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Accessibility
Subclonal cooperation drives metastasis by modulating local and systemic immune microenvironments

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ABSTRACT

Most human tumors are heterogeneous, composed of cellular clones with different properties present at variable frequencies. Highly heterogeneous tumors have poor clinical outcomes, yet the underlying mechanism remains poorly understood. Here, we show that minor subclones of breast cancer cells expressing IL11 and FIGF (VEGFD) cooperate to promote metastatic progression and generate polyclonal metastases composed of driver and neutral subclones. Expression profiling of epithelial and stromal compartments of monoclonal and polyclonal primary and metastatic lesions revealed that this cooperation is indirect, mediated through the local and systemic microenvironments. We identified neutrophils as a leukocyte population stimulated by the IL11-expressing minor subclone and showed that depletion of neutrophils prevents metastatic outgrowth. Single-cell RNA-seq of CD45+ cell populations from primary tumors, blood, and lungs demonstrated that IL11 acts on bone-marrow-derived mesenchymal stromal cells, which induce pro-tumorigenic and pro-metastatic neutrophils. Our results indicate key roles for non-cell-autonomous drivers and minor subclones in metastasis.
Tumors are mixtures of cells with distinct characteristics\textsuperscript{1}. High intratumor diversity increases the likelihood of disease progression\textsuperscript{2}, as different subclones respond differently to microenvironmental cues. Treatment of heterogeneous tumors favors selection of resistant subclones, leading to therapeutic failure. Heterogeneous tumors also display phenotypes different from those of individual clones; thus, intratumor heterogeneity has a significant impact on tumor progression and therapeutic resistance.

Metastatic disease is responsible for most cancer-associated mortality; therefore, understanding drivers of metastatic progression is key for improving clinical outcomes. Cancer genome sequencing studies identified limited genetic differences between primary and metastatic tumors and demonstrated extensive subclonal heterogeneity in both primary and distant lesions\textsuperscript{3,4}. However, the mechanism(s) by which polyclonal primary tumors produce polyclonal metastases remains elusive. Moreover, several recent studies implicated microenvironmental changes as key mediators of metastatic dissemination and outgrowth\textsuperscript{5,6}, highlighting the role of non-cell-autonomous factors in tumor evolution.

**Clonal cooperation drives polyclonal metastasis**

We have been investigating the effect of subclonal interactions on tumor phenotypes using a human breast cancer cell line (MDA-MB-468)-derived xenograft model of intratumor heterogeneity. We previously established that a minor subclone can drive tumor growth through non-cell-autonomous interactions, supporting long-term subclonal heterogeneity\textsuperscript{7}. Briefly, we tested 18 subclones, each expressing a secreted protein implicated in metastasis and angiogenesis, and found that polyclonal tumors with all 18 subclones grew the fastest, while in monoclonal tumors only IL11 and CCL5 were able to drive tumor growth. We also determined that a mixture of two subclones expressing IL11 (interleukin 11) and FIGF (FOS-induced growth factor, also known as VEGFD) was largely able to reproduce this phenotype. Omitting IL11\textsuperscript{+} cells from polyclonal tumors decreased tumor growth, suggesting that IL11 and FIGF may cooperate. In addition, both polyclonal tumors and tumors
comprised of only IL11 and FIGF subclones were highly metastatic, but the underlying mechanism remained undefined.

To dissect the molecular basis of this metastasis-driving subclonal cooperation, we first investigated the clonality of metastases of primary MDA-MB-468 tumors comprising IL11+ and FIGF+ driver subclones, as well as neutral subclones. Monoclonal or polyclonal mixtures of green fluorescent protein (GFP) and luciferase-expressing parental cells, red fluorescent protein (RFP) and V5-tagged IL11+ cells, and RFP+ FIGF+ cells were implanted into the mammary fat pads of immunodeficient NOG mice. We monitored primary tumor growth by weekly caliper measurements and macrometastatic lesions by weekly bioluminescence imaging. Polyclonal tumors initiated from 5% IL11+ and 5% FIGF+ RFP+ cells with 90% GFP+ parental cells grew faster and were more metastatic than monoclonal and parental tumors (Fig. 1a-c, Supplementary Table 1). Immunohistochemistry-based quantification of human cytokeratin+ (CK+) cells in the lungs revealed an increased number of metastatic lesions in mice with FIGF+ primary tumors (Fig. 1d,e) despite small primary tumors. However, most of these were micrometastases, detectable as single cells only by immunohistochemistry, while the lungs of mice with polyclonal primary tumors were filled with macrometastases emitting high bioluminescence-signal (Fig. 1b). The increased metastases by polyclonal tumors were not simply due to their faster growth, as this trend was still observed when primary tumors were surgically removed upon reaching 1 cm in diameter (Supplementary Fig. 1a, b). Furthermore, IL11+ monoclonal tumors grew faster than parental and neutral clones but were not as metastatic as polyclonal tumors. Thus, macrometastatic outgrowth is a phenotype unique to polyclonal tumors. Immunofluorescence demonstrated that metastases of polyclonal tumors were also mostly polyclonal, composed of V5-tag+ IL11 and FIGF driver and GFP+ parental subclones, the latter of which did not produce significant macrometastases on their own except for rare GFP+ monoclonal metastases (Fig. 1f,g). These results show that polyclonal tumors behave differently than monoclonal tumors and that IL11+ and FIGF+ subclones driving tumor growth and dissemination, respectively, cooperate to generate frequent large polyclonal metastases. This
cooperation is likely to be indirect, as close cell contact between driver and neutral subclones was not commonly observed and clonal distribution within metastatic lesions was random (Fig. 1g,h).

