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Original Research

Expression, regulation, function and clinical significance of B7-H6 on neutrophils in human gastric cancer

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ABSTRACT

Neutrophils are conspicuous components of gastric cancer (GC) tumors, increasing with tumor progression and poor patient survival. However, the phenotype, regulation, function and clinical relevance of neutrophils in human GC are presently unknown. We used flow cytometry analyses to examine levels and phenotype of neutrophils in samples from 50 patients with GC. Kaplan-Meier plots for patient survival were performed using the log-rank test, and multivariate analysis of prognostic factors for patient survival was performed using the Cox proportional hazards model. Neutrophils were isolated, stimulated and/or cultured for regulation and function assays. We found that GC patients showed a significantly higher neutrophil infiltration in tumors, and that neutrophil infiltration was positively associated with tumor progression but negatively correlated with patient survival. Most tumor-infiltrating neutrophils showed an activated $CD54^+$ phenotype and expressed high level B7-H6. Tumor tissue culture supernatants from GC patients inhibited neutrophil apoptosis and induced the expression of CD54 and B7-H6 on neutrophils in time-dependent and dose-dependent manners. Intratumoral $CD54^+$ neutrophils and $B7\text{-}H6^+$ neutrophils positively correlated with increased G-CSF detection *ex vivo*; and *in vitro* both G-CSF and tumor-derived G-CSF induced the expression of CD54 and B7-H6 on neutrophils via NF-κB signaling pathway activation. Furthermore, blockade of B7-H6 promoted the apoptosis of tumor-infiltrating and tumor-conditioned neutrophils, and shortened their lifespan. Importantly, intratumoral $B7\text{-}H6^+$ neutrophils increased with tumor progression and predicted poor patient survival. Our results illuminate a novel mechanism of B7-H6 expression on tumor-activated neutrophils in GC, and also suggest $B7\text{-}H6^+$ neutrophils would be novel potential biomarkers in GC.

Introduction

Gastric cancer (GC) is the fifth most common cancer worldwide as well as one of the leading causes of tumor death in many less-developed countries [1,2]. In the past few years, substantial advances have been achieved in the diagnosis and treatment of GC, however, the mortality of patients with GC remains high [3]. Although the pathogenesis of GC remains poorly understood, it is believed that the development and prognosis of GC are closely associated with the immune cells infiltrated in the GC environment [4,5].

In the GC environment, different immune cells are locally infiltrating, and neutrophils are the ones of the mostly infiltrated immune

Abbreviations: GC, gastric cancer; PBMCs, peripheral blood mononuclear cells; IL, interleukin; TTCS, tumor tissue culture supernatants; NTCS, non-tumor tissue culture supernatants; G-CSF, granulocyte colony stimulating factor; NF-κB, nuclear factor-κB.

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cells [6]. Currently, many studies focus on the prognosis of the neutrophil level in blood of GC patients, showing that the increased peripheral neutrophil/lymphocyte ratio predicts poor survival of GC patients [7]. As for locally infiltrating neutrophils of GC patients, other researchers have performed immunohistochemistry to identify close relationships between high tumor-infiltrating neutrophils and poor prognosis of GC patients [8,9]. These studies on peripheral and infiltrating neutrophils together suggest that neutrophils may play pathological roles in GC. Therefore, in human GC, characterization of pathological phenotype of neutrophils, and evaluation of their regulatory mechanism, function and clinical relevance of this phenotype of neutrophils, are essential for understanding the potential roles of neutrophils in GC immunopathogenesis.

B7-H6, also known as NCR3LG1, is discovered in 2009 and regarded as a new immune-regulatory protein of the B7 family [10]. Although B7-H6 is commonly identified as the ligand of human natural cytotoxicity receptor NKp30 [11,12], subsequent reports have shown that B7-H6 can exert immunopathogenic effects of immune escape in the tumor environment [13], and can be modified by the factors such as ADAM10 and ADAM17 in the tumor environment to foster immune escape [14]. Nowadays, B7-H6 has been found to be over-expressed in several human cancer types, such as small cell lung cancer [15] and oral squamous cell carcinoma [16]. Furthermore, B7-H6 represents a predictor of poor prognosis for bladder cancer [17] and esophageal squamous cell carcinoma [18], and has been reported to promote disease progression in cervical cancer [19] and glioma [20]. As for gastrointestinal cancers, although B7-H6 expression has no prognostic significance in human gastric carcinoma [21], B7-H6 was detected in 98 % of colorectal cancer and 77 % of GC tissue samples, and by generating a novel B7-H6-targeted IgG-like T cell-engaging antibody to perform *in vitro* and *in vivo* experiments showing increased antitumor activity against colorectal cancer cells, suggest that targeting tumor-associated B7-H6 may be provides a novel therapy for the treatment of gastrointestinal tumors [22]. Especially, B7-H6 expression on human primary neutrophils in GC and its underlying regulatory mechanism, function and clinical relevance have not yet been explored.

Herein, we show that neutrophils are highly enriched within the GC environment and that their enrichment is positively associated with GC tumor progression but is negatively correlated with GC patient survival. Moreover, we demonstrate that GC tumors inhibited neutrophil apoptosis, and that tumor-derived granulocyte colony stimulating factor (G-CSF) efficiently activates neutrophils and induces B7-H6 expression on neutrophils via nuclear factor-κB (NF-κB) signaling pathway activation. Furthermore, blockade of B7-H6 promotes the apoptosis of tumor-infiltrating and tumor-conditioned neutrophils, and shortens their lifespan. Importantly, higher intratumoral B7-H6^{+} neutrophil percentage and higher intratumoral B7-H6^{+} neutrophil number are associated with advanced tumor-node-metastasis (TNM) stage and poor overall survival in patients with GC.

Materials and methods

Patients and specimens

Fresh gastric tumor, peritumoral, and non-tumor (non-tumor tissues, at least 5 cm distant from the tumor site) tissues and autologous peripheral blood were obtained from GC patients who underwent surgical resection at the Southwest Hospital of Third Military Medical University. Tumor regions were mainly identified according to the preoperative imaging examination (such as Computed Tomography), in addition, the intraoperative visual examination and palpation were combined to determine the tumor areas. When necessary, fluorescence imaging technology was used (that was, the needle was used to puncture the tumor and injected an appropriate amount of contrast agent, and fluorescence imaging was stimulated under a fluorescence mirror to accurately identify the location, size and edge of the tumor). According to Gastric Cancer Diagnosis and Treatment Guidelines (2018 edition, China) and Japanese Gastric Cancer Treatment Guidelines (2018, 5th edition), the peri-tumoral regions were the gastric tissue within 5 cm of the tumor boundary, and the non-tumors were more than 5 cm from the tumor boundary. None of these patients had received chemotherapy or radiotherapy before surgery. Patients with infectious diseases, autoimmune disease, or multi-primary cancers were excluded. The clinical stages of tumors were determined according to the TNM classification system of the International Union Against Cancer (8th edition). *Helicobacter pylori* (*H. pylori*) infection was determined by serology test for specific anti-*H. pylori* antibodies. Antibodies and other reagents were listed in Table. S1. Clinical characteristics of GC patients were shown in Table. S2.

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded samples were cut into 5 μm sections. For immunohistochemical staining, the sections were incubated with rabbit anti-human CD15, and then were stained by horseradish peroxidase (HRP) anti-rabbit immunoglobulin G (IgG) followed by diaminobenzidine. All the sections were finally counterstained with hematoxylin and examined using a microscope (Nikon Eclipse 80i; Nikon).

Isolation of single cells from tissues of GC patients

Fresh tissues were washed 3 times with Hank's solution containing 1 % fetal bovine serum before being cut into small pieces. The specimens were then collected in RPMI 1640 containing 200 μg/ml collagenase I, 200 μg/ml collagenase II, 1 mg/ml collagenase IV and 10 mg/ml deoxyribonuclease (DNase) I and mechanically dissociated using the gentle MACS Dissociator (Miltenyi Biotec). Dissociated cell suspensions were further incubated for 1 h at 37°C under continuous rotation. The cell suspensions were then filtered through a 70 μm cell strainer (BD Labware). Cell viability, as determined by trypan blue exclusion staining, was typically >95 %.

