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RAPID COMMUNICATION

Neutrophil extracellular traps induced by interleukin 8 via CXCR1/2 promote the progression of gastric carcinoma through transcription factor IIB-related factor 1 and cyclin

Neutrophils constitute a significant portion of the immune cells present within the tumor microenvironment. Evidence generated by our group has confirmed neutrophils to be an adverse independent prognostic factor affecting the disease-free survival of gastric carcinoma (GC) patients.[1] Neutrophil extracellular traps (NETs), protein-covered DNA webs that interact with tumor cells in the tumor microenvironment, were detected in GC tissues and found to clinically associated with disease progression in our previous study.[2] NETosis could be mediated by a series of agonists, such as interleukin 8 (IL-8).[3] However, the mechanism of IL-8 in the progression of GC occurs via NETs remains unclear. In this study, we identified that IL-8-mediated NETosis can promote the proliferation, migration, and invasion of GC cells *in vitro*, which can be abrogated by NET degradation through deoxyribonuclease I (DNase I) or IL-8 suppression through neutralizing antibodies. Disruption of NETs by DNase I or blockade of the IL-8-CXC chemokine receptor 2 (CXCR2) axis could result in growth retardation in mouse models. We also confirmed that NETs may influence the biological behavior of GC cells by up-regulating the expression of transcription factor IIB-related factor 1 (*BRF1*) and cyclin *p21*/*p27*.

In vitro, confocal microscopy confirmed the presence of NETs containing neutrophils after treatment with IL-8 or CXCL1/2 (IL-8 homologs), which was attenuated by the administration of DNase I or CXCL1/2-neutralizing antibody (Fig. S1, 2A, B). Then, a large number of NETs captured GC cells when IL-8-, phorbol 12-myristate 13-acetate (PMA)-, and granulocyte colony-stimulating factor (G-CSF)-activated neutrophils were cocultured with SGC7901 (Fig. S3A, 2C), BGC823 (Fig. S3B, 2D), MKN45 (Fig. S4A, 2E), and AGS cells (Fig. S2F, 2H), and this effect was abolished by DNase I. Moreover, CXCL1/2 and PMA-induced NETs sequestered MFC cells, and the addition of CXCL1- and/or CXCL2-neutralizing antibodies and DNase I significantly decreased NET formation (Fig. S4B, 2G). Therefore, NETosis induced by IL-8 and its murine homologs led to the entrapment of GC cells, and these traps were destroyed by the administration of DNase I.

Next, species-matched IL-8-(IL-8 homologs), PMA-, and G-CSF-stimulated neutrophil conditional medium was collected and cocultured with GC cells (Fig. S5A). Colony formation and cell counting kit-8 (CCK-8) assays revealed that NETs conditional medium resulted in a significant increase in cell proliferation compared with that in the control group (Fig. S5B–E, 6). Conversely, the degradation of NETs using DNase I to lyse cell-free DNA prior to coculture with GC cells ameliorated this effect. Then, we performed Transwell chamber assays to determine whether NETs promote the invasion of GC cells (Fig. S5F). As expected, coculture with NETs increased the migration and invasion of SGC7901 and BGC823 cells (Fig. S5G, 7A, B), while the ability of NETs to promote the migration and invasion of GC cells was lost when the NET-DNA was digested by DNase I. *In vivo*, we inoculated MFC cells into the right axillary region of BALB/c mice subcutaneously to establish GC mouse models. We observed that tumorigenesis was decreased in DNase I-treated mice, which was demonstrated by significant growth retardation and decreased weights of tumors in the treatment group compared with the control group (Fig. S7C–E). Additionally, the DNase I-treated mice reduced plasma NE-DNA levels and decreased NET formation in

