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Metastatic growth instructed by neutrophil-derived transferrin

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The tumor-promoting functions of neutrophils have been mainly attributed to induction of tumor angiogenesis or suppression of anticancer immunity. However, a direct impact of neutrophils on tumor cell growth and metastasis remains largely uncharacterized. Here, we coupled a proteomic approach with a functional screen to interrogate the secretome of tumor-associated neutrophils. Surprisingly, the iron-transporting protein transferrin was identified as the major mitogen for tumor cells secreted by neutrophils. Depletion of neutrophils inhibited lung metastasis and transferrin production in the metastatic microenvironment. Deletion of transferrin receptor suppressed growth of lung-colonizing tumor cells. Also, media conditioned by neutrophils isolated from metastatic breast cancer patients stimulated growth of human breast cancer cells, an effect that was largely abolished by transferrin immunodepletion. We identified GM-CSF, which is produced primarily by tumor cells, as a selective inducer of de novo transferrin synthesis in neutrophils through the Jak/Stat5β pathway. GM-CSF neutralization or inhibition of Jak kinases curtailed neutrophil transferrin expression in vitro and in vivo as well as cancer metastasis. Thus, transferrin provides a mechanistic link between neutrophils and metastatic growth owing to the ability of tumor-infiltrating neutrophils to locally deliver this growth-promoting protein in response to GM-CSF stimulation. Our study identifies neutrophil-derived transferrin as a key regulator of metastatic tumor cell growth and a therapeutic target for antimetastatic treatment.

innate immunity | metastasis | tumor microenvironment | breast cancer | GM-CSF

Growing evidence suggests that neutrophils, an important component of the innate immunity, are actively involved in the development of cancer-associated inflammation and contribute to tumor growth (1), metastasis (2–5), and resistance to anticancer therapies (3, 6) (reviewed in ref. 7). Although some studies have also revealed tumoricidal properties of neutrophils (8), increased neutrophil levels in cancer patients often predict poor clinical outcomes. An elevated neutrophil-to-lymphocyte ratio (NLR) is associated with not only advanced tumor stages (9), higher risk of cancer recurrence (9), metastasis (9, 10), and worse patient survival (11), but also with lower response rates to cancer treatments (12). Moreover, presence of tumor-infiltrating neutrophils was reported to be associated with tumor metastasis and worse patient survival (13).

Given the importance of neutrophils in innate immunity (14), complete suppression of this cell population renders patients vulnerable to infections. A promising alternative is to identify the effectors that mediate the tumor-supporting functions of neutrophils and design therapeutics that specifically target these effectors. Previous work has recognized the immunosuppressive and proangiogenic functions of neutrophils in the tumor microenvironment (15–17). A direct impact of neutrophils on tumor cells, however, remains largely uncharacterized.

In this study, we decided to address this question using a systematic approach, by analyzing the secretome of neutrophils isolated from tumor-bearing mice. The cell secretome is comprised of proteins that are secreted by the cells into the extracellular environment and are responsible for cell–cell or cell–matrix communication (18). Recent studies have emphasized the value of the cancer secretome as a source of biomarkers and drug targets (19). However, little is known about the secretome of tumor-associated stromal cells. Characterizing the secretome of stromal cells may provide insights on the crosstalk between tumor and stromal cells and may also result in drug targets for cancer therapy. Here, we coupled proteomic profiling with a functional screen to interrogate the secretome of tumor-associated neutrophils, to identify potential neutrophil-derived mitogenic factors for tumor cells.

Results

Neutrophils Promote Cancer Metastasis Without Involvement of Adaptive Immunity and Tumor Angiogenesis. We previously reported that depletion of neutrophils through G-CSF neutralization inhibits lung metastasis of 4T1 mouse breast cancer cells (2). We confirm here that anti–G-CSF significantly curtails aberrant neutrophil accumulation in peripheral blood and lung of 4T1 tumor-bearing BALB/c mice (*SI Appendix*, Fig. S1 *A* and *B*) and suppresses lung metastasis (*SI Appendix*, Fig. S1*C*). No inhibition of tumor angiogenesis was observed in response to anti–G-CSF treatment in primary tumors or in metastatic lung tissues (*SI Appendix*, Fig. S1*D*), suggesting that modulation of tumor angiogenesis does not contribute to the prometastatic functions of neutrophils in our model. Subsets of neutrophils (MDSC) have been characterized as suppressors of immune responses (20), and this property has been frequently implicated in the protumoral functions of neutrophils (7). Interestingly, in $Rag1^{-/-}$ immunodeficient mice, metastatic development from 4T1 mammary tumors (*SI Appendix*, Fig. S1*E*) or following surgical resection of s.c. 3LL (Lewis lung carcinoma) tumors (*SI Appendix*, Fig. S1*F*) was inhibited by anti–G-CSF–

Significance

The current study uncovers a mechanistic link between neutrophils and cancer metastasis. Transferrin, an iron-transporting protein, is expressed at mRNA and protein levels in mouse and human neutrophils and is mainly responsible for neutrophil-secreted mitogenic activity on tumor cells. GM-CSF, a cytokine largely produced by tumor cells in the metastatic microenvironment, selectively acts on neutrophils to enhance transferrin gene expression, through activation of the Jak/Stat5β pathway. Accordingly, deletion of the transferrin receptor (Tfr1) in tumor cells, blockade of GM-CSF, or inhibition of Jak kinases inhibited mouse tumor lung metastasis. Our findings raise the possibility that inhibiting GM-CSF or the Jak/Stat5b pathway may benefit patients with metastatic diseases and high local neutrophil infiltration.

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The authors declare no conflict of interest.

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mediated neutrophil depletion. These findings indicate that neutrophils may also promote cancer metastasis through mechanisms unrelated to suppression of the adaptive immune system. Accordingly, neutrophil accumulation, but not microvessel density in the lungs, was decreased by anti–G-CSF in mice that have primary 3LL tumors surgically removed (*SI Appendix*, Fig. S1*G*).