**Indirect clonal cooperation via the microenvironment**

To explore mechanisms by which driver subclones cooperate to promote metastasis, we generated polyclonal tumors composed of fluorescently-labeled driver clones (10% FIGF-mCherry and 10% IL11-GFP cells) and neutral clones (40% Thy1.1 cell surface marker and 40% CFP [cyan fluorescent protein]-expressing cells). To be able to sort a sufficient number of cells of each fraction from these tumors, 10% driver clones was used in this experiment. As controls, we established tumors with 100% of each individual clone. Mice were terminated when the primary tumors reached ~2 cm. Primary and metastatic lesions were dissociated and fluorescently-labeled cancer and unlabeled murine cells were purified by FACS and analyzed by RNA-seq (Fig. 2a, Supplementary Fig. 1c, and Supplementary Table 2).

First, we investigated whether a common transcriptional program was induced by the driver subclones in polyclonal tumors. We compared the expression profiles of each subclone purified from polyclonal tumors to that of parental tumors (Supplementary Table 3). We found 36 commonly upregulated genes in fractions from tumors with driver clones (Fig. 2b), including *TMEM173* encoding STING, an immune modulator⁸, and several extracellular matrix (ECM) components. These expression changes suggested that driver subclones may promote metastasis by altering the tumor microenvironment. Therefore, we analyzed the differences in stromal cells (i.e., cells lacking human cancer cell markers) from monoclonal and polyclonal primary tumors and lungs. Since the parental cells did not generate metastases, we used lung tissue from mice with 100% parental cell tumors as controls in our comparisons. Principal component analysis demonstrated a trend toward clustering according to the tumor of origin, implying that tumor cells have a significant impact on stromal gene expression (Supplementary Fig. 2a,b and Fig. 2c,d). Using MetaCore⁹, we analyzed pathway and process network enrichment within upregulated genes in stromal cells of primary tumors capable of
seeding metastases (i.e., containing a driver subclone) compared to the stroma of parental tumors (Supplementary Tables 3 and 4). Interestingly, 9 of 10 top significantly enriched pathways were immune-related (Fig. 2e and Supplementary Fig. 2c). In contrast, all 10 top pathways enriched in non-cancer cells from lungs of mice with polyclonal tumors were related to vesicular transport and cell motility (Fig. 2e). FIGF and IL11 fractions of polyclonal tumors compared to 100% FIGF+ or IL11+ tumors also showed upregulation of angiogenesis-related genes (Supplementary Table 3). Immunohistochemistry of CD31 blood and LYVE1 lymphatic vessel markers confirmed this upregulation even in primary tumors (Supplementary Fig. 2d).

We also analyzed the relative frequencies of each of the four subclones within polyclonal tumors by FACS and found that IL11+ and FIGF+ subclones remained as minor fractions (Supplementary Fig. 2e). Thus, their ability to drive tumor growth and metastasis did not require clonal expansion indicating a non-cell-autonomous mechanism. Therefore, subclonal cooperation in primary tumors promotes metastasis by modulating the microenvironment of primary and metastatic lesions.

**Polyclonal tumors affect leukocytes**

To follow up on our finding of immune-related changes in the stroma of metastatic-capable primary tumors and to analyze leukocytes, we repeated the polyclonal and monoclonal xenograft experiment using luciferase-expressing cells to monitor metastases. Immunodeficient NOG mice retain innate immune and myeloid cells (albeit not fully functional), and myeloid-related pathways were highly upregulated in the primary tumors driving metastases. We assessed qualitative changes in blood leukocytes by FACS at three weeks after tumor initiation and at the six-week experimental endpoint, when primary tumor, lungs, and bone marrow were also collected (Fig. 3a and Supplementary Fig. 3a). The most significant differences were detected in neutrophils and monocytes (Fig. 3b and Supplementary Fig. 3b). The relative fraction of neutrophils was significantly higher in the blood, primary tumors, and lungs of mice with polyclonal and IL11+ monoclonal tumors, compared to mice bearing parental cell tumors, while the fraction of CD11c+ cells was significantly lower in the blood and lungs,
macrophages showed a slight decrease in primary tumors and lungs, and monocytes were decreased in the blood and primary tumors (Fig. 3b and Supplementary Fig. 3b).