Isolation of neutrophils

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient centrifugation using Ficoll-Paque Plus. Blood neutrophils were harvested after lysis of red blood cells with lysis solution from non-PBMCs. The cells were used unless their viability was determined >95 % and their purity was determined >95 %.

Preparation of TTCS and NTCS

Tumor tissue culture supernatants (TTCS) or non-tumor tissue culture supernatants (NTCS) were prepared by plating autologous tumor or non-tumor gastric tissues in 1 ml RPMI 1640 medium for 24 h. The supernatant was then centrifuged and harvested.

Neutrophil stimulation

Neutrophils from healthy donors were stimulated with 50 % TTCS or 50 % NTCS of the same GC patients, or 50 % TTCS with a neutralizing antibody against human G-CSF (10 μg/ml), or 50 % NTCS with human recombinant (hr) G-CSF (100 ng/ml) for 12 h, or were stimulated with TTCS (10 %, 20 %, or 50 %) or hr G-CSF (25, 50, or 100 ng/ml) for 12 h, or were stimulated with 50 % TTCS or hr G-CSF (100 ng/ml) for 3, 6, or 12 h. After stimulation, the cells were harvested for flow cytometric analysis and western blot. Neutrophils cultured with RPMI-1640 medium were used as controls. For the signaling pathway inhibition experiments, neutrophils were pretreated with BAY 11-7082 (an IκBα inhibitor), AG490 (a JAK inhibitor), FLLL32 (an STAT3 inhibitor), SB203580 (an MAPK inhibitor), SP600125 (a JNK inhibitor), SR1664 (a

PPARγ inhibitor), U0126 (an MEK-1 and MEK-2 inhibitor), Wortmannin (a PI3K inhibitor), or GSK-3β inhibitor (5 μl, 20 μM) for 1 h, then the cells were stimulated with 50 % TTCS or hr G-CSF (100 ng/ml) for 12 h and harvested as above. Since the inhibitor was dissolved in DMSO, parallel cell groups were treated with DMSO (5 μl) or culture media as controls.

Neutrophil apoptosis assay

Firstly, neutrophils from healthy donors were stimulated with 50 % TTCS or 50 % autologous NTCS, or 20 %, 40 %, or 80 % TTCS for 12 h, and then were harvested. Secondly, to generate tumor-conditioned neutrophils, neutrophils from healthy donors and cultured with 50 % TTCS for 12 h, and then washed with RPMI-1640 medium for 3 times. These tumor-conditioned neutrophils were cultured in the presence of human B7-H6 neutralizing antibody or control IgG (20 μg/ml) for 12 h, and then were harvested. Thirdly, neutrophils from autologous tumor and non-tumor tissues were sorted by fluorescence-activated cell sorter (FACS) (FACSAria III; BD Biosciences) by gating on $CD45^+CD11b^+CD66b^+CD15^+$ cells. These tissue-derived neutrophils were cultured for 12 h, and then were harvested; additionally, tumor-derived neutrophils were cultured in the presence of human B7-H6 neutralizing antibody or control IgG (20 μg/ml) for 12 h, and then were harvested. Neutrophil apoptosis was quantified using Annexin V Apoptosis Detection Kit or APO-Direct Apoptosis Detection Kit according to the manufacturer's instructions.

Flow cytometry

Cell surface markers were stained with specific or isotype control antibodies. Flow cytometric analysis was performed according to standard protocols. The cells were analyzed by multicolor flow cytometry with FACSCanto™ (BD Biosciences). Data were analyzed with Flowjo software (TreeStar) or FACSDiva software (BD Biosciences).

Real-time PCR

RNA of biopsy specimens was extracted with RNAiso Plus reagent. The RNA samples were reversed transcribed into cDNA with PrimeScript™ RT reagent Kit. Real-time PCR was performed on an IQ5 (Bio-Rad) with Real-time PCR Master Mix according to the manufacturer's specifications. The mRNA expression of genes was measured using the SYBR green method with the relevant primers (Table. S3). For human samples, human GAPDH mRNA level served as a normalizer, and its level in non-tumor tissues served as a calibrator. The relative gene expression was expressed as fold change of relevant mRNA calculated by the ΔΔCt method.

Western blots

Western blots were performed on 10–15 % SDS-PAGE gel transferred PVDF membranes using equivalent amounts of cell lysate protein for each sample. Five percent skimmed milk or three percent BSA was used for blocking the PVDF membranes. Human p65 and p-p-p65 were detected with anti-p65 and anti-p-p65 antibodies respectively. This was followed by incubation with HRP-conjugated secondary antibodies. Bound proteins were visualized using Super ECL plus Western blotting Kit.

ELISA

Human gastric tissues from specimens were collected, homogenized in 1 ml sterile Protein Extraction Reagent, and centrifuged. Tissue supernatants were collected for ELISA. Concentrations of G-CSF in the tissue supernatants or in the TTCS and NTCS from autologous tumor or non-tumor gastric tissues were determined using ELISA kits according to the manufacturer's instructions.

Statistical analysis

Results are expressed as mean±SEM. Student *t*-test was generally used to analyze the differences between two groups, but when the variances differed, the Mann-Whitney U test was used. For multigroup data analysis, an ANOVA analysis was used. Correlations between parameters were assessed using the Pearson correlation analysis and linear regression analysis as appropriate. Overall survival was defined as the interval between surgery and death. The known tumor-unrelated deaths (eg, accidental death) were excluded from the death record for this study. Cumulative survival time was calculated by the Kaplan-Meier method, and survival was measured in months; the log-rank test was applied to compare between 2 groups. Multivariate analysis of prognostic factors for patient survival was performed using the Cox proportional hazards model. SPSS statistical software (version 13.0) was used for all statistical analysis. All data were analyzed using 2-tailed tests, and $P < 0.05$ was considered statistically significant.

Results

Neutrophils are enriched in human GC environment with tumor progression and associated with poor patient survival

To evaluate the potential role of neutrophils in human GC, we analyzed neutrophil percentage within the total $CD45^+$ leukocytes in different samples at various stages. Peripheral blood samples from healthy donors were used as controls. Notably, patients with GC showed a higher neutrophil percentage in peripheral blood than healthy donors. Within the patient cohort, tumors contained a significantly higher neutrophil percentage than peritumoral and non-tumor tissues (Fig. 1A, B). Moreover, as the cancer progressed, we found that the percentage of neutrophils significantly increased in each of the tested samples (Fig. 1A). Similar observations were made when analyzing the total number of neutrophils per million total cells in each tissue (Fig. 1C). Furthermore, immunohistochemical staining also showed that neutrophils were accumulated in tumors (Fig. 1D), indicating a potential role for neutrophils in the GC microenvironment. In keeping with these findings, increased intratumoral neutrophil percentage and intratumoral neutrophil number were correlated with increased tumor size and advanced tumor stage (Fig. S1).

Next, we evaluated the clinical relevance of intratumoral neutrophils in GC. Comparing patients with high (≥7 % median level) versus low (<7 % median level) intratumoral neutrophil percentage level, the 29-month overall survival rates were significantly lower for those within the higher intratumoral neutrophil percentage group (Fig. 1E). Similar results were obtained when the patient cohort was stratified based on intratumoral neutrophil number (Fig. 1E). Importantly, the finding that intratumoral neutrophil percentage independently predicted patient survival was verified by multivariate analyses using a Cox proportional hazard model (Table. S4). Taken together, these findings suggest that increased intratumoral neutrophil infiltration is associated with tumor progression and poor survival for GC patients.