Characterization of Neutrophil Secretome. To explore the possibility that neutrophils directly promote metastatic tumor cell growth, we prepared serum-free conditioned media (CM) from Ly6g+ neutrophils isolated from the spleens of 4T1 tumor-bearing mice. Proteins in the neutrophil CM were purified by sequential chromatographic steps and tested for their ability to stimulate 4T1 tumor cell growth as described in *Materials and Methods* (Fig. 1*A*). At each purification step, only one peak of absorbance, composed of three to four contiguous fractions, showed mitogenic activity. After the last reverse-phase column, the mitogenic fractions (38, 39, and 40), the adjacent negative (37 and 41), and distant negative (32 and 46) fractions (Fig. 1*B*) were subjected to mass spectrometry analyses. A list of 25 candidate proteins (*SI Appendix*, Fig. S2*A*) was generated (see *Materials and Methods*). Most of these proteins were intracellular and likely released into the CM due to cell death and thus were excluded from further investigation. Among the secreted proteins, only transferrin [recombinant human transferrin or mouse apo-transferrin (iron-free)] (Fig. 1*C*), but not hepatocyte growth factor (HGF), macrophage migration inhibitory factor (MIF), orosomucoid 1, or galectin-1 (*SI Appendix*, Fig. S2*B*), was able to stimulate 4T1 cell growth. Transferrin is an 80-kDa glycoprotein responsible for transporting iron into proliferative cells via binding to transferrin receptor (Tfr1) (21). Interestingly, lactoferrin, another iron-binding protein that shares structural similarity with transferrin, does not stimulate 4T1 cell growth (Fig. 1*C*). Indeed, transferrin levels in the eluted fractions were tightly correlated with the mitogenic activity (Fig. 1*D*). Transferrin was then immunodepleted from the mitogenic fractions, which resulted in almost complete loss of the activity (Fig. 1*E*). Moreover, Tfr1 deletion in 4T1 cells abrogated the mitogenic responses to recombinant transferrin or the bioactive fraction (Fig. 1*F*). Together, these results indicate that transferrin is responsible for the mitogenic activity of neutrophil secretome on tumor cells.

Presence of transferrin in pulmonary neutrophils was further verified by intracellular flow cytometry analyses (*SI Appendix*, Fig. S2*C*). It is noteworthy that transferrin was undetectable without the fixation/permeabilization step (*SI Appendix*, Fig. S2*D*), arguing against the possibility that the staining was due to absorption of extracellular transferrin on neutrophil cell membrane. Moreover, colocalization of transferrin and Ly6g staining in lung tissues of 4T1 tumor-bearing mice was visualized by immunostaining (*SI Appendix*, Fig. S2*E*).