Neutrophil influx has been associated with IL11 signaling and inflammatory bursts\textsuperscript{10}; therefore, we analyzed bone marrow CD45\textsuperscript{+} cells for IL11R\textalpha expression. Approximately 20-30\% of CD45\textsuperscript{+} cells of mice bearing IL11\textsuperscript{+} or FIGF\textsuperscript{+} monoclonal and polyclonal tumors were IL11R\textalpha\textsuperscript{(Fig. 3c)}, and the fraction was significantly higher in mice with IL11\textsuperscript{+} or FIGF\textsuperscript{+} monoclonal and polyclonal tumors, compared to mice with parental tumors (Fig. 3d). Hence, the presence of minor metastasis-promoting subclones within tumors alters the immune system, particularly the neutrophil population.

In breast cancer patients, distant metastases are usually recurrences after surgical removal of primary tumors, and some studies suggested that the surgery itself may induce metastatic lesions\textsuperscript{11,12}. Thus, in another experiment, we performed mock or primary tumor removal surgery but did not observe any effects of surgery on metastatic burden in lungs (Supplementary Fig. 4a-c). Therefore, surgery-related wound healing and inflammation are not inherently responsible for the observed immune-related changes or increased metastasis in our polyclonal tumors.

**IL11 and FIGF cooperate to induce metastasis-promoting systemic changes**

Next, we investigated whether the metastasis-promoting subclones exert their effects via local or systemic changes. We injected a 1:1 mixture of MDA-MB-468 cells expressing doxycycline (DOX)-inducible, HA-tagged IL11 and FIGF into the right mammary fat pad and a slow-growing, weakly metastatic patient-derived xenograft (PE1) into the contralateral mammary fat pad. Mice were randomized into DOX +/- groups to induce IL11 and FIGF expression. The IL11\textsuperscript{+}FIGF\textsuperscript{+} tumors grew faster than parental tumors, as expected, although the difference was not as profound as in polyclonal tumors with constitutive expression. The growth of PE1 primary tumors was not affected by the contralateral IL11+FIGF+ tumor (Fig. 4a), but lung metastases were significantly increased in DOX+ mice (Fig. 4b,c). Using immunohistochemistry for the HA-tag, we found that metastatic lesions were a
mixture of HA\(^+\) and HA\(^-\) cells, with the latter presumably representing PE1 cells (Fig. 4c). To confirm that these negative cells were not MDA-MB-468 cells that lost HA-tagged IL11 and FIGF expression, we performed fluorescence in situ hybridization (FISH) for EGFR and human-specific CEP8, since MDA-MB-468 cells have an EGFR amplification not detected in PE1, while CEP8 marks all human cells. This analysis clearly demonstrated that metastases were a mixture of PE1 and EGFR\(^{\text{amp}}\) IL11\(^+\)/FIGF\(^+\) MDA-MB-468 cells (Fig. 4e). Single-cell quantification of the FISH signal showed a significant correlation between the numbers of IL11\(^+\)/FIGF\(^+\) (EGFR\(^{\text{amp}}\)) and PE1 (CEP8\(^{\text{only}}\)) cells, implying potential local interactions between these populations (Fig. 4f). Therefore, IL11 and FIGF promote metastasis via systemic effects, potentially mediated by neutrophils.

**Depletion of neutrophils affects metastases**

To determine whether neutrophils are required for the increased metastasis driven by IL11 and FIGF, we treated mice with a neutrophil-depleting Ly6G antibody\(^{13}\). To determine the optimal timing to initiate treatment, we used mathematical modeling to predict the onset of metastasis based on our bioluminescence data (see Methods for details). Our computational framework considers two types of cells – those residing in the primary tumor and those that have left the primary site (Supplementary Fig. 4d). Each time a cell leaves (at a dissemination rate \(q\)), it establishes a new metastatic colony. Growth and dissemination rates were estimated from experimental data (Supplementary Fig. 4b), dependent on the experimental condition (Supplementary Fig. 4e). Briefly, to estimate the metastatic rate \(q\), we minimized the distance between experimental results and model predictions for the distribution of the size of the first metastasis six weeks after the start of the experiment and the primary tumor size when the first metastasis is detected. We identified the metastatic rate as \(10^{-7} < q < 10^{-6}\) per cell per week and, using this, defined the timing of the first metastatic cell leaving the primary site as 5.18±2.45 days for IL11\(^+\) monoclonal tumors and 5.11±2.45 days for polyclonal tumors when \(q = 10^{-7}\), and 1.33±1.05 and 1.68±0.91 days, respectively when \(q = 10^{-6}\) (Supplementary Fig. 4f-k). Thus, we concluded that to prevent metastatic seeding, treatment should be initiated immediately after tumor inoculation.
We initiated polyclonal xenografts with 10% DOX-inducible IL11+ and FIGF+ and 90% parental cells and assigned animals to receive DOX with a neutrophil-blocking anti-Ly6G antibody or an isotype control, administered for four weeks (Supplementary Fig. 5a). We confirmed the efficacy of anti-Ly6G antibody treatment after two weeks by assessing blood neutrophil counts (Supplementary Fig. 5b-d). At 6 weeks, we found that neutrophil depletion did not affect primary tumor growth (Fig. 4g,h), but significantly decreased CK+ metastatic cells in the lungs of mice compared to isotype control (Fig. 4i,j). Comparing to untreated animals achieved borderline significance. Unexpectedly, anti-Ly6G treatment increased the metastatic load in -DOX mice with tumors not expressing IL11 and FIGF (Fig. 4i,j). These results demonstrate that systemic depletion of neutrophils decreases metastatic spread (or outgrowths) of polyclonal tumors driven by IL11+ and FIGF+ subclones and can have opposing effects on metastases depending on factors produced by the primary tumor. Furthermore, injection of an isotype control antibody itself can enhance metastasis. Therefore, the phenotype and functional properties of neutrophils depend on the systemic host environment.

