a, c, e), high CD66b density; right (b, d, f), low CD66b density. **D,** Cumulative OS and PFS curves of patients. The patients were divided into 2 groups according to the median $CD66b^+$ neutrophil density.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

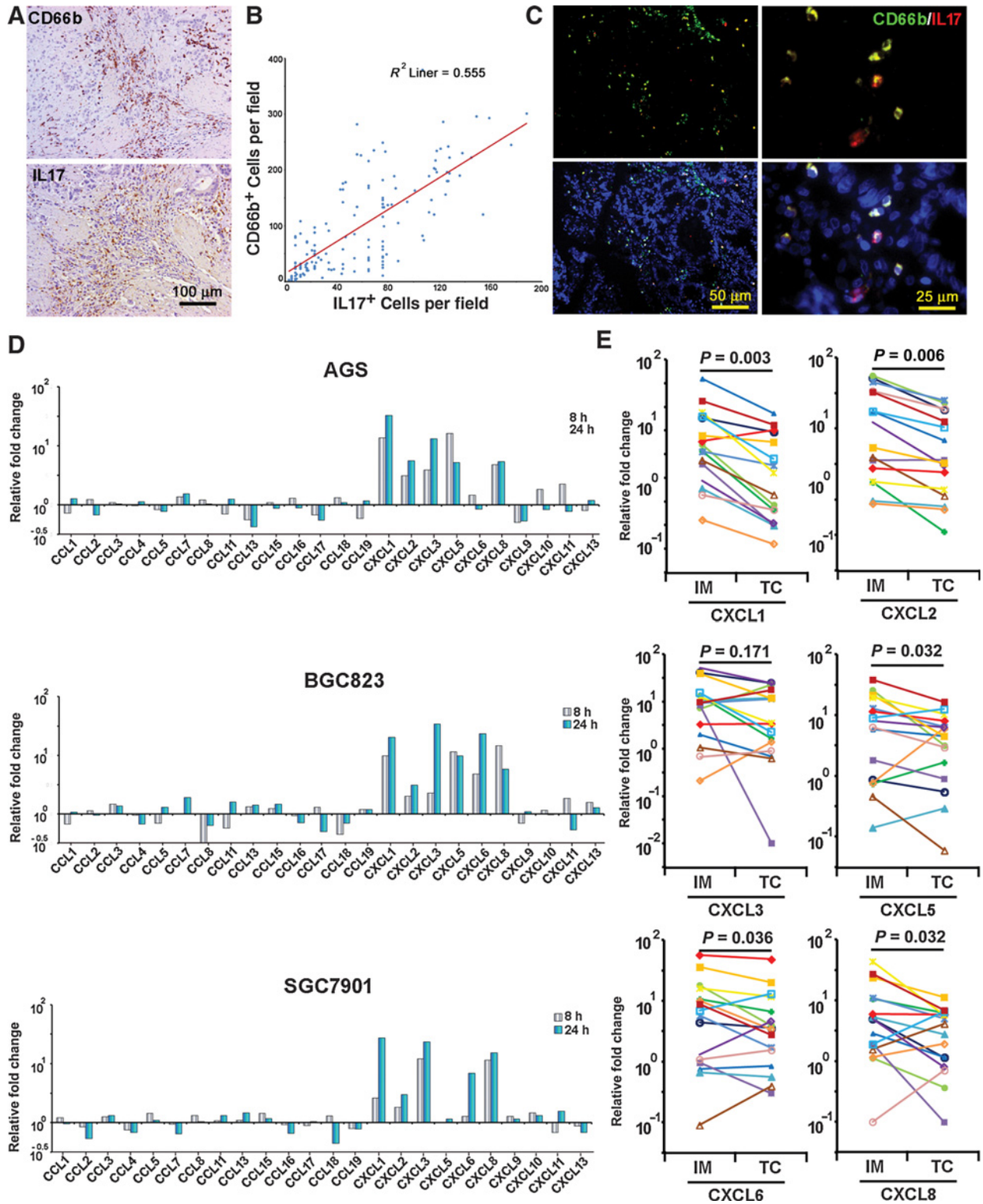

Figure 2.