B7-H6 expression and activation of neutrophils are correlated in human GC environment

We next analyzed the immuno-phenotyping of these enriched intratumoral neutrophils. First, we found that peripheral neutrophils from GC patients expressed little neutrophil activation marker CD54 (Fig. 2A-C). Next, we found that intratumoral neutrophils expressed significantly higher level of CD54 than those on peritumoral and non-tumor tissue neutrophils (Fig. 2A-C), suggesting an activation of neutrophils in the GC environment. Interestingly, intratumoral neutrophils from GC patients expressed significantly higher level of immune-

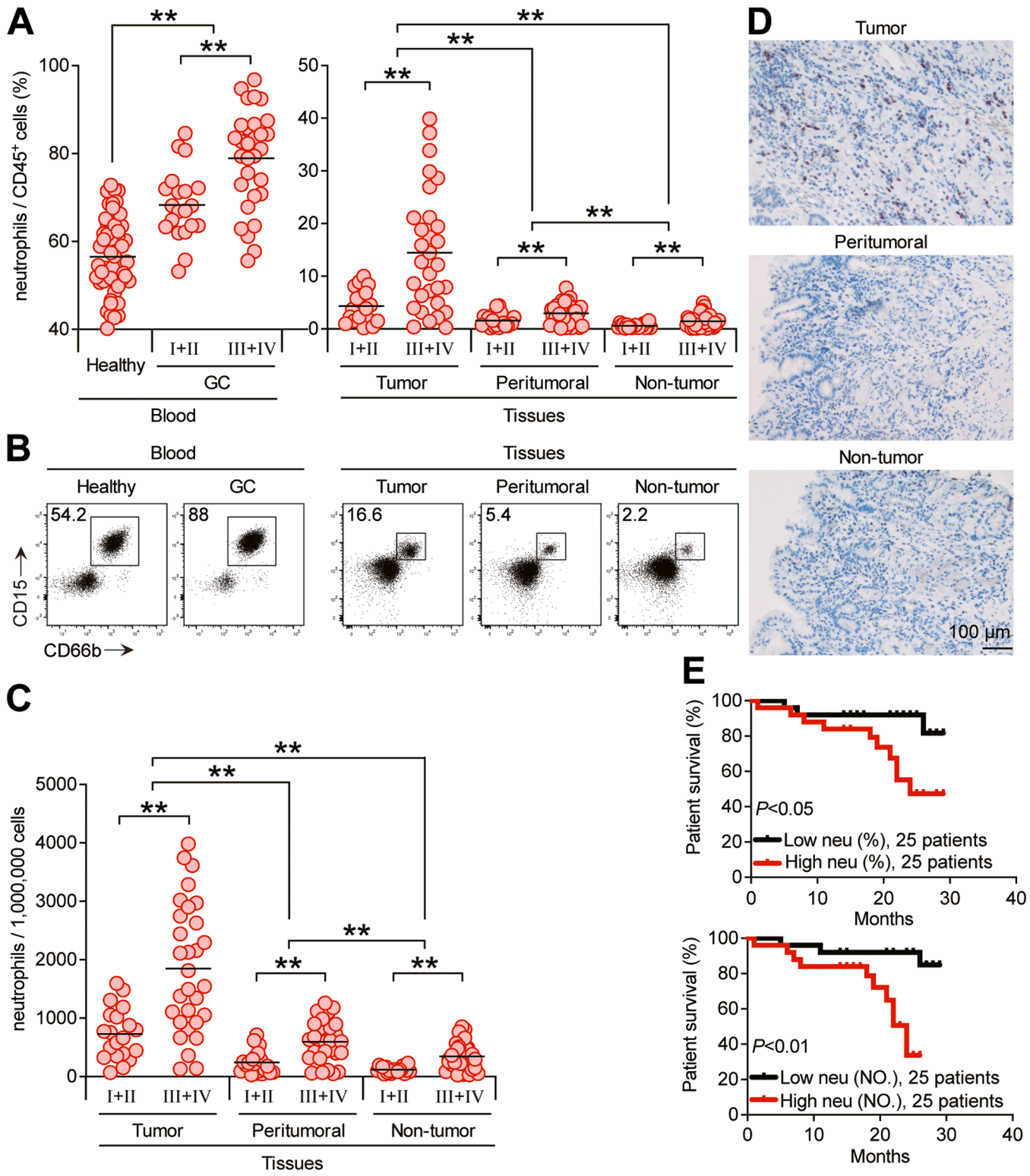

Fig. 1. Neutrophils are enriched in human GC with tumor progression and associated with poor patient survival. (A) The percentage of neutrophils in $CD45^+$ cells among TNM stages (*I*+II vs III+IV) in each tissue of patients with GC by gating on $CD45^+CD11b^+CD66b^+CD15^+$ cells. Cumulative results from 50 GC patients and 60 healthy donors are shown. (B) Dot plots of surface molecule staining for neutrophils gating on $CD45^+CD11b^+$ cells. Results are expressed as the percentage of neutrophils in $CD45^+$cells. (C) The number of neutrophils per million total cells among TNM stages (*I*+II vs III+IV) in each tissue of patients with GC by counting. Cumulative results from 50 GC patients are shown. (D) Representative analysis of $CD15^+$ (brown) neutrophil distributions in tissues of GC patients by immunohistochemical staining. Scale bars: 100 microns. (E) Kaplan-Meier plots for overall survival of 50 GC patients by median neutrophil percentage (7 %) or median neutrophil number (1082 per million) (25 patients with low or high neutrophil percentage/number respectively). The horizontal bars in panels A and C represent mean values. Each ring or dot in panels A and C represents 1 patient. $^*P < 0.05$, $^{**}P < 0.01$, n.s $P > 0.05$ for groups connected by horizontal lines. neu (%): neutrophil percentage. neu (NO.): neutrophil number.