**Single-cell analysis of CD45+ cell populations**

To further investigate the effects of IL11+ and FIGF+ subclones on leukocytes, we repeated the above experiment with polyclonal xenografts composed of 10% DOX-inducible IL11+ and FIGF+ and 90% parental cells. Six weeks after tumor inoculation, we purified CD45+ cells from blood, primary tumors, and lungs by FACS, and performed single-cell RNA-seq (scRNA-seq)\(^\text{13}\). Clustering analysis revealed a minor fraction of CK+EGFR+ contaminating cancer cells that were removed from subsequent analyses (Supplementary Fig. 6a-c), as well as numerous identifiable leukocyte subpopulations (Fig. 5a, Supplementary Fig. 6d-g, and Supplementary Table 5). CD45+ cells from lungs, blood, and primary tumors contributed to the distinct cell clusters to different extents (Fig. 5b). For example, M1 macrophages were found only in primary tumors, whereas M2 macrophages were only present in lung metastases. Since our previous results indicated that neutrophils contribute to metastasis, we compared the single-cell transcriptomic profiles of blood and lung neutrophils from mice with or without
DOX. IL11 and FIGF induced upregulation of several pathways, including TGFβ and JAK/STAT signaling, associated with pro-tumorigenic and pro-metastatic immune signatures, in neutrophils from lungs but not from blood (Fig. 5c and Supplementary Tables 6 and 7). Despite this change in lung neutrophils, we did not detect IL11 or FIGF receptor expression in this cell population by scRNA-seq. However, cells with IL11RA transcripts were clearly present in a separate cluster (Fig. 5d), not classified as either neutrophils or any other leukocyte subpopulation. These IL11RA+ cells express IL6ST encoding GP130, a coreceptor necessary for IL11 signaling, and STAT3, a downstream IL11 effector (Supplementary Fig. 6f). Based on gene expression in this cluster (Supplementary Table 8), which also included ECM and development-related proteins, we denoted this population as IL11-responsive mesenchymal stromal cells (MStrCs). Although this population does not express the classic mesenchymal stem cells (MSC) markers, its signature is rich in stem cell-related genes, implying that it could be a previously uncharacterized MSC-like cell type. Prior studies have described interactions between MSCs and leukocytes mediated by various cytokines and chemokines\textsuperscript{14}. Thus, we identified secreted factors from our MStrC-specific expression signatures using gene-enrichment analysis and analyzed them in different conditions (Fig. 5e). Comparison of lung MStrCs from -/+ DOX animals revealed that the induction of IL11/FIGF expression in the primary tumors upregulated the expression of chemoattractants for pro-metastatic neutrophils including CXCL12, CXCL14, and CXCL1 (Fig. 5e).

To validate the presence of CD45\textsuperscript{+}IL11RA\textsuperscript{+} cells in lungs and to assess co-expression of MStrC markers with IL11RA we performed immunofluorescence and FACS. CD45\textsuperscript{+}IL11RA\textsuperscript{+} cells were relatively common in the spleen and present as a rare subpopulation with mesenchymal morphology in the lungs (Fig. 5f). Rare IL11RA\textsuperscript{high} cells co-expressing PLXDC2 and ANTRXR1, markers of these cells based on scRNA-seq (Supplementary Table 8), were also identified in the lungs by FACS representing 0.5-1% of cells (Fig. 5g and Supplementary Fig. 6h-l). Immunofluorescence analysis of lungs demonstrated the presence of rare IL11RA\textsuperscript{+}PLXDC2\textsuperscript{+} cells with mesenchymal morphology (Fig. 5h). Lastly, we sorted cells from lungs of mice with IL11\textsuperscript{+}/FIGF\textsuperscript{+} tumors by IL11RA expression and analyzed their gene expression profiles. Our MStrC signature was significantly enriched in IL11RA\textsuperscript{high} samples.
(Fig. 5i) and enrichment for cell adhesion and ECM remodeling-related process networks was noted (Supplementary Table 9).