Invasive margin IL17$^+$ cells in gastric cancer recruit neutrophils via epithelial-derived CXC chemokines. **A** and **B,** Association of CD66b$^+$ and IL17$^+$ cells in the invasive margin of HCC ($n = 245$). **C,** Analysis of IL17 and CD66b distribution in gastric cancer samples by confocal microscopy. One of 10 representative micrographs is shown. **D,** Fold changes of chemokine mRNA levels in IL17-treated gastric cancer cells compared with untreated gastric cancer cells, as analyzed by real-time PCR. A representative result from 3 separate experiments is shown. **E,** Fold changes in CXC chemokine mRNAs in gastric cancer invasive margin or tumor center tissues ($n = 16$) compared with paired nontumoral tissues were determined by real-time PCR. $P < 0.05$ was considered statistically significant.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

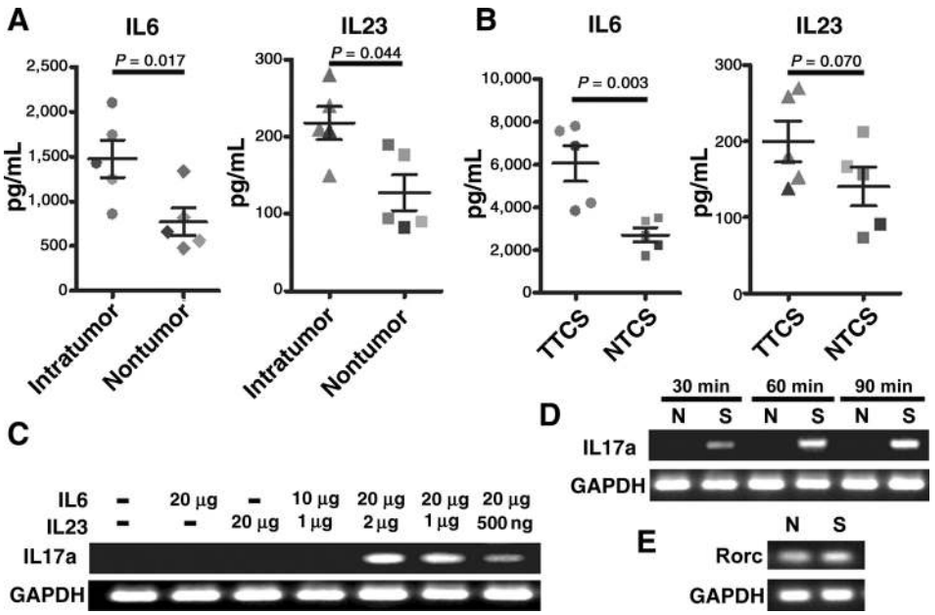

Figure 3.
Effects of IL6 and IL23 on IL17$^+$ neutrophil induction. **A,** Statistical analysis of concentrations of IL6 and IL23 in monocytes from tumor and nontumor culture supernatants as determined by ELISA ($n = 5$). **B,** statistical analysis of the concentrations of IL6 and IL23 in monocytes induced by TTCS and NTCS as determined by ELISA ($n = 5$). **C,** Neutrophils were stimulated for 1 hour with recombinant human (rh) IL6 and/or rhIL23 at the indicated concentrations per milliliter, and Il17a gene expression was detected by quantitative PCR. **D,** Il17a gene expression in neutrophils incubated for 30, 60, and 90 minutes with 20 μg/mL rhIL6 and 2 μg/mL rhIL23 and analyzed by quantitative PCR. **E,** RORC expression in nonstimulated neutrophils (N) or after incubation with rhIL6 and IL23.

quantitative PCR in unstimulated neutrophils or after incubation with recombinant human IL6 and IL23. These results indicate that IL6 and IL23 may also induce the production of IL17 by RORγt in neutrophils. Together, these findings suggest that IL6 and IL23 play an essential role in TAM-mediated IL17$^+$ neutrophil induction *in vitro* and suggest that a similar process might operate *in vivo*.