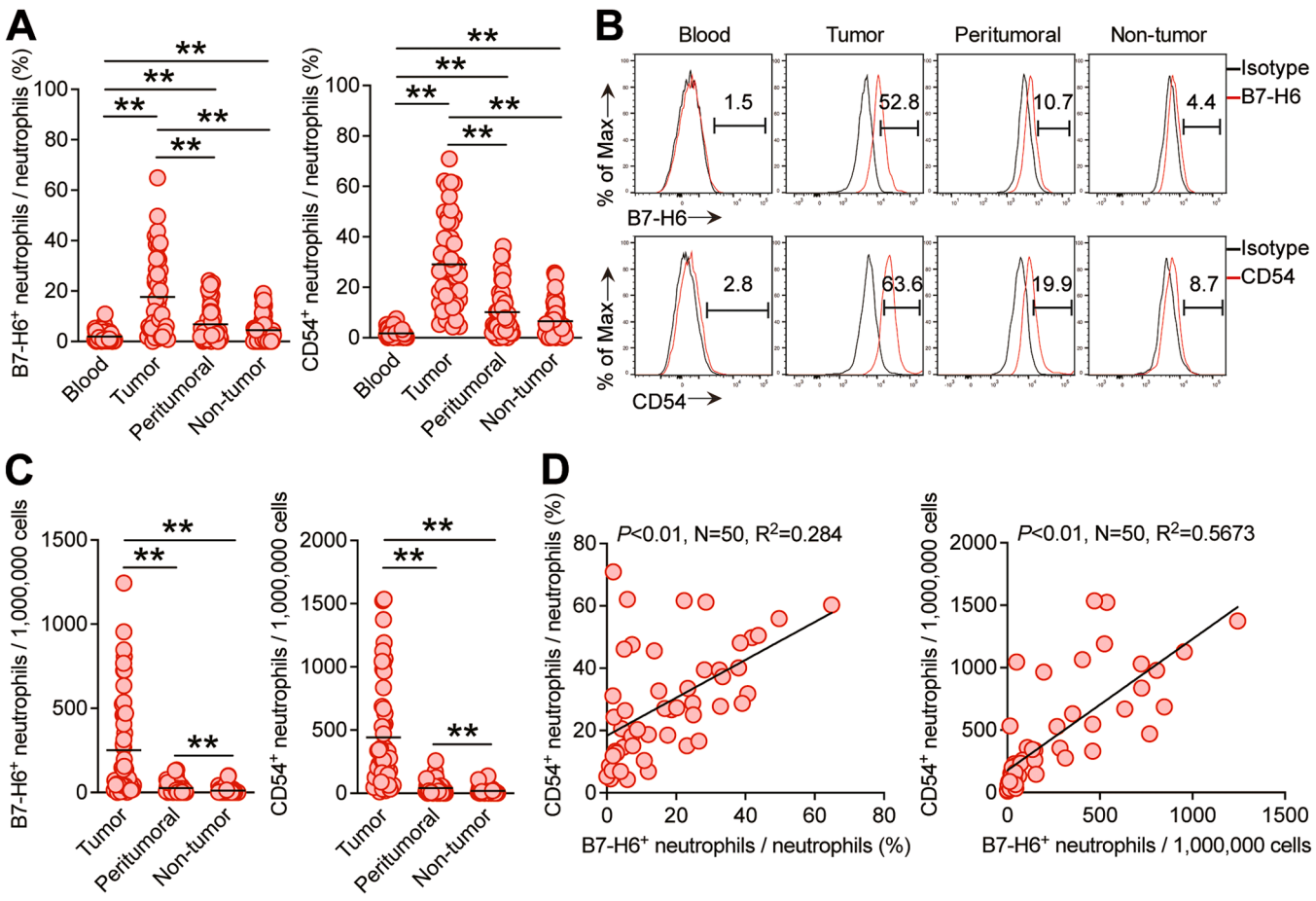

Fig. 2. B7-H6 expression and activation of neutrophils are correlated in human GC environment. (A) Statistics analysis of the percentage of $CD54^+$neutrophils and $B7\text{-}H6^+$neutrophils in neutrophils in each sample of patients with GC ($n = 50$). (B) Expression of molecules CD54 and B7-H6 on neutrophils. Results are expressed as the percentage of $CD54^+$neutrophils and $B7\text{-}H6^+$neutrophils in neutrophils. Color histograms represent staining of neutrophil activation marker CD54 and immune-regulatory molecule B7-H6; black, isotype control. (C) Statistics analysis of the number of $CD54^+$ neutrophils and $B7\text{-}H6^+$ neutrophils per million total cells in each sample of patients with GC ($n = 50$). (D) The correlations between $CD54^+$neutrophils and $B7\text{-}H6^+$neutrophils in GC tumors were analyzed. Results are expressed as the percentage of $CD54^+$neutrophils and $B7\text{-}H6^+$neutrophils in neutrophils or the number of $CD54^+$ neutrophils and $B7\text{-}H6^+$ neutrophils per million total cells in tumor tissues of patients with GC. The horizontal bars in panels A and C represent mean values. Each ring or dot in panels A, C and D represents 1 patient. $^*P < 0.05$, $^{**}P < 0.01$, n.s $P > 0.05$ for groups connected by horizontal lines.

regulatory molecule B7-H6 than those on peritumoral and non-tumor tissue neutrophils (Fig. 2A-C), while peripheral neutrophils expressed less B7-H6 (Fig. 2A-C). Moreover, significant correlations were found between the levels of CD54 and B7-H6 expression on neutrophils in GC tumors (Fig. 2D). The above data indicate that tumor-infiltrating neutrophils exhibit an activated and highly B7-H6-expressing phenotype.

Human GC environments inhibit neutrophil apoptosis and maintain neutrophil activated and highly B7-H6-expressing phenotype

The results described above suggested that GC environments trigger the accumulation of neutrophils. Thus, we hypothesized that GC environment may sustain neutrophil lifespan by apoptosis inhibition. To test this hypothesis, neutrophils from healthy donors were stimulated respectively with NTCS and TTCS from GC patients, then we assessed the survival of neutrophils after exposure to NTCS and TTCS, and found that neutrophils exposed to TTCS exhibited a delayed onset of apoptosis when compared to those exposed to NTCS from autologous GC patients (Fig. 3A). We also found that TTCS-conditioned neutrophils exhibited a delayed onset of apoptosis in a dose-dependent manner (Fig. 3B). These findings together imply that GC environment is involved in the inhibiting the apoptosis of neutrophils.

Meanwhile, we hypothesized that GC environments might contribute to the activated and highly B7-H6-expressing phenotype of neutrophils. Consistent with our hypothesis, neutrophils from healthy donors were stimulated respectively with NTCS and TTCS from GC patients, and the observations revealed a significantly up-regulated expression of CD54 and B7-H6 on TTCS-conditioned neutrophils compared to NTCS-conditioned neutrophils (Fig. 3C). We also found that TTCS-conditioned neutrophils up-regulated the expression of CD54 and B7-H6 in a time-dependent manner (Fig. 3D) as well as in a dose-dependent manner (Fig. 3E). These findings together imply that GC environment is involved in the activation of neutrophils and B7-H6 expression on neutrophils.

G-CSF activates neutrophils and induces B7-H6 expression on neutrophils

Tumor microenvironment can possess various soluble inflammatory factors, including cytokines with potential pro-inflammatory effects. To see which cytokines might activate neutrophils and induce B7-H6 expression on neutrophils, we first screened pro-inflammatory cytokines in human GC environments by real-time PCR (Fig. 4A), and stimulated normal neutrophils with top 15 highly-expressed cytokines including TGF-β, TNF-α, G-CSF, M-CSF, GM-CSF, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17A, IL-17F, IL-21, IL-23, IL-33 etc. We found that only G-CSF remarkably up-regulated the expression of B7-H6 on neutrophils