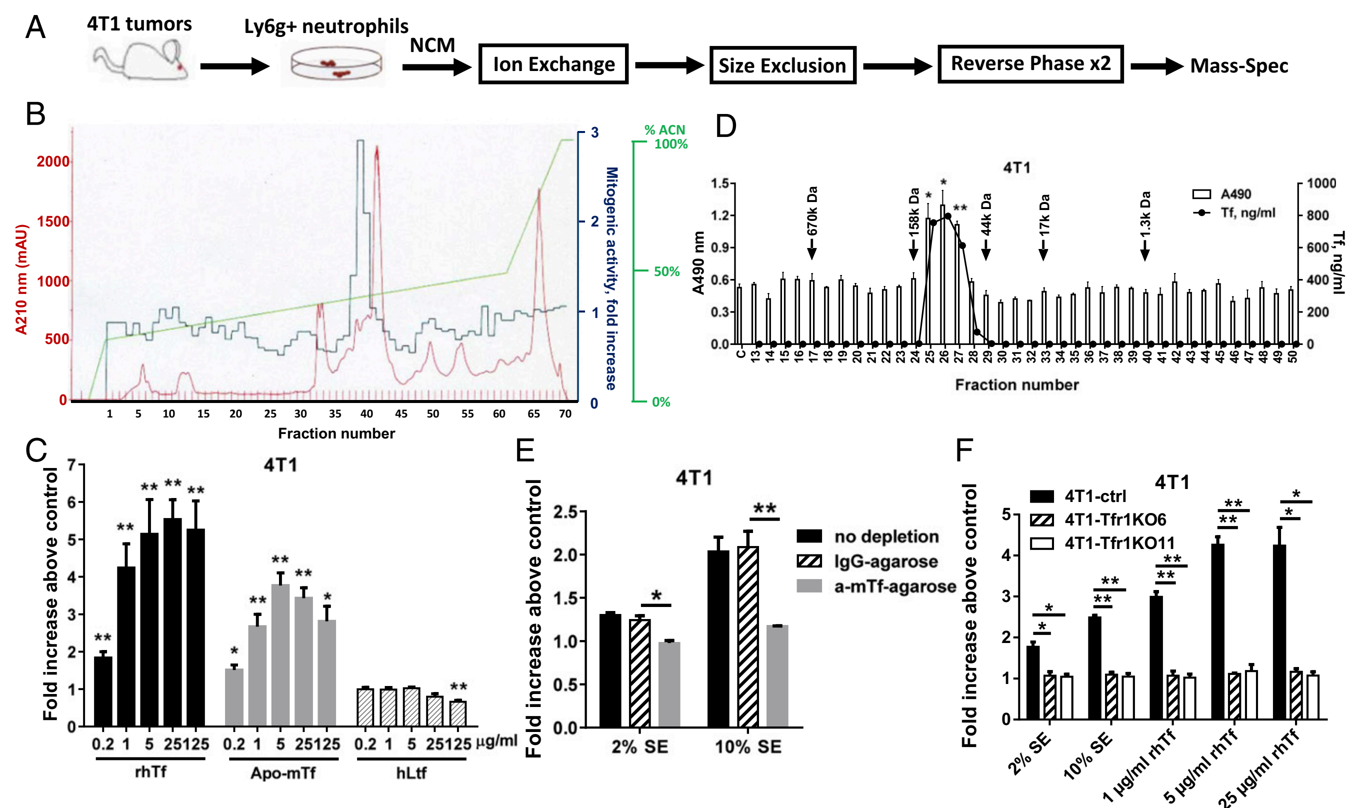

Fig. 1. Transferrin mediates the mitogenic activity of neutrophil secretome on tumor cells. (*A*) Neutrophil conditioned medium (NCM) was purified by sequential chromatographic steps. At each step, fractions were tested for mitogenic activity on 4T1 cells. (*B*) Mitogenic activity of fractions from the last reverse-phase column. Green line indicates the concentration of acetonitrile in the elution buffer. Red line indicates absorbance at 210 nm (A210 nm) in eluted fractions. Blue line indicates the mitogenic activity. (*C*) Induction of 4T1 cell growth with recombinant human transferrin (rhTf), apo-mouse Tf (Apo-mTf), and human lactoferrin (hLtf). Results are expressed as fold-changes compared with PBS treatment, $n = 3$. (*D*) Alignment between 4T1 cell growth (bar, measured at A490 nm) and transferrin concentrations in the fractions (line) from size-exclusion chromatography of bioactive HiTrap Q fractions, $n = 3$. Arrows indicate molecule weight markers. (*E*) The mitogenic fraction from size-exclusion (SE) chromatography was immunodepleted by a-mTf agarose, IgG-agarose, or left untreated (no depletion). The effects of these treatments on 4T1 cell growth were tested. Results are expressed as fold-changes compared with 4T1 cells without any treatment, $n = 3$. (*F*) The 4T1 cells with (4T1-Tfr1KO6 and 4T1-Tfr1KO11) or without (4T1-ctrl) Tfr1 gene deletion were treated with rhTf or the mitogenic size-exclusion fraction (SE). Results on cell growth are expressed as fold-changes compared with controls, $n = 3$. *$P < 0.05$; **$P < 0.01$.

We compared lung transferrin production between tumor-free and 4T1 tumor-implanted mice, with or without anti–G-CSF treatment, by measuring transferrin levels in whole lung cell conditioned medium (LCM). Our results show that LCM from tumor-bearing mice contains significantly higher levels of transferrin than tumor-free mice (*SI Appendix*, Fig. S2*F*). Depletion of neutrophils from tumor-bearing mice significantly reduced transferrin levels in LCM (*SI Appendix*, Fig. S2*F*).

Tfr1 Is Essential for Metastatic Growth. Stable overexpression of Tfr1 in 4T1 cells significantly promoted tumor growth (*SI Appendix*, Fig. S3*A*) and lung metastasis (*SI Appendix*, Fig. S3*B*). Conversely, Tfr1 deletion significantly inhibited primary tumor growth (*SI Appendix*, Fig. S3*C*) and lung metastasis (*SI Appendix*, Fig. S3*D*). In both experiments, lung metastasis was examined when primary tumors from control and mutant groups reached comparable sizes. Tfr1 is critical for metastatic growth but not for lung colonization of metastatic tumor cells, since Tfr1-deleted tumor cells possessed equivalent ability to colonize lung, yet showed severely compromised subsequent cell proliferation (*SI Appendix*, Fig. S4). In the 3LL model, Tfr1 deletion abrogated transferrin-induced tumor cell growth in vitro (*SI Appendix*, Fig. S5*A*) and inhibited lung metastasis of tail vein-injected tumor cells (*SI Appendix*, Fig. S5*B*).

We compared Tfr1 expression in human breast cancer versus normal tissues, by analyzing data deposited in Oncomine (22). Using the thresholds described in *Materials and Methods*, upregulation of Tfr1 mRNA in breast cancer (compared with normal tissues) was found in 9 of the 14 eligible datasets. Moreover, higher Tfr1 mRNA levels were associated with higher tumor grades/stages (*SI Appendix*, Fig. S6*A*). The prognostic value of Tfr1 gene in breast cancer was analyzed by the Kaplan–Meier Plotter (23). Elevated Tfr1 gene expression predicted significant worsening of overall survival (OS; $n = 1402$, $P = 0.0066$) and distant metastasis-free survival (DMFS; $n = 1746$, $P = 0.02$) in breast cancer patients (*SI Appendix*, Fig. S6*B*).

GM-CSF Selectively Induces Transferrin Production in Neutrophils. The ability of pulmonary neutrophils to secrete transferrin increased as metastasis progressed. Neutrophils isolated during the late stage (4 wk posttumor implantation) of metastatic progression secreted significantly more transferrin compared with neutrophils at the premetastatic (2 wk) or early (3 wk) stages (*SI Appendix*, Fig. S7*A*). This finding raises the possibility that transferrin expression in neutrophils may be modulated by tumor cells. Indeed, while 4T1 cell conditioned medium (4T1CM) contains no transferrin, it can stimulate transferrin secretion from Ly6g+ neutrophils (*SI Appendix*, Fig. S7*B*). To identify the factor(s) potentially implicated in such induction, a panel of 31 cytokines/growth factors was screened for the ability to stimulate neutrophil transferrin secretion. These cytokines were chosen based on expression by 4T1 cells and/or involvement in breast cancer progression (24, 25). We found that only GM-CSF and IL-3 significantly increased transferrin levels in neutrophil CM (Fig. 2*A*). Intriguingly, the receptors for GM-CSF and IL-3 share a common beta subunit (CSFR2b) (26), implying a similar mechanism by which these two cytokines regulate transferrin expression. Immunoneutralization of GM-CSF, but not IL-3, significantly suppressed recombinant GM-CSF or 4T1 CM-induced neutrophil transferrin production (*SI Appendix*, Fig. S7*B*). Combination of anti–GM-CSF and anti–IL-3 neutralizing antibodies did not further reduce transferrin levels, compared with anti–GM-CSF alone (*SI Appendix*, Fig. S7*B*). Moreover, GM-CSF neutralization significantly inhibited recombinant GM-CSF or 3LL CM-induced transferrin production, by neutrophils isolated from 3LL tumor-bearing mice (*SI Appendix*, Fig. S7*C*). These data indicate that in our models, GM-CSF is the major tumor-secreted factor that induces neutrophil transferrin production. Notably, neither LPS nor fMLP, a bacterial chemotactic peptide, stimulated transferrin production from mouse neutrophils (Fig. 2*A*). GM-CSF induced transferrin secretion in a time-dependent manner (*SI Appendix*, Fig. S7*D*). Furthermore, GM-CSF enhanced transferrin levels in LCM prepared from 4T1 tumor-bearing mice, but not tumor-free mice or neutrophil-depleted tumor-bearing mice (Fig. 2*B*). Transferrin expression was previously reported in macrophages (27) and hepatoma cells (28). Interestingly, GM-CSF treatment did not affect transferrin production from bone marrow-derived macrophages (BMMs), F4/80+ pulmonary macrophages isolated from 4T1 tumor-bearing mice, or Hepa1-6 hepatoma cells (*SI Appendix*, Fig. S7*E*).