Peritumoral stromal neutrophils promote angiogenesis at the invasive margin via MMP-9 signaling

MMP-9 participates in tumor development by inducing an angiogenic switch (28, 29). To understand the mechanism by which neutrophils facilitate tumor angiogenesis, we initially examined the distribution of microvessels in gastric cancer tissues by staining for the vascular endothelial marker CD34. As shown in Fig. 4A, angiogenesis was most active at the invading edge, which was situated close to the invasive margin with abundant MMP-9$^+$/CD66b$^+$ cells (Figs. 1B and 4B). We next investigated the expression of MMP-9 in serial sections of human gastric cancer tissues. In all samples analyzed ($n = 245$), both CD66b$^+$ and MMP-9$^+$ cells were present throughout the tissue but were predominant in the peritumoral stroma surrounding the cancer nests (Fig. 4B). There was a positive association between the densities of CD66b$^+$ cells and MMP-9$^+$ cells in the invasive margin ($n = 245$; linear regression, $r = 0.792$; $P < 0.01$; Supplementary Fig. S2). We further confirmed by confocal microscopy that most (85% ± 7.1%; $n = 10$) of the MMP-9 protein was expressed by CD66b$^+$ neutrophils in the tumor (Fig. 4C). Moreover, when cultured with tumor supernatants, neutrophils secreted significantly higher levels of MMP-9 than gastric cancer cells, macrophages, or T cells (Fig. 4D). Exposure of neutrophils to 15% AGS-derived supernatant resulted in sustained release of MMP-9 that lasted for at least 48 hours (Fig. 4D) as well as inhibition of neutrophil apoptosis (Supplementary Fig. S3). Consistent with the findings in the tumor samples, these data suggest that neutrophils in the invasive margin are major sources of MMP-9 in gastric cancer tissues.

Tumor-educated neutrophils promote angiogenesis via MMP-9

Angiogenesis is a prerequisite for cancer development. To clarify the role of neutrophils in gastric cancer angiogenesis, we first performed *in vitro* angiogenic tube formation assays using HUVECs. Culture supernatants from neutrophils or untreated gastric cancer (AGS) cells had only a marginal effect on capillary-like structures. In contrast, culture supernatants from AGS cells that had been exposed to neutrophils significantly promoted angiogenic tube formation (Fig. 4E); this effect was attenuated by an anti-MMP-9 antibody (Fig. 4F). A similar effect was observed in the CAM animal model (Fig. 4G). The group of eggs ($n = 5$ in each group) treated with culture supernatants from AGS cells that had been exposed to neutrophils exhibited more marked signs of angiogenesis than the other 2 groups in the CAM assay. These data suggest that soluble factors derived from neutrophils, including MMP-9, play an important role in angiogenic tube formation. The potential role of MMP-9$^+$ neutrophils in tumor angiogenesis was further supported by real-time PCR analysis of the expression of angiogenesis-associated genes in the invasive margin of gastric cancer tissues. Compared with the tumor center, a marked upregulation of a set of proangiogenic genes was observed in the invasive margin of gastric cancer tissues with a greater accumulation of peritumoral neutrophils (Fig. 4H). Thirteen genes exhibited significant 2-fold upregulation (59.1% of 22 differentially expressed genes). Consistent with these results, exposure of AGS cells to neutrophils induced rapid upregulation of several proangiogenic genes, with an expression profile comparable to that of cells observed in the invading edges of gastric cancer tissues (Fig. 4I).

High infiltration of peritumoral stromal neutrophils is correlated with increased vasculature in gastric cancer

VEGF mediates signaling, and angiogenesis can contribute to the pathogenesis of many types of cancers (30). We hypothesized that VEGF plays a pivotal role in angiogenesis during gastric cancer development; therefore, we studied the roles of VEGF and CD34