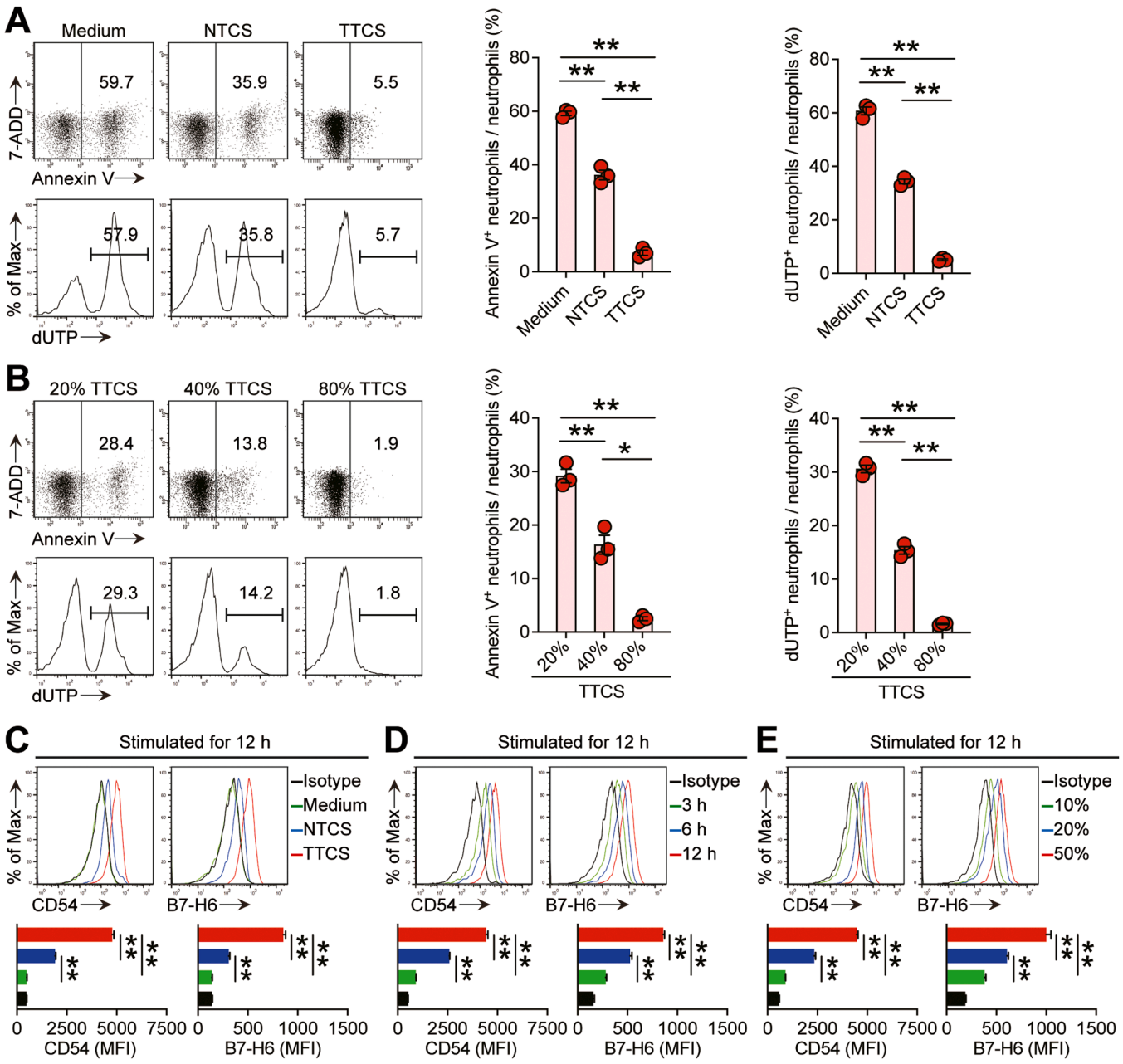

Fig. 3. Human GC environments maintain neutrophil survival, activation and B7-H6 expression. (A) Representative data and statistics analysis of annexin V^+ or deoxyuridine triphosphate nucleotides $(dUTP)^+$ apoptotic neutrophils exposed to 50 % TTCS and 50 % NTCS from autologous GC patients for 12 h ($n = 3$). (B) Representative data and statistics analysis of annexin V^+ or $dUTP^+$ apoptotic neutrophils exposed to 20 %, 40 % or 80 % TTCS from GC patients for 12 h ($n = 3$). (C) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to 50 % TTCS and 50 % NTCS from GC patients, or to medium control for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (D) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to 50 % TTCS from GC patients for 3, 6, or 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (E) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to 10 %, 20 %, or 50 % TTCS from GC patients for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. $^*P < 0.05$, $^{**}P < 0.01$ for groups connected by horizontal lines.

(Fig. 4B and Fig. S2). We also found that G-CSF up-regulated B7-H6 expression on neutrophils in a time-dependent manner (Fig. 4C) as well as in a dose-dependent manner (Fig. 4D). Similar observations were made when analyzing the induction of neutrophil activation marker CD54 on neutrophils by G-CSF (Fig. 4B-D). Moreover, significant correlations were found between the concentrations of G-CSF and the levels of $CD54^+$ neutrophils (Fig. 4E) as well as between the concentrations of G-CSF and the levels of $B7\text{-}H6^+$neutrophils (Fig. 4F) in GC tumors analyzed. To sum up, the above data indicate that G-CSF activates neutrophils and induces B7-H6 expression on neutrophils.

Tumor-derived G-CSF activates neutrophils and induces B7-H6 expression on neutrophils via activating NF-κB signaling pathway

To test the neutrophils' B7-H6 induction by G-CSF derived from GC tumors, we next cultured neutrophils with TTCS altogether with G-CSF neutralizing antibodies. We found that G-CSF blocking could inhibit the B7-H6 expression on neutrophils exposed to TTCS (Fig. 5A). On the other hand, we cultured neutrophils with NTCS altogether with human recombinant G-CSF, and found an increased B7-H6 expression on neutrophils exposed to G-CSF (Fig. 5B). Similar observations were seen when analyzing the neutrophils' CD54 induction in these culture

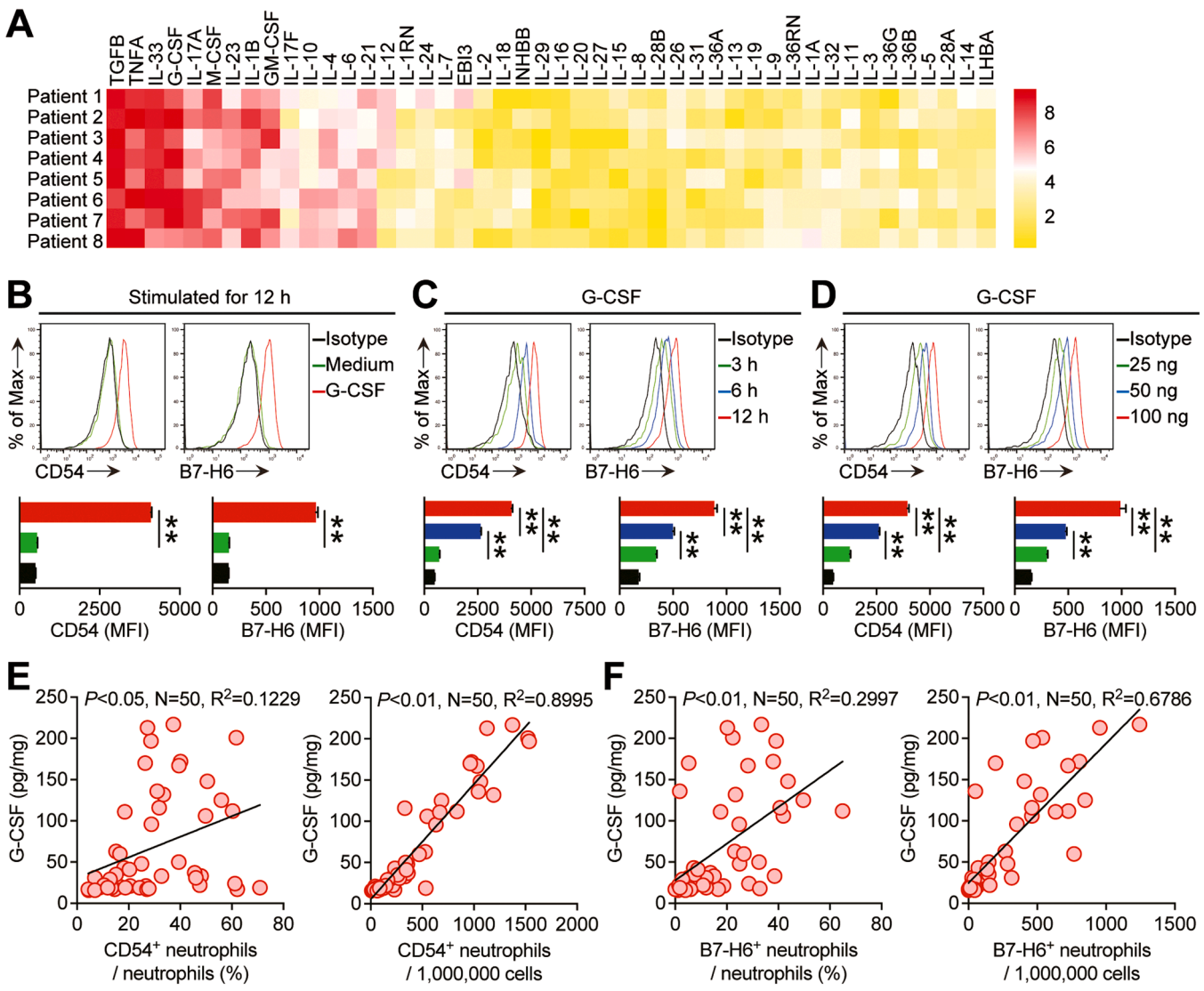

Fig. 4. G-CSF activates neutrophils and induces B7-H6 expression on neutrophils. (A) Clustering of real-time PCR data for the expression of pro-inflammatory cytokine genes in tumor tissues from 8 GC patients. (B) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to G-CSF (100 ng/ml), or to medium control for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (C) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to G-CSF (100 ng/ml) for 3, 6, or 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (D) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to G-CSF (25, 50, or 100 ng/ml) for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (E) The correlations between G-CSF and CD54$^+$neutrophils in GC tumors were analyzed. Results are expressed as the percentage of CD54$^+$neutrophils in neutrophils or the number of CD54$^+$neutrophils per million total cells and G-CSF concentration in GC tumor tissues. (F) The correlations between G-CSF and B7-H6$^+$neutrophils in GC tumors were analyzed. Results are expressed as the percentage of B7-H6$^+$neutrophils in neutrophils or the number of B7-H6$^+$neutrophils per million total cells and G-CSF concentration in GC tumor tissues. Each ring or dot in panels E and F represents 1 patient.

systems above (Fig. 5A, B). We further found that G-CSF was significantly increased in tumor tissues or TTSC when compared to that in non-tumor tissues or NTCS (Fig. 5C). The data above suggest that GC tumor-derived G-CSF activates neutrophils and induces B7-H6 expression on neutrophils.