In summary, our scRNA-seq data and its subsequent validation by FACS, immunofluorescence, and bulk RNA-seq uncovered an interaction loop among different subpopulations of bone marrow-derived cells that mediates the pro-metastatic effects of tumor cell-secreted IL11 and FIGF (Fig. 5j). The expression of IL11 and FIGF in a minor subpopulation of cancer cells in polyclonal tumors alters these MStrCs in the lungs, leading to the activation of “effector” neutrophils, which are crucial for metastatic progression.

**IL11-driven signatures in human metastatic breast cancer**

To investigate the clinical relevance of our findings, we analyzed the expression of IL11 and IL11-driven neutrophil and MStrC signatures in breast cancer metastases. IL11 is amplified in a subset of primary breast tumors, and this amplification is more commonly observed in metastases (cBioPortal\textsuperscript{15,16}, Fig. 6a). Based on our data, IL11 and FIGF can drive significant systemic changes even when expressed by minor subclones making them difficult to detect. Thus, we explored whether gene signatures of IL11/FIGF-activated neutrophils and MStrCs, defined by our single-cell RNA data, are present in distant metastases in the Metastatic Breast Cancer Project (MBCP) dataset\textsuperscript{17}, which contains RNA expression data of 156 distant metastatic lesions. Since most of the systemic changes were IL11-driven in our experimental system, we classified metastatic lesions into IL11\textsuperscript{high} and IL11\textsuperscript{low} groups based on mRNA levels. We then compared the IL11\textsuperscript{high} and IL11\textsuperscript{low} metastases for the expression of gene signatures activated by IL11/FIGF in lung and blood neutrophils and in MStrCs (Supplementary Table 6). Signatures for activated lung and blood neutrophils and lung MStrCs were significantly higher in IL11\textsuperscript{high} than IL11\textsuperscript{low} metastases (Fig. 6b). In contrast, gene signature of IL11/FIGF-activated cancer cells, derived from genes expressed by neutral clones in polyclonal tumors, did not correlate with IL11 expression in metastases (Fig. 6b, bottom panel). Analyzing the expression of these gene signatures in matched primary and metastatic lesions using the MBCP dataset showed that activated neutrophil
and MStrC signatures were significantly higher in metastases, while cancer cell signatures did not show a difference (Fig. 6c, left). To eliminate potential bias due to differences in tissue type used for RNA extraction (FFPE for primary tumors and frozen for metastases), we also compared the MBCP metastases to ER+ tumors in the TCGA data with essentially the same findings (Fig. 6c, right). Therefore, IL11/FIGF-driven microenvironmental changes are also detected in human breast cancer patients and are associated with distant metastases.

DISCUSSION

Polyclonal metastasis of breast cancer has been described in several recent studies18,19. Here, we provide evidence that in polyclonal tumors, minor subpopulations can drive metastatic progression and outgrowth of polyclonal metastases that also contain neutral subclones (non-metastatic on their own) by modulating local and systemic microenvironments.

In our study, we focused on two secreted factors expressed by minor subclones that cooperate to drive metastasis, selected based on our prior data7. IL11 belongs to the IL6 family of cytokines and has been shown to play a role in progression and resistance in multiple cancer types including prostate and colon cancer20,21. In breast cancer, IL11 has been implicated in therapeutic resistance22 and bone metastasis23, as well as a marker of poor outcomes24. FIGF is a ligand for VEGFR2 and VEGFR3 and can stimulate both angiogenesis and lymphangiogenesis25,26. Our results show that FIGF enhances dissemination, as evidenced by increased numbers of CK+ cells in the lungs, but is not sufficient to drive macrometastatic outgrowths. In our model, IL11 and FIGF secreted by minor subclones have profound effects on the metastatic ability of breast cancer cells. While IL11 acts on leukocytes to induce metastasis-permissive changes in the microenvironment of distant organs, FIGF acts more locally by increasing lymphangiogenesis and vascular permeability. However, because these subclones represent only 1-5% of tumor cells, their presence and their secreted factors may be difficult to detect in clinical samples, making it challenging to predict their metastasis-promoting effects in human tumors. Nevertheless, our analysis of human metastatic breast tumors suggests that the systemic changes in
the immune and other bone marrow-derived cells induced by even low levels of these secreted factors can be detected and potentially exploited for patient stratification and treatment.