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

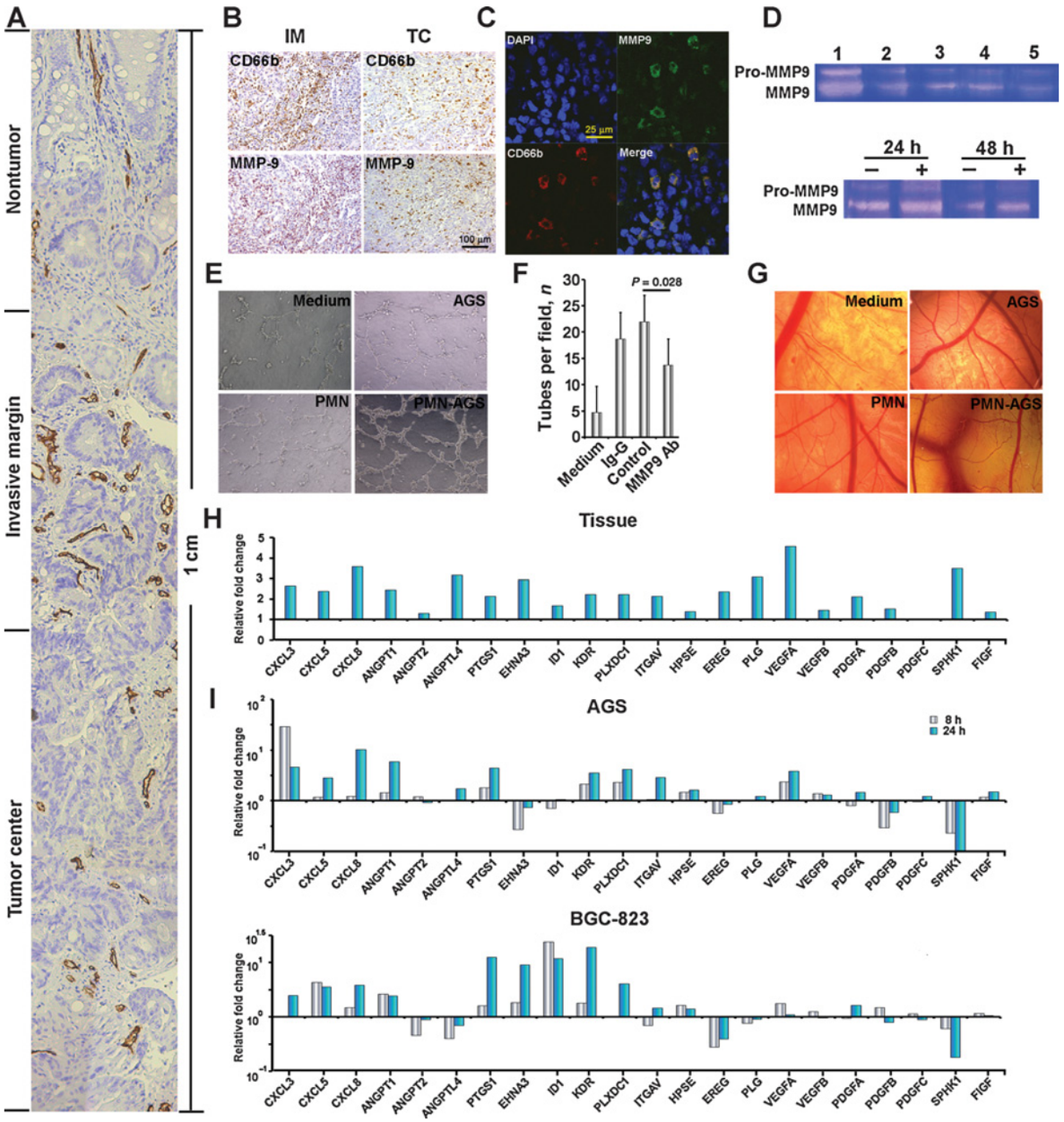

Figure 4.