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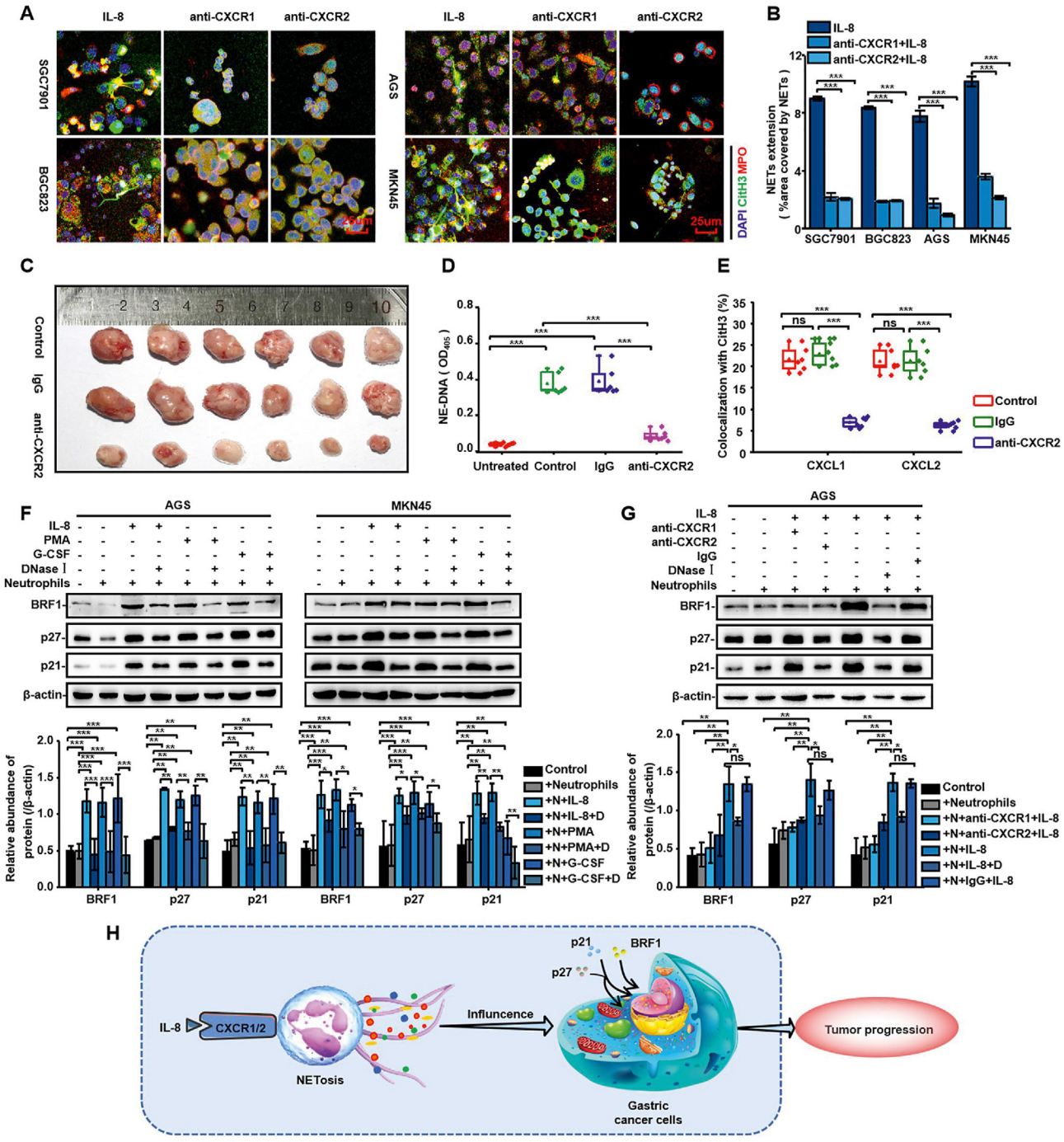

Figure 1 NETs induced by IL-8 via CXCR1/2 promote GC progression through *BRF1* and cyclin *p21*/*p27*. **(A, B)** Blocking CXCR1/2 inhibited IL-8-mediated NETosis. Magnification, 63 × ; scale bar, 25 μm. One-way ANOVA with Tukey's test, ***$P < 0.001$. Data are representative of three experiments (B). **(C)** CXCR2 inhibition resulted in the retardation of tumor growth in the treatment group versus the control and IgG groups. $n = 6$ mice/group. **(D)** CXCR2 inhibition reduced the mean OD_{405} value of plasma NET levels. One-way ANOVA with Tukey's test, ***$P < 0.001$. $n = 6$ mice/group. **(E)** Anti-CXCR2 treatment directly affected the colocalization of IL-8 homologs and CitH3. One-way ANOVA with Tukey's test, ***$P < 0.001$. NS, no significance. **(F)** NETs significantly increased the expression of *BRF1* and *p21*/*p27* in AGS and MKN45, and the effect was abolished by the addition of DNase I. The bar graphs show the quantification results for the Western blot bands. The data shown are representative of three experiments with similar results. One-way ANOVA with Tukey's test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **(G)** CXCR1/2 inhibition of IL-8-mediated NETs reduced the expression of *BRF1* and *p21*/*p27* in AGS cells. The data shown are representative of three experiments with similar results. One-way ANOVA with Tukey's test, *$P < 0.05$, **$P < 0.01$. NS, no significance. **(H)** The mechanisms of NETs as pro-tumorigenic agents that potentiate GC progression.

tumor tissues (Fig. S7F—I). Taken together, our results indicated that the priming of neutrophils toward NETosis promotes the proliferation, migration, and invasion of GC cells *in vitro* and that NET presence promotes tumor growth *in vivo*. The therapeutic targeting of NETs by DNase I can suppress tumor growth.