To determine the cellular localization of GM-CSF in the metastatic microenvironment, we purified Ly6g+ neutrophils, F4/80+ macrophages, B220+ B cells, CD4+ and CD8+ T cells, CD45+ hematopoietic cells, and CD45− cells (metastatic tumor cells and nonhematopoietic stromal cells) from lungs of 4T1 tumor-bearing mice and measured GM-CSF mRNA levels in these cell types as well as in cultured 4T1 tumor cells. GM-CSF mRNA was expressed by all of the cell types examined here (Fig. 2*C*), yet the highest levels were in CD45− cells and cultured 4T1 tumor cells (Fig. 2*C*). Immunostaining of perfused lung sections confirmed that GM-CSF is expressed by both metastatic tumor cells and stromal cells (Fig. 2*C*).

We investigated whether blockade of GM-CSF affects transferrin levels and cancer metastasis. Given the multifaceted function of GM-CSF in various immune cells, we used $Rag1^{-/-}$ immunodeficient mice to eliminate the effects of GM-CSF neutralization on adaptive immunity. Treatment of 4T1 (Fig. 2*D*) or 3LL (*SI Appendix*, Fig. S8*A*) tumor-bearing $Rag1^{-/-}$ mice with anti–GM-CSF inhibited transferrin production in the lung. To emphasize the effect of GM-CSF on metastatic growth, primary 4T1 or 3LL tumors were first established and then surgically removed and treated with anti–GM-CSF or isotype control. Anti–GM-CSF significantly inhibited metastatic development (Fig. 2*E* and *SI Appendix*, Fig. S8*B*).

GM-CSF Induces de Novo Synthesis of Transferrin mRNA and Protein. Despite the fact that neutrophils are terminally differentiated and are considered inert in gene expression, transferrin mRNA was detectable in freshly isolated Ly6g+ neutrophils to a level comparable to BMMs, J774 macrophages, and F4/80+ lung macrophages and significantly higher than CD4+, CD8+, or B220+ lymphocytes (*SI Appendix*, Fig. S9*A*). The positive control, the Hepa1-6 hepatoma cell line, had the highest expression. Notably, transferrin mRNA expression was absent in 4T1 tumor cells and was significantly higher in CD45+ versus CD45− cells in metastatic lungs (*SI Appendix*, Fig. S9*A*), suggesting that transferrin in the metastatic microenvironment is mainly expressed by the stromal cells rather than tumor cells. Using two sets of real-time PCR primers that target different regions of transferrin mRNA, we found that GM-CSF induces transferrin (but not lactoferrin) mRNA in a time-dependent manner and that the induction is markedly stronger than that elicited by G-CSF (*SI Appendix*, Fig. S9*B*). GM-CSF can also stimulate transferrin expression in FACS-sorted neutrophils (*SI Appendix*, Fig. S9*C*). It is noteworthy that induction of transferrin mRNA was not observed in neutrophils treated with Q-VD.OPH, a pan-caspase inhibitor that promotes neutrophil survival to a level comparable to or higher than GM-CSF treatment (*SI Appendix*, Fig. S10). Additionally, combination of GM-CSF and Q-VD.OPH treatment did not further enhance transferrin gene expression, compared with GM-CSF alone (*SI Appendix*, Fig. S10). Thus, the induction of transferrin mRNA by GM-CSF is not likely due to improved neutrophil survival.

To investigate the requirement of gene transcription or translation for GM-CSF–induced transferrin production, Ly6g+ neutrophils were treated with actinomycin D (ActD) or cycloheximide (CHX) to block gene transcription or translation, respectively. Treatment with either ActD or CHX significantly inhibited GM-CSF–induced transferrin protein production (*SI Appendix*, Fig. S9*D*), suggesting that the increased transferrin production requires