Invasive margin neutrophils regulate tumor angiogenesis via MMP-9. **A,** Analysis of capillary distribution in gastric cancer samples by immunohistochemical staining for CD34. One of 10 representative micrographs is shown. **B,** Adjacent sections of paraffin-embedded gastric cancer stained with an anti-CD66b or an anti–MMP-9 antibody ($n = 245$). Different levels of neutrophil infiltration and MMP-9 protein can be observed in the invasive margin and tumor center. **C,** Analysis of MMP-9 distribution in gastric cancer samples by confocal microscopy. One of 10 representative micrographs is shown. **D,** Detection of MMP-9 activity by gelatin zymography. Top, 1×10^6 human neutrophils (1), macrophages (2), T cells (3), AGS cells (4), and BGC-823 cells (5) were pretreated for 12 hours with 30% supernatant from AGS cells and then washed and resuspended in 0.5 mL of serum-free M199 for 12 hours. Bottom, 1×10^6 neutrophils were untreated or exposed to 30% supernatant from AGS cells for the indicated times and then washed and resuspended in 0.5 mL of serum-free M199 for 12 hours. Then, the culture supernatants were harvested for gelatin zymography. The results represent 3 separate experiments. **E,** Soluble factors derived from neutrophil-treated tumor cells induce angiogenic tube formation. The tube formation assay was performed using HUVECs in the absence (medium) or presence of serum-free conditioned medium from neutrophils, AGS cells, or AGS cells exposed to neutrophil culture supernatant. The results represent 3 separate experiments. **F,** Anti-MMP9 antibody inhibits tube formation by HUVECs. The tube formation assay was performed using HUVECs in the absence (medium) or presence of serum-free conditioned medium from AGS cells exposed to neutrophil culture supernatant alone (control) or supplemented with an MMP-9 blocking antibody or a control antibody (isotype). The results represent the mean ± SEM of 3 separate experiments. **G,** PMNs promoting angiogenesis were observed in the CAM animal model. **H,** Fold changes of angiogenesis-related mRNA levels in gastric cancer invasive margin tissues with high infiltration of peritumoral neutrophils ($n = 16$) compared with those of tumor center tissues with no or low infiltration of neutrophils as determined by real-time PCR. The data with significant differences are shown. **I,** Fold changes of angiogenesis-related mRNAs in gastric cancer cells exposed to neutrophil culture supernatant for the indicated times compared with those of untreated gastric cancer cells as determined by real-time PCR. The results represent 3 separate experiments. PMN, polymorphonuclear neutrophils.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

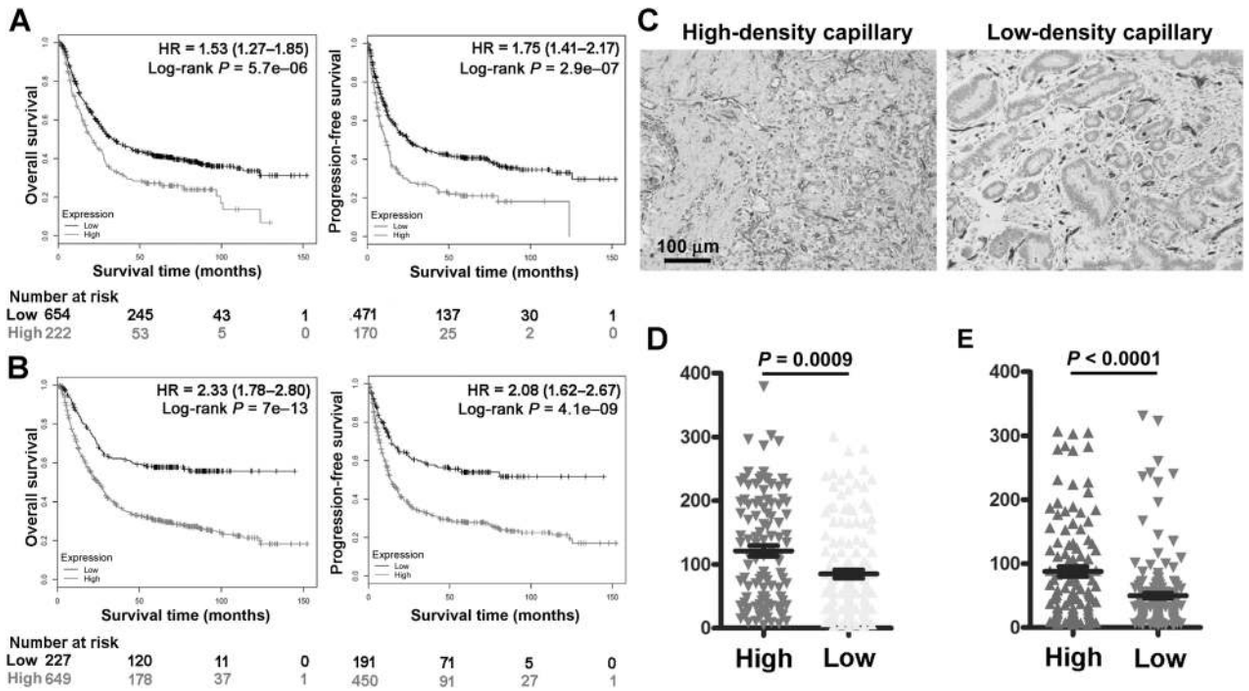

Figure 5.