We identified neutrophils as the leukocyte population required for IL11- and FIGF-induced polyclonal metastasis. A caveat of our approach is the use of immunocompromised animals, which did not allow us to study the roles of lymphoid cells in metastatic cooperation. Thus, it is possible that other immune cells may also play a role in the metastatic process. Neutrophils have been implicated in metastatic progression by promoting the premetastatic niche\textsuperscript{27-31}. Furthermore, high pre-operative neutrophil-to-lymphocyte ratio is associated with poor prognosis in breast cancer patients\textsuperscript{32}. Neutrophils have also been shown to have antitumor effects and inhibit seeding of cancer cells in the lungs\textsuperscript{33,34}. In our experiments, depending on whether or not the tumors expressed IL11 and FIGF, depleting neutrophils could either decrease or enhance lung metastasis. These seemingly contradictory roles for neutrophils could potentially be explained by our observation, based on scRNA-seq data, that there are multiple different types of neutrophils, potentially pro- and anti-tumorigenic, depending on anatomical location and gene expression patterns. Our findings are therefore in agreement with reports that the context and temporal infiltration of monocytes and neutrophils is critical to outcome\textsuperscript{31,35,36} and that heterogeneity of not only tumor cells but also immune cells must be considered. Another point to consider is that injection of isotype control antibody unexpectedly increased metastatic burden potentially by triggering an inflammatory response. The magnitude of neutrophil-depletion effect on metastasis may therefore be very sensitive to the systemic environment of the host.

The use of single-cell expression profiling of CD45\textsuperscript{+} cells in our study allowed us to uncover interactions between different leukocytes subpopulations. We found that leukocytes may not be the direct cellular targets of IL11, but indirectly affected via factors secreted by MStrCs responsive to IL11. Interestingly, these cells also express \textit{PLXDC2} and \textit{ANTXR1}, which are highly expressed in tumor-associated endothelial cells\textsuperscript{37,38}. Thus, these IL11RA\textsuperscript{+} mesenchymal cells may represent progenitors that can give rise to multiple cell types. Further characterization of these cells and their effector leukocytes will shed new light on the biology of pro-tumorigenic neutrophils. An improved understanding of neutrophil
subtypes and the development of tools to target pro-metastatic neutrophils, in combination with cancer cell-targeting drugs, could potentially be used to prevent breast cancer metastasis.

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AUTHOR CONTRIBUTIONS

D.P.T. and M.J. performed xenograft, molecular profiling, and immunohistochemical experiments, and data analyses. M.B.E. and N.W.H. analyzed RNA-seq data. A.M. helped with study conception and xenograft experiments. N.L.K. and K.C.M. assisted with immunohistochemical staining. M.K. generated low-input RNA-seq libraries. Y.Q., Z.C., M.A., and C.G.A. performed FACS analyses. T.L. and S.S. assisted with animal experiments. K.N.Y. carried out mathematical modeling. A.L. and K.W.W. assisted with single-cell RNA-seq libraries generation. S.C. analyzed single-cell RNA-seq data. O.C. and N.W. provided MBCP cohort data and performed analyses. K.P. supervised with help from F.M., S.S.M., and R.F. All authors helped to design the study and write the manuscript.

AUTHOR INFORMATION
RNA-seq and scRNA-seq data have been deposited in the NCBI GEO database with the accession number GSE109281. The following secure token has been created to allow review of record GSE109281 while it remains in private status: cnehiokwzjaflmn

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: K.P. received research support from and was a consultant to Novartis Oncology during the execution of this study. K.P. also serves on the Scientific Advisory Board of Mitra Biotech.
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FIGURE LEGENDS

Figure 1. Minor driver clones lead to polyclonal metastasis. a, Primary tumor growth kinetics, n=10 tumors, 5 animals per group. Mean +/- SD are shown. b, Representative bioluminescence images. c, Quantification of lung macrometastases based on in vivo bioluminescence at week 6, n = 5 animals per group, except IL11 and polyclonal group: n=4 animals. Mean +/- SD is shown. All areas with detected luminescence were quantified and primary tumor area was excluded from the analysis. d, Representative images of cytokeratin staining in the lungs. Scale bar 100 μm. Staining was repeated twice with similar results. e, Quantification of human cytokeratin+ cells in the lungs. On average, 3,500 cells were counted per field, 3 fields per sample. Lungs of 5 mice per group were analyzed. Percent of positive cells per field is shown, with mean +/- SD. p values indicate statistical significance based on unpaired two-tailed t-test in all panels. f, Representative images of the histology (hematoxylin-eosin staining, upper panels) and clonal composition (GFP and V5 immunofluorescence) of primary tumors and metastases. Scale bar 100 μm. Staining was repeated twice with similar results. g, Quantification of parental GFP+ and IL11/FIGF-expressing V5+ cells in lung metastatic lesions from four polyclonal tumor-bearing animals, six lesions per animal were quantified. h, Frequency of parental GFP+ and IL11/FIGF-expressing V5+ cells per lesion, n=24 lesions in lungs of 4 animals. Pearson correlation R^2 and two-tailed t-test p value are indicated. See also Supplementary Table 1 for raw data.