Next, we tried to test the signaling pathways of these neutrophils' B7-H6 induction. We then pre-treated neutrophils with corresponding inhibitors and cultured neutrophils with TTCS, and found that only blocking NF-κB signaling with BAY 11-7082 could inhibit the B7-H6 expression on neutrophils exposed to TTCS (Fig. 5D and Fig. S3). Similar observations were seen when analyzing the neutrophils' B7-H6 induction by G-CSF (Fig. 5E). Similar observations were seen when analyzing the neutrophils' CD54 induction in these culture systems above (Fig. 5D, E). Furthermore, we found an increased phosphorylation of p65, a direct NF-κB signaling pathway downstream substrate, in TTCS-conditioned neutrophils compared to that in NTCS-conditioned neutrophils, and found that blocking G-CSF could abolish this p65' phosphorylation in TTCS-conditioned neutrophils (Fig. 5F and Fig. S4). Overall, these data suggest, in the GC environment, GC tumor-derived G-CSF activates neutrophils and induces B7-H6 expression on neutrophils by activating NF-κB signaling pathway.

Blockade of B7-H6 promotes the apoptosis of tumor-conditioned and tumor-derived neutrophils, and shortens their lifespan

Given the better survival and the higher B7-H6 expression of tumor-conditioned neutrophils (Fig. 3), we hypothesized that B7-H6 itself might play important roles in the survival of neutrophils in GC. First, we cultured neutrophils with TTCS to generate tumor-conditioned neutrophils, then cultured them in the presence of human B7-H6 neutralizing antibody. We found that tumor-conditioned neutrophils exposed to B7-H6 neutralizing antibody exhibited an increased onset of apoptosis

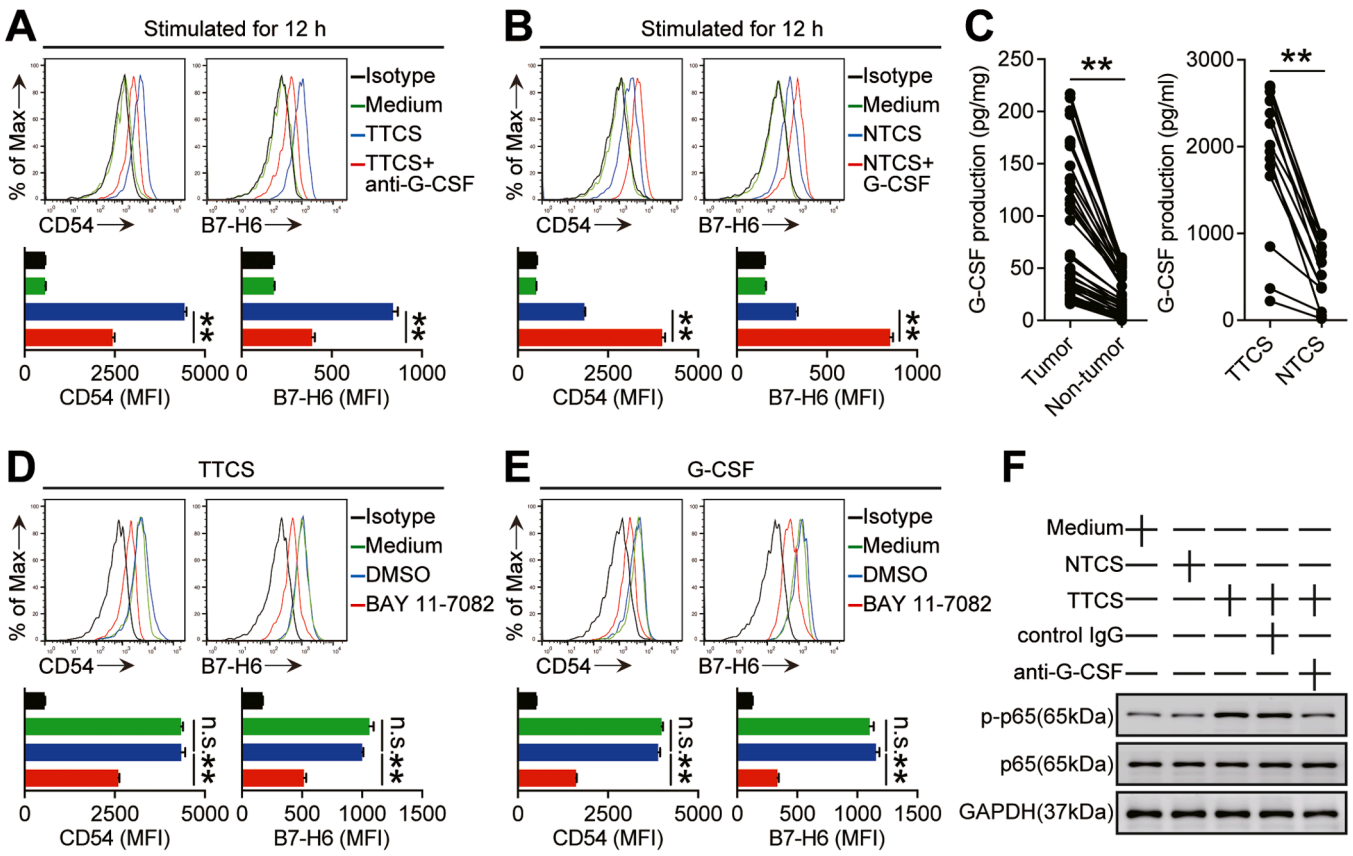

Fig. 5. Tumor-derived G-CSF activates neutrophils and induces B7-H6 expression on neutrophils via activating NF-κB signaling pathway. (A) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to 50 % TTCS with anti-G-CSF antibody for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (B) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to 50 % NTCS with G-CSF for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (C) G-CSF concentration between autologous tumor and non-tumor tissues ($n = 50$) or between autologous TTCS and NTCS ($n = 15$) was analyzed. (D) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to 50 % TTCS with or without NF-κB signaling pathway inhibitor BAY 11-7082 for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (E) Representative data and statistical analysis of the expression of CD54 and B7-H6 on neutrophils exposed to G-CSF with or without NF-κB signaling pathway inhibitor BAY 11-7082 for 12 h ($n = 3$). black, isotype control. MFI: mean fluorescence intensity. (F) The p65 and p-p65 in neutrophils exposed to 50 % TTCS and 50 % NTCS from GC patients, or to medium control, or to 50 % TTCS with anti-G-CSF antibody or control IgG were analyzed by western blot. Western blot results are run in parallel and contemporaneously. Each ring or dot in panel B represents 1 patient. $^{**}P < 0.01$ for groups connected by horizontal lines.

when compared to those exposed to control IgG (Fig. 6A), suggesting a delayed apoptosis of GC tumor-conditioned neutrophils via B7-H6. To further confirm the similar role of B7-H6 on tumor-derived neutrophils, neutrophils from tumor and non-tumor tissues of autologous GC patients were therefore isolated and cultured in the presence of human B7-H6 neutralizing antibody. We found that tumor-derived neutrophils exhibited a delayed onset of apoptosis compared to non-tumor-derived neutrophils (Fig. 6B), which could be significantly attenuated by blockade of B7-H6 (Fig. 6C), suggesting a delayed apoptosis of tumor-derived neutrophils in GC via B7-H6. These findings together imply that neutrophil survival is prolonged by inhibiting apoptosis via B7-H6 in GC.

Intratumoral B7-H6$^+$ neutrophils correlate with advanced GC tumor stage and predict poor GC patient survival

We finally tested the clinical association and the prognosis of these increased neutrophils' B7-H6 expression in GC patients. First, the percentage of intratumoral B7-H6$^+$ neutrophils in GC patients with advanced GC was significantly higher than that in GC patients with early GC (Fig. 7A). Next, comparing patients with high (≥12.85 % median level) versus low (<12.85 % median level) intratumoral B7-H6$^+$ neutrophil percentage level, the 29-month overall survival rates were significantly lower for those within the higher intratumoral B7-H6$^+$ neutrophil percentage group (Fig. 7A). Similar results were obtained when the patient cohort was stratified based on intratumoural B7-H6$^+$ neutrophil number (Fig. 7B). Moreover, increased intratumoral B7-H6$^+$ neutrophil percentage and intratumoral B7-H6$^+$ neutrophil number were correlated with increased tumor size and advanced tumor stage (Fig. S5). Overall, these data suggest that intratumoral B7-H6$^+$ neutrophils correlate with advanced tumor stage and poor survival in patients with GC.