The accumulation of peritumoral stromal neutrophils coincides with increased MVD at the invasive margin of gastric cancer. **A** and **B,** Kaplan–Meier analysis of OS and PFS stratified by VEGF (**A**) and CD34 (**B**) expression in gastric cancer. **C,** Paraffin-embedded gastric cancer samples stained with an anti-CD34 antibody. Patients were divided into 2 groups on the basis of the MVD in the invasive margin of gastric cancer. **D** and **E,** Association between MVD and CD66b$^+$ cell densities in the invasive margin and tumor center.

in gastric cancer survival by Kaplan–Meier analysis. As shown in Fig. 5A and B, high levels of VEGF and CD34 in patients with gastric cancer indicated poor prognosis, consistent with previous reports (30). The above observations suggested that neutrophils might act to reprogram the microvessels involved in mediating angiogenesis. To test this hypothesis, we classified the 245 patients into 2 groups according to MVD in the invasive margin (Fig. 5C). Patients with high MVDs at the invading edge had more invasive margin neutrophils than those with low MVDs ($n = 245$; $P = 0.0009$; Fig. 5D), and similar results were observed in the tumor center ($n = 245$; $P < 0.0001$; Fig. 5E).

Discussion

Despite the generally immunosuppressed status of patients with cancer, substantial evidence indicates that inflammatory reactions at the tumor site can promote disease progression (31, 32). Neutrophils can produce IL17, and it has been suggested that IL17 participates in adaptive immunity and promotes tumor progression by fostering angiogenesis in many types of tumors (3, 33). Our investigation suggests that high levels of IL17$^+$ neutrophils are present in gastric cancer tissues and that IL17 induces the migration of neutrophils into gastric cancer via cancer cell–derived CXC chemokines. Further investigation revealed that neutrophils stimulate the proangiogenic activity of tumor cells both *in vitro* and *in vivo*. These findings imply that neutrophils in tumor tissue, particularly those producing IL17, do not contribute to the host defense against the malignancy but instead are rerouted in a tumor-promoting direction by inducing angiogenesis. This conclusion is supported by our observations that CD66b$^+$ cells in gastric cancer tumor tissue but not nontumoral gastric tissue are positively associated with lymph node metastasis and TNM stage.

Little is known regarding the mechanisms underlying the polarization of IL17$^+$ neutrophils and their roles in gastric cancer progression. We observed that neutrophils were present predominantly in the invasive margin. In addition, IL17 was secreted mostly by neutrophils, and IL17$^+$ neutrophils may be promoted by TAMs in the same area. The data from both clinical sample analysis and experimental studies suggested that functional IL17$^+$ cells in the invasive margin stimulated cancer cells to produce CXC chemokines that induced neutrophil trafficking to the tumors. The accumulated neutrophils in the invasive margin were the major source of MMP-9, which in turn triggered the angiogenic switch in the invasive margin. These data provide direct evidence that neutrophils play an important role in human tumor progression by serving as a link between the proinflammatory response and angiogenesis in the tumor milieu. These findings are consistent with the general view that the tumor microenvironment promotes tumor progression by stimulating angiogenesis and tissue remodeling.

Gaps remain in our understanding of neutrophil plasticity and the switch between pro- and antitumor effects (5, 7, 34). The present study demonstrates that IL17$^+$ neutrophils are a critical mediator of the recruitment of neutrophils to gastric cancer cells based on the following findings. First, we observed that both IL17$^+$ cells and neutrophils accumulate in the invasive margin and that the densities of these 2 cell types in the invasive margin

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

are significantly correlated. Second, exposure of gastric cancer cells to IL17 resulted in marked upregulation of several CXC chemokines that attract neutrophils, including CXCL8 (IL8). Third, consistent with these *in vitro* data, a high degree of infiltration of neutrophils in the invasive margin was associated with greater expression of CXC chemokines compared with that in tumor center tissues. This observation is supported by a recent report that $IL17^+$ T cells recruit neutrophils in hepatocellular carcinoma by releasing CXC chemokines (3).