Figure 2. Driver clone-induced changes in the stroma of primary and metastatic tumors. a, Schematic outline of the experimental design. b, Overlap of differentially expressed genes in cancer cell fractions from polyclonal primary tumors (n = 3) compared to parental cell line tumors (n = 3). c, d, Principal component analysis of RNA-seq profiles of stromal cells from mono- and polyclonal primary tumors (c) and non-tumor cells from metastatic lungs (d). n=3 independent tumors per group. e, MetaCore GO Processes overrepresented in expression profiles from stroma of primary tumors (n=3) and metastasis-bearing lung cells (n=3). Color scale corresponds to -log (p-value) of significance of enrichment, calculated by MetaCore Enrichment Analysis test.
Figure 3. The effect of polyclonal tumors on the immune system. In all bar graphs: Box-and-whisker plots show mean (midline), 25th-75th percentile (box) and 5th-95th percentile (whiskers). p value of unpaired two-tailed t-test. a, Schematic outline of the experimental design. b, Analysis of myeloid cells from the blood, primary tumors, and lungs of mice bearing parental, 100% FIGF or 100% IL11 monoclonal or polyclonal tumors; n = 5 per group. c, Fraction of bone marrow CD45+ cells expressing IL11RA; n = 5 per group. d, Fraction of neutrophils among CD45+IL11RA+ bone marrow cells; n = 5 per group. See also Supplementary Table 1 for raw data.

Figure 4. Systemic effects of metastasis-driver subclones. a-d, Effects of driver subclones (IL11 and FIGF under a DOX-inducible promoter) on the growth and metastasis of contralaterally injected patient-derived xenografts. a-b, Primary tumor growth kinetics (a) and final tumor weight (b); n = 5 animals per group; representative of two independent experiments. PE1 – non-metastatic patient-derived xenograft. DOX – doxycycline. Mean ± SD shown. Two-tailed unpaired t-test p values are shown. c, Representative immunohistochemical images of the indicated staining. d, Fraction of cytokeratin+ cells. On average, 2,900 cells were counted per field, three fields per sample. Lungs of five mice per group were analyzed. Data shown are representative of two independent experiments. Box-and-whisker plots show mean (midline), 25th-75th percentile (box), 5th-95th percentile (whiskers) and outliers (dot). Two-tailed unpaired t-test p values are shown. e, Representative images of EGFR and CEP8 FISH on primary tumor and lung metastasis under DOX treatment. Scale bar 25 μm. Staining was repeated twice with similar results. f, Correlation between numbers of cells that are EGFRamp or have CEP8 only FISH signal per metastatic lesion. 3-4 fields of view were quantified in lungs from 4 animals; total of 16 lesions analyzed. Pearson correlation R² and two-tailed t-test p value are indicated. g-j, Neutrophil depletion in mice bearing polyclonal tumors with minor driver subclones. g-h, Tumor growth kinetics (g) and final tumor weight (h, n = 5 per group). Dashed line marks the anti-Ly6G and
isotype antibody treatment duration. Mean ± SD shown. i, Representative images of cytokeratin staining in lungs of treated mice. Scale bar 100 μm. j, Quantification of cytokeratin⁺ cells. On average, 2,600 cells were counted per field, three fields per sample. Lungs of five mice per group were analyzed. Box-and-whisker plots show mean (midline), 25⁻⁷⁵ percentile (box), 5⁻⁹⁵ percentile (whiskers) and outliers (dot). p values indicate statistical significance of the observed differences defined by unpaired two-tailed t-test. See also Supplementary Table 1 for raw data.

Figure 5. Single-cell RNA-seq data analysis of CD45⁺ cells. a-b, tSNE clustering of single-cell expression profiles. Each dot represents a single cell (n=7,704), and the distance between cells is a measure of their transcriptional similarity. a, Twelve distinct clusters of immune were identified according to expression of cell type-specific marker genes or expression signatures (Supplementary Fig. 7-8 and Supplementary Table 5). Colors denote distinct clusters of cells. b, Contribution of cells from different samples to the distinct clusters. Cells are colored according to their sample of origin. c, Gene expression signature analysis of lung neutrophils from mice with (+DOX) or without (-DOX) IL11/FIGF-expressing tumors. NES – normalized enrichment score. d, The expression of IL11RA in the single cells analyzed (n=7,704). e, Secreted factors among cluster-specific differentially expressed genes in lung mesenchymal stromal cells. Genes highlighted in green and red are characteristic of antitumorigenic neutrophils and neutrophil chemoattractants, respectively. f, Immunofluorescence analysis of CD45 and IL11RA in spleen and lungs of mice with IL11/FIGF-expressing tumors. White arrows highlight rare CD45⁺IL11RA⁺ cells with mesenchymal morphology in the lungs. Staining was repeated twice with similar results. g, Flow cytometry analysis of lungs of tumor-free mice stained with AF55-conjugated IL11RA, and AF488-conjugated PLXDC2 and ANTXR1 (TEM8) antibodies. Highlighted populations show IL11RA⁺PLXDC2⁺ as well as IL11RA⁺ANTXR1⁺ double positive cells. h, Immunofluorescence analysis depicting rare IL11RA⁺ and PLXDC2⁺ cells with mesenchymal morphology in the lungs of +DOX-isotype control-treated mice. Staining was repeated twice with similar results. i, Mesenchymal stromal cell signature enrichment in lung cells FACS-sorted for IL11RA
expression from tumor-bearing mice (n=3 animals). P values of the Gene Set Enrichment Analysis (Wald test with Benjamini-Hochberg multiple comparison correction) are shown. j, Model of metastatic cascade driven by IL11/FIGF-expressing minor primary tumor subclones.