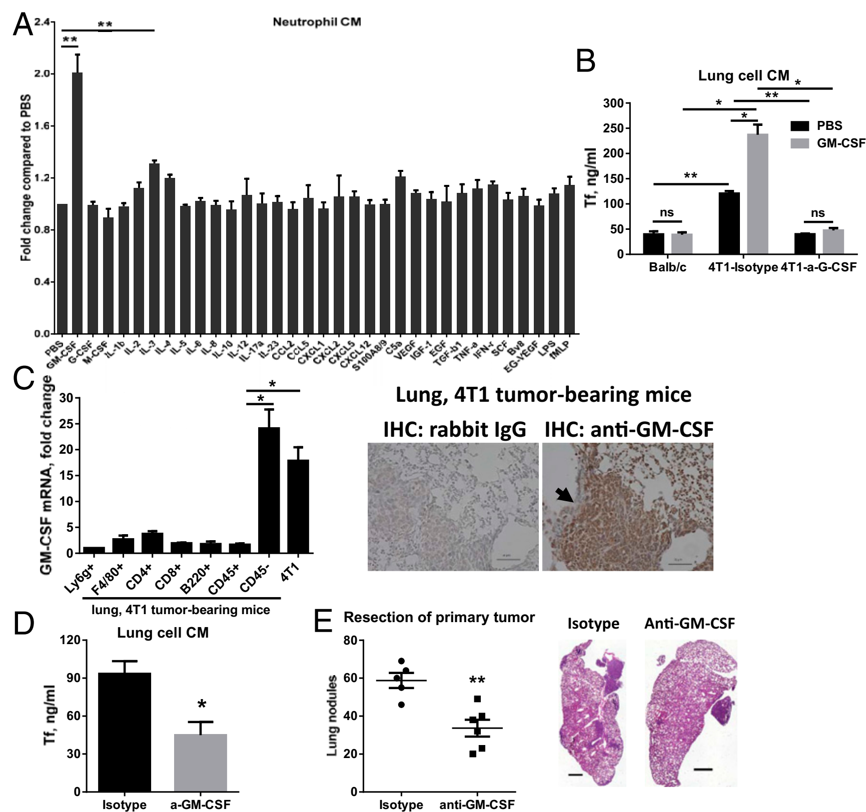

Fig. 2. GM-CSF promotes neutrophil transferrin production and cancer metastasis. (*A*) Ly6g+ splenic neutrophils were treated for 12 h, and transferrin levels in neutrophil CM were determined by ELISA, $n = 3$. C5α, complement component 5α. (*B*) Lung cell CM was prepared from tumor-free BALB/c or 4T1 tumor-bearing mice with or without a-G-CSF treatment. GM-CSF or PBS was added during in vitro culture. Transferrin levels were determined by ELISA, $n = 3$. (*C*, *Left*) Real-time PCR analyses of GM-CSF mRNA in isolated lung cell populations and 4T1 tumor cells. Expression levels in Ly6g+ neutrophils were set to 1, $n = 3$. (*C*, *Right*) Immunostaining of GM-CSF in perfused lung sections of 4T1 tumor-bearing mice. Arrow indicates metastatic tumors. (Scale bar, 50 μm.) (*D*) The 4T1 tumor-bearing Rag1$^{-/-}$ mice were treated with isotype or an anti–GM-CSF neutralizing antibody. Transferrin levels in lung cell CM were determined by ELISA, $n = 4$. (*E*) Rag1$^{-/-}$ mice with primary 4T1 tumors surgically removed were treated with isotype or anti–GM-CSF. (*Left*) Quantification of metastatic lung nodules, $n = 5–6$. (*Right*) Representative H&E staining of lung sections. (Scale bar, 1,000 μm.) $*P < 0.05$; $**P < 0.01$; ns, not statistically significant.

de novo synthesis of transferrin mRNA and subsequent translation. Moreover, ActD, but not CHX, significantly inhibits up-regulation of transferrin mRNA by GM-CSF treatment (*SI Appendix*, Fig. S9*D*), indicating that GM-CSF–initiated signaling directly stimulates transcription of transferrin gene, without involving translation of a secondary protein. These findings show that the transferrin gene is actively transcribed and translated in neutrophils.

Neutrophil Transferrin Expression Is Governed by the Jak/Stat5β Pathway. To further dissect the mechanisms of GM-CSF–induced transferrin expression, Ly6g+ neutrophils were treated with inhibitors of various signaling pathways before GM-CSF stimulation, and transferrin mRNA levels were then examined. GM-CSF–induced transferrin expression was dramatically inhibited by two Jak kinase inhibitors (AZD1480 and baricitinib) (Fig. 3*A*), but not by inhibitors of the MEK (cobimetinib), Akt/mTOR (dactolisib), Src (dasatinib), NF-κb (Bay 11–7082 and SC-514), p38 (SB203580), and JNK (SP600125) pathways (*SI Appendix*, Fig. S9*E*). Indeed, GM-CSF stimulated phosphorylation of Stat5, a downstream substrate of Jak kinases, which was inhibited by AZD1480 (Fig. 3*B*). In comparison, treatment of neutrophils with G-CSF only stimulated p-Stat3, but not p-Stat5 (Fig. 3*B*). Based on these results, we hypothesized that GM-CSF acts through the Jak/Stat5 signaling pathway to regulate neutrophil transferrin expression.

To test this possibility, we utilize MPRO cells, a promyelocytic leukemia cell line that is capable of differentiating into neutrophils upon treatment with all-trans retinoid acid (ATRA). MPRO neutrophils were transfected with siRNAs to selectively knockdown expression of Jak family members, Stat3, Stat5α, or Stat5β. Knockdown efficiency was confirmed through real-time PCR and/or Western blot analyses (*SI Appendix*, Fig. S11 *A* and *B*). Our results demonstrate that silencing Jak1, Jak2, or Stat5β, but not Jak3, Tyk2, Stat5α, or Stat3, significantly inhibits GM-CSF–induced transferrin expression (Fig. 3*C*). This is consistent with a previous report that GM-CSF only induces DNA binding of Stat5β, but not Stat5α, in human neutrophils (29). As expected, silencing Jak1, Jak2, or Stat5β suppressed GM-CSF–induced p-Stat5 (*SI Appendix*, Fig. S11*C*). Therefore, GM-CSF induces neutrophil transferrin expression through activation of Jak1/2 and Stat5β.

Induction of transferrin gene expression by the Jak/Stat5β pathway is likely neutrophil-specific. Transferrin expression did not change when Hepa1-6 hepatoma cells were transfected with siRNAs targeting Jak1, Jak2, or Stat5β (*SI Appendix*, Fig. S11*D*). In contrast, siRNA against HNF4α, a known transcription factor for hepatocyte transferrin expression (30), inhibited transferrin gene expression in Hepa1-6 cells (*SI Appendix*, Fig. S11*D*), but not in neutrophils (Fig. 3*C*).