There is substantial evidence that human neutrophils release TIMP-free MMP-9, a potent catalytic stimulator of angiogenesis (35), although MMP-9 can be produced by many cell types. Our results suggest that neutrophils are major sources of MMP-9 in gastric cancer and that tumor-activated neutrophils exhibit delayed apoptosis and sustained release of MMP-9. Neutrophil-derived factors, including MMP-9, stimulated proangiogenic activity in gastric cancer cells. Other studies have also correlated high neutrophil infiltration with increased VEGF expression and high MVD in cancer tissues (3). In addition, exposure to IL17 upregulated several neutrophil chemoattractants in tumor cells, which could lead to a positive feedback loop to recruit more neutrophils into the tumor.

Current knowledge of polymorphonuclear neutrophils (PMN) in cancer progression provides strong support for our findings (5). Our research had limitations: first, some *in vitro* data of how PMNs influence tumor angiogenesis only suggest the potential mechanism, whereas the exact mechanism has still not been elucidated; second, tumors may contain abnormal granulocytic-lineage myeloid-derived suppressor cells (MDSC), and whether normal tumor-infiltrated PMNs or abnormal MDSCs promote tumor angiogenesis is not investigated here. Unfortunately, we had insufficient power to address these issues in the current study.

Emerging evidence indicates that the inflammatory "context" rather than the inflammation *per se* determines the ability of proinflammatory factors to facilitate or prevent tumor growth, and our results provide new insights into the role of $IL17^+$ neutrophils in human tumor progression. Recent research in mouse models has demonstrated that neutrophils can have antitumorigenic or protumorigenic functions, depending on the tumor microenvironment (7, 9, 16, 33), and this mechanism may also contribute to the protumorigenic role of neutrophils in gastric cancer development. Understanding the mechanisms that selectively modulate the functional activities of neutrophils may provide a novel strategy for anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y.-M. Jiang, Y. Yang, Q. Zhang, G.-X. Li
Development of methodology: T.-J. Li, Y.-M. Jiang, L. Huang, G.-X. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-M. Jiang, Y.-F. Hu, L. Huang, J. Yu, L.-Y. Zhao, T.-Y. Mou, H. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-M. Jiang, Y.-F. Hu, L. Huang, J. Yu, L.-Y. Zhao
Writing, review, and/or revision of the manuscript: T.-J. Li, Y.-M. Jiang, Y.-F. Hu, L. Huang, L.-Y. Zhao, H.-J. Deng, H. Liu, Y. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.-J. Li, Y.-M. Jiang, Y.-F. Hu, J. Yu, L.-Y. Zhao, H.-J. Deng, T.-Y. Mou, H. Liu, Q. Zhang
Study supervision: Y.-M. Jiang, Q. Zhang, G.-X. Li
Other (contributed with sampling of tissue and clinical information): Y.-F. Hu

Grant Support

This work was supported by grants from: The State Key Projects on Infection Diseases of China (the 12th Five-Year Plan Period)2012ZX10002017-005, 2012ZX10002016-011, 2012ZX10002010-001-007; National Natural Science Foundation of China, 81372243, 81370575, 81370555; Natural Science Foundation of Guangdong Province, S20120011190; Specialized Research Fund for the Doctoral Program of Higher Education, 20120171110082; Science and Technology Planning Project of Guangzhou, 201400000001-3; Public welfare in Health Industry, National Health and Family Planning Commission of China (201402015, 201502039); Key Clinical Specialty Discipline Construction Program.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 26, 2016; revised July 28, 2016; accepted August 15, 2016; published OnlineFirst September 12, 2016.