Discussion

Neutrophils have been well characterized in their role for promoting tumor progression [23,24], and origins of tumor-associated neutrophils have been fully identified [25]. However, the inducible phenotypes of neutrophils in the tumor environment and their underlying regulatory and functional mechanisms have not already been explored. In this study, to our knowledge, this is the first demonstration of a statistically significant correlation between prevalent high B7-H6$^+$ neutrophils and tumor progression or poor prognosis in human tumors; it is also the first demonstration for tumor-derived G-CSF to induce B7-H6 expression on neutrophils to prolong their lifespan within the tumor environment.

Although tumor-infiltrating neutrophils are reported to be increased with poor prognosis of patients with GC [26,27], very little is currently known about the phenotype of tumor-infiltrating neutrophils, as well as

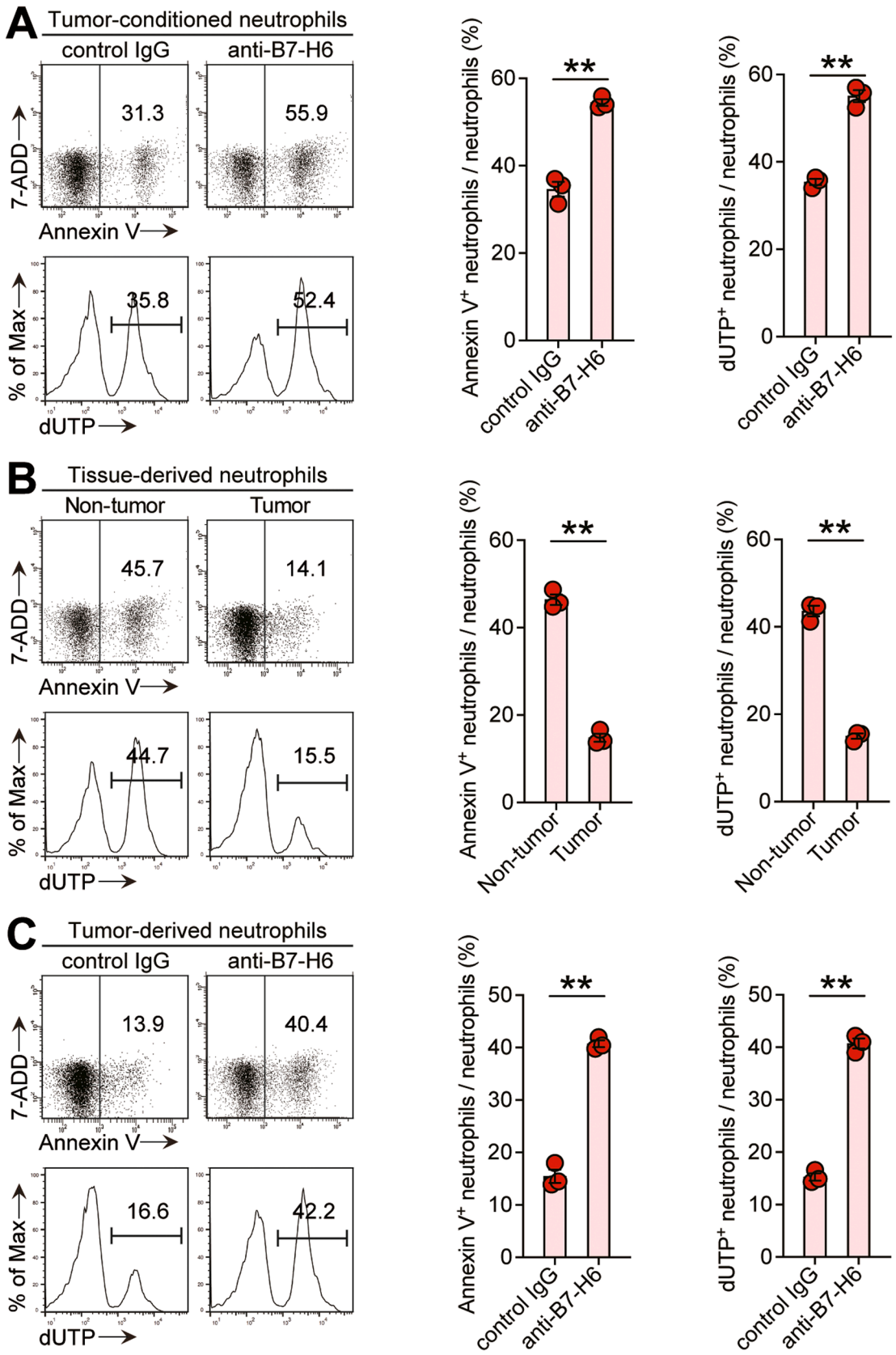

Fig. 6. Blockade of B7-H6 promotes the apoptosis of tumor-conditioned and tumor-derived neutrophils, and shortens their lifespan. (A) Representative data and statistics analysis of annexin V^+ or deoxyuridine triphosphate nucleotides $(dUTP)^+$ apoptotic tumor-conditioned neutrophils exposed to B7-H6 neutralizing antibody or control IgG for 12 h ($n = 3$). (B) Representative data and statistics analysis of annexin V^+ or $dUTP^+$ apoptotic neutrophils derived from autologous tumor and non-tumor tissues cultured for 12 h ($n = 3$). (C) Representative data and statistics analysis of annexin V^+ or $dUTP^+$ apoptotic tumor-derived neutrophils exposed to B7-H6 neutralizing antibody or control IgG for 12 h ($n = 3$). $^{**}P < 0.01$ for groups connected by horizontal lines.