We then investigated the impact of Jak kinase inhibition on neutrophil transferrin expression in vivo. Treatment of 4T1 (Fig. 3*D*) or 3LL (*SI Appendix*, Fig. S8*C*) tumor-bearing mice with 50 mg/kg AZD1480 inhibited p-Stat5 and transferrin expression in neutrophils and transferrin production in the lungs. Accordingly, metastatic growth after surgical removal of primary 4T1 (Fig. 3*E*) or 3LL (*SI Appendix*, Fig. S8*D*) tumors was significantly delayed by AZD1480 treatment, consistent with a previous report (31).

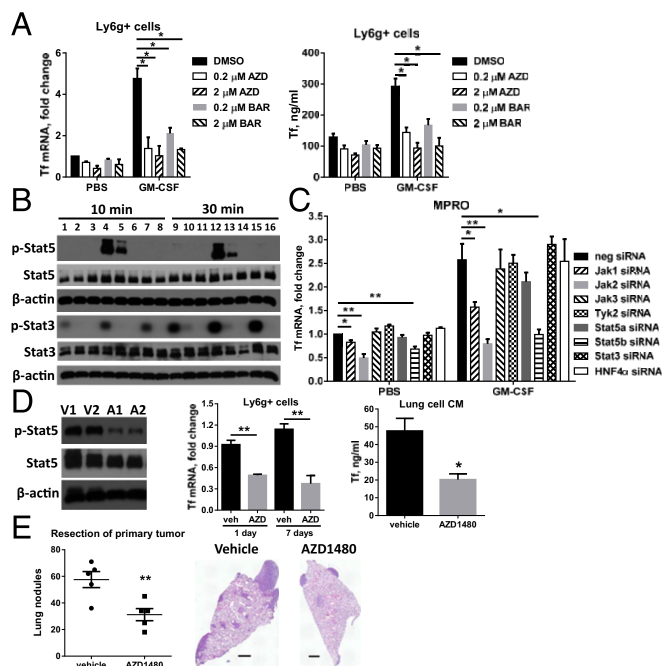

Fig. 3. Neutrophil transferrin expression is governed by the GM-CSF/Jak/Stat5β pathway. (*A*) Ly6g+ splenic neutrophils were treated with DMSO, AZD1480, or baricitinib (BAR), in the presence or absence of GM-CSF for 6 h (for Tf mRNA expression, *Left*) or 12 h (for Tf ELISA, *Right*), *n* = 3. (*B*) Western blot analyses of splenic neutrophil cell lysates. Lanes 1 and 9, PBS+DMSO; 2 and 10: PBS+0.2 μM AZD1480; 3 and 11: PBS+2 μM AZD1480; 4 and 12: GM-CSF+DMSO; 5 and 13: GM-CSF+0.2 μM AZD1480; 6 and 14: GM-CSF+2 μM AZD1480; 7 and 15: G-CSF+DMSO; 8 and 16: G-CSF+2 μM AZD1480. Cytokines were added for either 10 min (lanes 1–8) or 30 min (lanes 9–16). (*C*) ATRA-differentiated MPRO neutrophils were transfected with indicated siRNAs for 72 h before treatment with PBS or GM-CSF for 6 h. Transferrin mRNA levels were analyzed by real-time PCR. Expression levels in PBS and negative (neg) siRNA-treated cells were set to 1, *n* = 3. (*D*) The 4T1 tumor-bearing mice were treated with vehicle or 50 mg/kg AZD1480. (*Left*) Western blot analyses of isolated lung neutrophils. A, AZD1480; V, vehicle. Results from two mice per group are shown. (*Middle*) Transferrin mRNA levels in Ly6g+ neutrophils isolated after 1 or 7 d of AZD1480 treatment, *n* = 3. (*Right*) Transferrin levels by ELISA in lung cell CM prepared from vehicle or AZD1480-treated mice, *n* = 3. (*E*) Mice with surgically removed primary 4T1 tumors were treated with vehicle or 50 mg/kg AZD1480. (*Left*) Quantification of metastatic lung nodules, *n* = 5. (*Right*) Representative H&E staining of lung sections. (Scale bar, 1,000 μm.) $^*P < 0.05$; $^{**}P < 0.01$.

Transferrin Secreted by Human Neutrophils Stimulates Tumor Cell Growth. We isolated neutrophils from peripheral blood of breast cancer patients with metastatic diseases. We found that human neutrophils also express transferrin mRNA and protein, which can be augmented by GM-CSF and suppressed by Jak kinase inhibitors (Fig. 4*A*). GM-CSF also induced transferrin production in neutrophils from nonmetastatic breast cancer patients and healthy donors (*SI Appendix*, Fig. S12*A*). GM-CSF stimulated p-Stat5 in human neutrophils, which was abolished by AZD1480 (Fig. 4*B*). TNF-α, LPS, and fMLP also significantly stimulated transferrin production from human neutrophils (*SI Appendix*, Fig. S12*B*). MDA-MB-231 tumor cell conditioned media enhanced transferrin production by human neutrophils, which was significantly suppressed by anti–GM-CSF (Fig. 4*C*). Treatment with human neutrophil CM stimulated growth of human breast cancer cells, an effect that was largely abolished by immunodepletion of transferrin (Fig. 4*D* and *SI Appendix*, Fig. S12*C*). Knockout of Tfr1 expression in SUM159 cells dramatically inhibited human neutrophil CM-induced SUM159 cell growth (Fig. 4*D*). Collectively, these results demonstrate that transferrin produced by human neutrophils acts through Tfr1 to stimulate tumor cell growth.