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
2. Chung AS, Wu X, Zhuang G, Ngu H, Kasman I, Zhang J, et al. An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. Nat Med 2013;19:1114–23.
3. Kuang DM, Zhao Q, Wu Y, Peng C, Wang J, Xu Z, et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. J Hepatol 2011;54:948–55.
4. Zhang JP, Yan J, Xu J, Pang XH, Chen MS, Li L, et al. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. J Hepatol 2009;50:980–9.
5. Powell DR, Huttenlocher A. Neutrophils in the tumor microenvironment. Trends Immunol 2016;37:41–52.
6. Tazzyman S, Niaz H, Murdoch C. Neutrophil-mediated tumour angiogenesis: subversion of immune responses to promote tumour growth. Semin Cancer Biol 2013;23:149–58.
7. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. Nature 2015;528:413–7.
8. Yen J, White RM, Stemple DL. Zebrafish models of cancer: progress and future challenges. Curr Opin Genet Dev 2014;24:38–45.
9. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 2009;16:183–94.
10. Predina J, Eruslanov E, Judy B, Kapoor V, Cheng G, Wang LC, et al. Changes in the local tumor microenvironment in recurrent cancers may explain the failure of vaccines after surgery. Proc Natl Acad Sci U S A 2013;110:E415–24.
11. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res 2009;69:1553–60.
12. LeBert DC, Squirrell JM, Rindy J, Broadbridge E, Lui Y, Zakrzewska A, et al. Matrix metalloproteinase 9 modulates collagen matrices and wound repair. Development 2015;142:2136–46.
13. Pase L, Layton JE, Wittmann C, Ellett F, Nowell CJ, Reyes-Aldasoro CC, et al. Neutrophil-delivered myeloperoxidase dampens the hydrogen peroxide burst after tissue wounding in zebrafish. Curr Biol 2012;22:1818–24.
14. de Oliveira S, Reyes-Aldasoro CC, Candel S, Renshaw SA, Mulero V, Calado A. Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. J Immunol 2013;190:4349–59.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022

15. Cedervall J, Zhang Y, Huang H, Zhang L, Femel J, Dimberg A, et al. Neutrophil extracellular traps accumulate in peripheral blood vessels and compromise organ function in tumor-bearing animals. Cancer Res 2015;75:2653–62.
16. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest 2013;123:3446–58.
17. Lin AM, Rubin CJ, Khandpur R, Wang JY, Riblett M, Yalavarthi S, et al. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunolgy 2011;187:490–500.
18. Keijsers RR, Hendriks AG, van Erp PE, van Cranenbroek B, van de Kerkhof PC, Koenen HJ, et al. *In vivo* induction of cutaneous inflammation results in the accumulation of extracellular trap-forming neutrophils expressing RORgammat and IL-17. J Invest Dermatol 2014;134:1276–84.
19. Taylor PR, Roy S, Leal SMJr, Sun Y, Howell SJ, Cobb BA, et al. Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORgammat and dectin-2. Nat Immunol 2014;15:143–51.
20. Karthikeyan RS, Vareechon C, Prajna NV, Dharmalingam K, Pearlman E, Lalitha P. Interleukin 17 expression in peripheral blood neutrophils from fungal keratitis patients and healthy cohorts in southern India. J Infect Dis 2015;211:130–4.
21. Taylor PR, Leal SMJr, Sun Y, Pearlman E. Aspergillus and Fusarium corneal infections are regulated by Th17 cells and IL-17-producing neutrophils. J Immunol 2014;192:3319–27.
22. Benevides L, da Fonseca DM, Donate PB, Tiezzi DG, De Carvalho DD, de Andrade JM, et al. IL17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment. Cancer Res 2015;75:3788–99.
23. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87–108.
24. Zhuang Y, Peng LS, Zhao YL, Shi Y, Mao XH, Chen W, et al. CD8(+) T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer. Gastroenterology 2012;143:951–62.e8.
25. Wang R, Zhao N, Li S, Fang JH, Chen MX, Yang J, et al. MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42. Hepatology 2013;58:642–53.
26. Punt S, Fleuren GJ, Kritikou E, Lubberts E, Trimbos JB, Jordanova ES, et al. Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer. Oncoimmunology 2015;4:e984539.
27. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 2008;8:618–31.
28. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737–44.
29. Bekes EM, Schweighofer B, Kupriyanova TA, Zajac E, Ardi VC, Quigley JP, et al. Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. Am J Pathol 2011;179:1455–70.
30. Fuchs CS, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, et al. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. Lancet 2014;383:31–9.
31. Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. Gastroenterology 2011;140:1807–16.
32. Wang K, Kim MK, Di Caro G, Wong J, Shalapour S, Wan J, et al. Interleukin-17 receptor a signaling in transformed enterocytes promotes early colorectal tumorigenesis. Immunity 2014;41:1052–63.
33. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature 2015;522:345–8.
34. Liang W, Ferrara N. The complex role of neutrophils in tumor angiogenesis and metastasis. Cancer Immunol Res 2016;4:83–91.
35. Ardi VC, Kupriyanova TA, Deryugina EI, Quigley JP. Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. Proc Natl Acad Sci U S A 2007;104:20262–7.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/23/6/1575/2302159/1575.pdf by guest on 26 August 2022