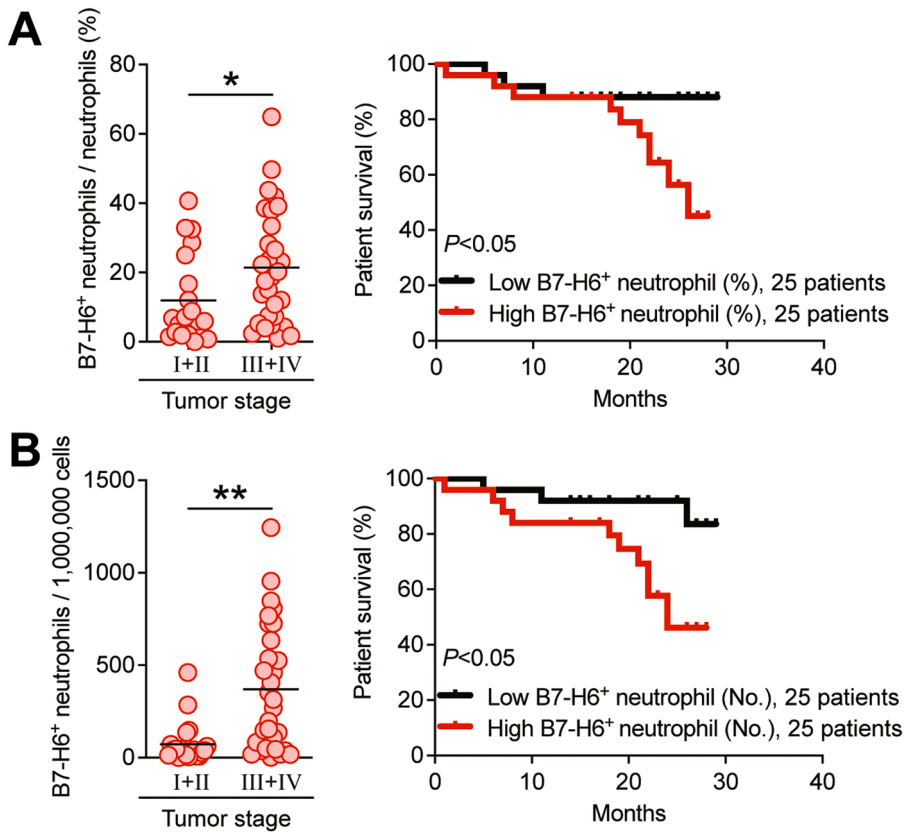

Fig. 7. Intratumoral B7-H6$^+$ neutrophils correlate with advanced tumor stage and poor survival in patients with GC. (A) Intratumoral B7-H6$^+$ neutrophil percentage among TNM stages was compared. Kaplan-Meier plots for overall survival of 50 GC patients by median intratumoral B7-H6$^+$ neutrophil percentage (12.85 %) (25 patients with low or high intratumoral B7-H6$^+$ percentage respectively). (B) Intratumoral B7-H6$^+$ neutrophil number among TNM stages was compared. Kaplan-Meier plots for overall survival of 50 GC patients by median intratumoral B7-H6$^+$ neutrophil number (96 per million) (25 patients with low or high intratumoral B7-H6$^+$ number respectively). The horizontal bars in panels A and B represent mean values. Each ring or dot in panels A and B represents 1 patient. *$P < 0.05$, **$P < 0.01$, n.s $P > 0.05$ for groups connected by horizontal lines.

its regulation, function and clinical relevance in human GC. Based on these previous observations, we have now expanded the profiling of tumor-infiltrating neutrophils, showing that within GC they are phenotypically distinct from their counterparts in blood, peritumoral or non-tumor tissues. Firstly, we confirm that tumor-infiltrating neutrophils exhibit an activated phenotype characterized by the increase of molecule CD54, compared with peripheral, peritumoral or non-tumor cohorts [28], which is consistent with the functional human neutrophils in their adherence, aggregation and phagocytosis [29,30], suggesting that activated phenotype of tumor-infiltrating neutrophils is an accompanying sign with immune-regulatory functions. Furthermore, we identify that these activated neutrophils express high level molecule B7-H6, a new immune-regulatory protein of the B7 family, indicating that the role of tumor-infiltrating neutrophils is likely to be modulating immune function. Most interestingly, we demonstrate that blockade of B7-H6 of tumor-conditioned neutrophils promotes their apoptosis and shortens their lifespan, suggesting that the main role of B7-H6 on tumor-infiltrating neutrophils is most likely to be maintaining survival themselves within the tumor environment.

Survival of neutrophils within the tumor environment is the premise to modulate immune function by these neutrophils. Many mechanisms including autophagy, ferroptosis and apoptosis, are involved in the survival of tumor-infiltrating neutrophils [31]. In hepatocellular carcinoma, increased autophagy sustains the survival of neutrophils to exert pro-tumorigenic effects by releasing matrix metalloproteinase-9 [32]. In breast cancer, neutrophils resist ferroptosis and promote tumor metastasis through aconitate decarboxylase 1 [33]. Here, in human GC, our findings present a novel mechanism by which GC tumor-induced B7-H6 promotes neutrophils survival, likely through inhibiting apoptosis. B7-H6-mediated anti-apoptosis in tumor cells is one of the main mechanisms contributing to tumor progression [34]. In bladder cancer, the proliferation, invasion and migration abilities of tumor cells are significantly inhibited by knocking down the expression of B7-H6, and the cell cycle arrest is induced at the G1 phase, which accelerates the apoptosis [17]. In cervical cancer, B7-H6 knockdown also suppresses the invasive, migratory and proliferative abilities of tumor cells, and promotes G1 cell cycle arrest and apoptosis [35]. In B-cell non-Hodgkin lymphoma, silencing of B7-H6 increases tumor cell apoptosis and sensitivity to vincristine and dexamethasone [36]. In glioma, after knocking down B7-H6, the expression levels of proteins involved in cell apoptosis such as Bcl-2 associated X protein in tumor cells are increased [37]. These data indicate that B7-H6-regulated apoptosis plays an important role in the pathogenesis and chemosensitivity of tumors. Nowadays, it is unclear how B7-H6 might impact the survival of tumor-associated neutrophils. Usually, neutrophil apoptosis is initiated via intrinsic and extrinsic signaling pathways [38]. As for intrinsic pathway of apoptosis, pro-apoptotic dimers of the Bcl-2 family initiates the activation of caspase-9, and caspase-9 drives the activation of caspase-3, which is the central effector of the apoptotic process [39]. On another hand, extrinsic apoptosis pathway is mostly activated via the engagement of cell surface death receptors, which activate caspase-8, similarly leading to the activation of caspase-3 [40]. It has been reported that B7-H6 plays anti-apoptotic roles in tumorigenesis through the signal transducer and activator of transcription 3 (STAT3) pathway [41] that is demonstrated to suppress caspase cascades and block apoptosis initiation by activating apoptosis suppressors in tumor cells [42]. Thus, B7-H6 may play roles of

anti-apoptosis by regulating the caspase cascades in tumor-associated neutrophils, which needs further investigation. In this study, we not only have identified a previously unrecognized role of anti-apoptosis for neutrophil-associated B7-H6 in GC, but also have shown that B7-H6 expression is readily induced upon stimulation by tumor tissue culture supernatants *ex vivo* on neutrophils.

B7-H6 can be expressed on various types of cells. In tumors, B7-H6 is strongly expressed on various tumor cell lines [43] as well as in primary tumor cells such as ovarian carcinoma [13] and breast cancer [44]. Furthermore, it has also been reported that B7-H6 can be expressed on both tumor cells and stromal cells in human ovarian cancer [45]. Mechanistically, B7-H6 expression on tumor cells has been shown to be induced by lipopolysaccharide [37] or regulated by bromodomain-containing protein 4 [46]. In human GC tumors, we are now the first to report the high expression of B7-H6 on tumor-infiltrating neutrophils, which may emphasize the importance of B7-H6-associated pathway in tumor-related immune responses. The up-regulation of B7-H6 often occurs during infection or inflammation. It has been shown that human immunodeficiency virus type 2 infection effectively induces the up-regulation of B7-H6 on human natural killer cells [47]. It has also been shown that B7-H6 expression on $CD14^{+}CD16^{+}$ monocytes can be effectively induced by pro-inflammatory cytokine IL-1β [48]. Our results are consistent with the later study showing cytokine-inducing effect on neutrophils' B7-H6 expression induced by G-CSF in GC. Furthermore, within GC environment, we show a higher production of G-CSF in tumors than that in non-tumor tissues and positive correlations between G-CSF production and B7-$H6^{+}$ neutrophils. Importantly, we further identify G-CSF as a novel pro-inflammatory factor to induce B7-H6 on neutrophils in GC, and show that GC tumor-derived G-CSF effectively activates NF-κB signaling pathway to up-regulate this neutrophil' B7-H6 expression.

Importantly, our findings also shed light on the clinical relevance of B7-$H6^{+}$neutrophils in GC. Specifically, we have shown that increased frequencies and numbers of intratumoral B7-$H6^{+}$neutrophils predict lower rates of GC patient survival. Given that the clinical outcome for GC patients remains poor and that few prognostic factors currently exist for this disease following surgery, intratumoral B7-$H6^{+}$neutrophils may prove useful clinical markers for GC.

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Informed Consent

Not applicable

Ethical Approval

The study was approved by the Ethics Committee of Southwest Hospital of Third Military Medical University (KY202220). Written informed consent was obtained from all patients recruited into the study.

CRediT authorship contribution statement

Pan Wang: Investigation. **Peng Zhu:** Writing – review & editing, Supervision. **Zheng-yan Li:** Resources, Funding acquisition. **Yong-liang Zhao:** Resources. **Fang-yuan Mao:** Investigation, Funding acquisition. **Liu-sheng Peng:** Investigation. **Shou-lu Luo:** Investigation. **Ping Luo:** Investigation. **Yu-gang Liu:** Supervision, Writing – review & editing. **Mao Chen:** Writing – review & editing, Supervision. **Yuan Zhuang:** Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2025.101149.

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