Discussion

Analysis of the secretome of tumor-associated neutrophils led us to discover that the iron-transporting protein transferrin is largely responsible for neutrophil-derived mitogenic activity on tumor cells. This finding substantiates the hypothesis that neutrophils cannot only promote tumor angiogenesis and suppress anticancer immunity but can also directly stimulate tumor cell growth. Depletion of neutrophils or deletion of transferrin receptor in tumor cells inhibited lung metastasis in our mouse models. Another key finding of our study was the identification of a neutrophil-specific mechanism for regulation of transferrin gene expression. We found that GM-CSF can induce transferrin expression through activation of the Jak/Stat5β pathway. Accordingly, GM-CSF neutralization or Jak inhibition suppressed neutrophil transferrin expression and mouse tumor lung metastasis.

Our understanding of the role of GM-CSF in cancer has considerably evolved. While initial studies emphasized the anticancer function of GM-CSF through supporting maturation and activation of dendritic cells, macrophages, and other aspects of the anticancer immunity, accumulating evidence has revealed numerous mechanisms through which GM-CSF facilitates cancer development and progression. GM-CSF expression levels were found to be positively correlated with the metastatic potential of tumor cell lines (32). Recently, another study reported that obesity induces pulmonary neutrophilia that promotes breast cancer metastasis, in a GM-CSF– and IL-5–dependent manner (33). However, the mechanism by which GM-CSF and neutrophils promote metastasis in that context remains unclear. Here, we show that GM-CSF stimulates expression of transferrin, a potent tumor cell mitogen, in neutrophils. Anti–GM-CSF treatment reduces transferrin production in the lung

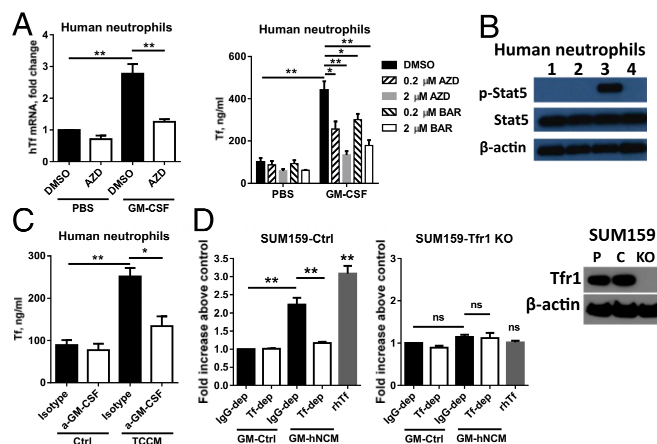

Fig. 4. Transferrin secreted by neutrophils isolated from metastatic breast cancer patients stimulates tumor cell growth. (*A*) Transferrin mRNA (*Left*) and protein (*Right*) analyses in circulating human neutrophils treated with DMSO, AZD1480, or baricitinib (BAR), in the presence or absence of GM-CSF, *n* = 3. (*B*) Western blot analyses of human neutrophils. Lane 1, PBS+DMSO; 2, PBS+2 μM AZD1480; 3, GM-CSF+DMSO; 4, GM-CSF+2 μM AZD1480. (*C*) Human neutrophils were treated with control media (Ctrl) or MDA-MB-231 CM (TCCM) in the presence or absence of anti-human GM-CSF. Transferrin release was determined by ELISA, *n* = 3. (*D*) Media conditioned by GM-CSF–stimulated human neutrophils (GM-hNCM) or GM-CSF–supplemented control media (GM-Ctrl) was immunodepleted by anti-hTf (Tf-dep) or an isotype IgG (IgG-dep). SUM159-Ctrl (*Left*) or SUM159-Tfr1KO (*Middle*) cells were treated as indicated. Twenty-five micrograms per milliliter rhTf was added as positive controls, *n* = 3. (*Right*) Western blot analyses of Tfr1 in parental (P), control (C), and Tfr1-knocked out (KO) SUM159 cells. $^*P < 0.05$; $^{**}P < 0.01$; ns, not statistically significant.

and inhibits cancer metastasis. In light of our data and previous observations (2), it is conceivable that G-CSF and GM-CSF work in concert to promote the prometastatic functions of neutrophils: primary tumor-secreted G-CSF systemically increases neutrophil production and recruitment to the metastatic sites (e.g., lung). GM-CSF, expressed mainly by metastatic tumor cells, induces activation of the Jak/Stat5β pathway in neutrophils and enhances transferrin production in the metastatic microenvironment, which in turn favors metastatic growth in a paracrine manner (*SI Appendix*, Fig. S12*D*). G-CSF or, to a lesser extent, GM-CSF is used to enhance myeloid recovery in cancer patients after chemotherapy (34). However, despite the success of this therapy, G-CSF or GM-CSF administration may risk fueling cancer progression by enhancing neutrophil accumulation and transferrin expression in the metastatic microenvironment.

Earlier work suggested that transferrin or its receptor might be potential therapeutic targets for cancer therapy (35). However, very little progress has been made in the clinical translation of this hypothesis, possibly due to the concern that systemic targeting of this pathway may result in major toxicity, given its critical role in iron delivery to normal cells (21). Inactivation of the Tfr1 gene results in embryonic lethality in mice due to severe anemia and impaired neurological development (36). A promising alternative is to either selectively target Tfr1 in tumor cells or to lower transferrin levels in surrounding tissues. Indeed, our study has revealed neutrophils as an unexpected source of transferrin within the metastatic microenvironment. The identification of the GM-CSF/Jak/Stat5β pathway as a neutrophil-specific regulatory mechanism for transferrin gene expression should make it possible to target this axis for cancer therapy, without interfering with its homeostatic functions.

Finally, although the models that we employed emphasize a role in metastasis, it is conceivable that the mechanism that we identified also plays a role in primary tumor growth in other contexts. It is tempting to speculate that it may also be implicated in neutrophil-dependent tissue repair or remodeling processes.

Materials and Methods

Biospecimens were collected by the Moores Cancer Center Biorepository and Tissue Technology shared resource from consented patients under a University of California, San Diego Human Research Protections Program Institutional Review Board approved protocol (HRPP no. 090401). Animal studies were approved and performed in compliance with guidelines by the University of California, San Diego Institutional Animal Care and Use Committee.