We also discovered that CXCL1/2 (IL-8 homologs) were highly enriched in mouse tumor tissues (Fig. S8A). Since IL-8 plays a role in different biological processes by binding its receptors CXCR1 and CXCR2, we sought to examine whether NETs function through the IL-8-CXCR1/2 axis in GC progression. We found that CXCR1/2 blockade suppressed IL-8-mediated NETosis (Fig. 1A, B), and CXCR1/2 inhibition ameliorated NET-induced proliferation *in vitro* (Fig. S9A—C). Considering the characteristic that murine CXCL1 and CXCL2 only bind murine CXCR2 *in vivo*, the experimental group mice were treated with anti-CXCR2 antibody via tail vein starting 1 day before tumor inoculation subcutaneously.[4] Mice treated with anti-CXCR2 therapy displayed significantly decreased tumor growth, which was grossly appreciable as smaller and lighter tumors than the control and IgG groups (Fig. 1C; Fig. S9D, E). Besides, there was a significant increase in NETs and circulating plasma levels of NE-DNA 21 days after tumor injection, both of which were attenuated by the administration of anti-CXCR2 (Fig. 1D). Decreased colocalization of CXCL1/2 and CitH3 in tumor tissues with anti-CXCR2 therapy was also observed, which means that the blockade of chemokine receptors can affect the recruitment of the appropriate ligand and subsequently suppress the formation of NETs (Fig. 1E; Fig. S8B). These results suggested that CXCR1/2 inhibition ameliorated the NET-induced proliferative effect and that the IL-8-CXCR1/2 axis mediated NETosis in the GC microenvironment.

Our previous finding demonstrated that high *BRF1* expression and myeloperoxidase-positive neutrophil infiltration in tumor tissues are both associated with poor prognosis in GC.[1] NETosis might be promoted by activating the c-Jun terminal kinase upstream of *BRF1*. These findings led us to speculate that NETs might promote tumor progression by affecting the expression of *BRF1* in GC cells. On the basis that cytoplasmic *p21/p27* drives tumorigenesis via cell cycle acceleration and PMA-activated neutrophils increase *p21* expression in cocultured colon carcinoma cells, we also examined *p21/p27* in GC cells exposed to NET media. Then, we observed that NETs could augment the expression of *BRF1* and *p21/p27* in GC cells. As expected, this up-regulation was disrupted by both DNase I (Fig. 1F) and anti-CXCR1/2 treatment (Fig. 1G). The abrogation of CXCL1/CXCL2-stimulated NETosis in murine neutrophils also had the same effects on MFC cells (Fig. S10A). Similar to the above Western blotting analysis, NETs increased *BRF1* expression in AGS cells at the mRNA level, which was also inhibited by DNase I and anti-CXCR1/2 (Fig. S10B).

In a prostate cancer model, altered neutrophil infiltration was observed within the tumor microenvironment upon elevation of *BRF1*.[5] Here, we designed a Transwell experiment to allow the cocultured GC cells to pass the membrane to the lower chamber (Fig. S8C). We found when *BRF1* knockdown GC cells were cocultured with different stimulant-activated neutrophil medium, the formation of NETosis was decreased in comparison with the untreated and negative control groups (Fig. S11A, 8D, E). Furthermore, transient down-regulation of *BRF1* reduced the proliferation of GC cells (Fig. S11B), and *p21/p27* decreased accordingly (Fig. S11C). Conversely, *BRF1*-overexpressing MKN45 cells promoted NETosis when cocultured with different stimulant-activated neutrophil medium (Fig. S11D, 8F), and the *p21/p27* expression of *BRF1*-overexpressing GC cells was increased (Fig. S11E). Taken together, our results indicate that NETs promote tumor progression by affecting the expression of *BRF1* and cyclin *p21/p27* in GC cells.

Generally, our study demonstrates the importance of IL8-CXCR1/2-mediated NETs in GC progression. NETs promote tumor progression by affecting the expression of *BRF1* and cyclin *p21/p27* in GC cells (Fig. 1H). DNase I and anti-CXCR2 therapy administration can effectively inhibit tumor growth, indicating that they may be novel therapeutic interventions for GC patients.

Ethics declaration

The studies involving human donors were reviewed and approved by the Ethics Committee of Anhui Medical University (No. 20200089). All animal experiments were approved by the Institutional Animal Care and Use Committee of Anhui Medical University (No. LLSC20200149).

Author contributions

KG and HW conceived and supervised the study. QW and YZ designed and conducted experiments. WD, YW, CF, and XL analyzed and interpreted the data. Some experiments were supervised by WD, XW, HW, and ZQ. QW and YZ wrote the manuscript. All authors reviewed, edited, and approved the final manuscript.

Conflict of interests

The authors have declared no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.03.025.

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