To establish primary tumors in mice, 4T1 tumor cells were injected in the mammary fat pads, while 3LL tumor cells were s.c. implanted. Mouse lung metastasis was assessed by manual counting of metastatic nodules and H&E staining of lung sections. All antibodies were administrated intraperitoneally every other day, and AZD1480 was administrated daily through oral gavage. To prepare neutrophil CM, Ly6g+ mouse neutrophils were isolated and cultured in serum-free DMEM (for proteomic characterization) or RPMI-1640 with 1% FBS (for other experiments) for 12 h. Detailed Procedures for in vitro experiments, animal studies, and statistical analysis are described in the *SI Appendix*.

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MEDICAL SCIENCES

1. Houghton AM, et al. (2010) Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nat Med* 16:219–223.
2. Kowanetz M, et al. (2010) Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci USA* 107:21248–21255.
3. Acharyya S, et al. (2012) A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell* 150:165–178.
4. Coffelt SB, et al. (2015) IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 522:345–348.
5. Wculek SK, Malanchi I (2015) Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature* 528:413–417.
6. Shojaei F, et al. (2007) Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol* 25:911–920.
7. Liang W, Ferrara N (2016) The complex role of neutrophils in tumor angiogenesis and metastasis. *Cancer Immunol Res* 4:83–91.
8. Granot Z, et al. (2011) Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 20:300–314.
9. Tong Z, et al. (2017) Predictive value of preoperative peripheral blood neutrophil/lymphocyte ratio for lymph node metastasis in patients of resectable pancreatic neuroendocrine tumors: A nomogram-based study. *World J Surg Oncol* 15:108.
10. Orditura M, et al. (2016) Neutrophil to lymphocyte ratio (NLR) for prediction of distant metastasis-free survival (DMFS) in early breast cancer: A propensity score-matched analysis. *ESMO Open* 1:e000038.
11. Templeton AJ, et al. (2014) Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: A systematic review and meta-analysis. *J Natl Cancer Inst* 106:dju124.
12. Templeton AJ, et al. (2016) Change in neutrophil-to-lymphocyte ratio in response to targeted therapy for metastatic renal cell carcinoma as a prognosticator and biomarker of efficacy. *Eur Urol* 70:358–364.
13. Zhao JJ, et al. (2012) The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. *PLoS One* 7:e33655.
14. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A (2012) Neutrophil function: From mechanisms to disease. *Annu Rev Immunol* 30:459–489.
15. Murdoch C, Muthana M, Coffelt SB, Lewis CE (2008) The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 8:618–631.
16. De Palma M, Biziato D, Petrova TV (2017) Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer* 17:457–474.
17. Zhou J, Nefedova Y, Lei A, Gabrilovich D (2018) Neutrophils and PMN-MDSC: Their biological role and interaction with stromal cells. *Semin Immunol* 35:19–28.
18. Patel S, Ngounou Wetie AG, Darie CC, Clarkson BD (2014) Cancer secretomes and their place in supplementing other hallmarks of cancer. *Adv Exp Med Biol* 806: 409–442.
19. Xue H, Lu B, Lai M (2008) The cancer secretome: A reservoir of biomarkers. *J Transl Med* 6:52.
20. Veglia F, Perego M, Gabrilovich D (2018) Myeloid-derived suppressor cells coming of age. *Nat Immunol* 19:108–119.
21. Gkouvatsos K, Papanikolaou G, Pantopoulos K (2012) Regulation of iron transport and the role of transferrin. *Biochim Biophys Acta* 1820:188–202.
22. Rhodes DR, et al. (2004) ONCOMINE: A cancer microarray database and integrated data-mining platform. *Neoplasia* 6:1–6.
23. Lánczky A, et al. (2016) miRpower: A web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. *Breast Cancer Res Treat* 160:439–446.
24. Nicolini A, Carpi A, Rossi G (2006) Cytokines in breast cancer. *Cytokine Growth Factor Rev* 17:325–337.
25. Kano A (2015) Tumor cell secretion of soluble factor(s) for specific immunosuppression. *Sci Rep* 5:8913.
26. Broughton SE, et al. (2012) The GM-CSF/IL-3/IL-5 cytokine receptor family: From ligand recognition to initiation of signaling. *Immunol Rev* 250:277–302.
27. Stecher VJ, Thorbecke GJ (1967) Sites of synthesis of serum proteins. OI. Serum proteins produced by macrophages in vitro. *J Immunol* 99:643–652.
28. Papaconstantinou J, Hill RE, Gibson WH, Rao EY (1978) Synthesis and secretion of transferrin by cultured mouse hepatoma cells. *Differentiation* 10:139–145.
29. Al-Shami A, Mahanna W, Naccache PH (1998) Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b. *J Biol Chem* 273:1058–1063.
30. Schaeffer E, Guillou F, Part D, Zakin MM (1993) A different combination of transcription factors modulates the expression of the human transferrin promoter in liver and Sertoli cells. *J Biol Chem* 268:23399–23408.
31. Xin H, et al. (2011) Antiangiogenic and antimetastatic activity of JAK inhibitor AZD1480. *Cancer Res* 71:6601–6610.
32. Takeda K, Hatakeyama K, Tsuchiya Y, Rikiishi H, Kumagai K (1991) A correlation between GM-CSF gene expression and metastases in murine tumors. *Int J Cancer* 47: 413–420.
33. Quail DF, et al. (2017) Obesity alters the lung myeloid cell landscape to enhance breast cancer metastasis through IL5 and GM-CSF. *Nat Cell Biol* 19:974–987.
34. Mehta HM, Malandra M, Corey SJ (2015) G-CSF and GM-CSF in neutropenia. *J Immunol* 195: 1341–1349.
35. Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML (2006) The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. *Clin Immunol* 121:144–158.
36. Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC (1999) Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nat Genet* 21:396–399.