Figure 6. IL11 is associated with metastasis in breast cancer patients. a, Frequency of genetic alterations of the IL11 locus in cBioPortal breast cancer dataset. b, Expression of IL11/FIGF-activated signatures of lung and blood neutrophils, mesenchymal stromal cells and primary cancer cells in human breast cancer metastases with high and low IL11 levels. n=156. Cancer cell signature was derived from neutral clone isolated from polyclonal primary tumors. c, Gene expression signatures of IL11/FIGF-activated cells in the Metastatic Breast Cancer Project dataset (matched primary (n=58) and metastatic lesions (n=156); left) and in the ER+ TCGA primary tumors (n=868) versus MBCP metastatic lesions (n=156; right). Box-and-whisker plots show mean (midline), 25th-75th percentile (box) and 5th-95th percentile (whiskers). ANOVA p values and two-sided Welch’s test p values are shown. See also Supplementary Table 1 for raw data.
**Region 1**

**Region 2**

**Fig. 1**

**Primary tumor diameter (mm)**

- Parental
- FIGF
- IL11
- Polyclonal

Table 1

| Region       | Metastasis area (cm²) |
|--------------|-----------------------|
| Parental     | FIGF                  |
|              | IL11                  |
|              | Polyclonal            |

Table 2

| Region       | Cytokeratin+ cells (%) |
|--------------|------------------------|
| Parental     | FIGF                   |
|              | IL11                   |
|              | Polyclonal             |

Table 3

| Region       | Cell count (%) |
|--------------|----------------|
| Parental     | FIGF           |
|              | IL11           |
|              | Polyclonal     |

**Graph A**

- Metastasis area (cm²)
- Primary tumor diameter (mm)
- Cytokeratin+ cells (%)

**Graph B**

- Luminescence
- Radiance (p/sec/cm /sr²)
- Color Scale
  - Min = 5.82e5
  - Max = 6.45e6

**Graph C**

- Parental
- FIGF
- IL11
- Polyclonal

**Graph D**

- Parental
- FIGF
- IL11
- Polyclonal

**Graph E**

- Parental
- FIGF
- IL11
- Polyclonal

**Graph F**

- Primary tumor
- Lung metastasis
- Liver metastasis
- Lymph node metastasis

**Graph G**

- IL11/FIGF-V5+
- Mouse ID

**Graph H**

- IL11/FIGF-V5+ cell number
- Parental GFP+ cell number
Fig. 2

(a) FIGF-mCherry, IL11-GFP, Thy1.1 (neutral), CFP (neutral)

(b) RNA-seq analysis

(c) PC1 vs PC2 vs PC3

(d) PC1 vs PC2 vs PC3

(e) FIGF IL11 Polyclonal

Stroma from primary tumor

Response to toxic substance
Cation homeostasis
Inorganic ion homeostasis
Response to inorganic substance
Myeloid cell differentiation
Response to metal ion
Ion homeostasis
Homeostatic process
Response to drug
Cellular homeostasis
Myeloid leukocyte activation
Inflammatory response
Immune system process
Leukocyte activation
Immune effector process
Leukocyte degranulation
Leukocyte mediated immunity
Cell activation
Defense response
Neuronal dense core vesicle transport
Response to rotenone
Dense core granule transport
Anterograde dendritic transport
Dense core granule cytoskeletal transport
Dense core granule localization
Regulation of translational termination
Mammary fat pad injection → Blood (3 weeks) → Blood (endpoint) → Primary tumor → Lung → Bone marrow

**b**

- **Neutrophils**
  - CD45^+CD11b^+ cells (%)
  - Blood (3 weeks) vs. Blood (endpoint): p=0.006, p=0.009, p=3x10^-8
  - Primary tumor vs. Lungs: p=0.0004

- **Monocytes**
  - CD45^+CD11b^+ cells (%)
  - Blood (3 weeks) vs. Blood (endpoint): p=0.0005
  - Primary tumor vs. Lungs: p=0.0005

- **CD11c^+**
  - CD45^+ cells (%)
  - Blood (3 weeks) vs. Blood (endpoint): p=0.0008
  - Lungs: p=0.0004

**c**

- **IL11RA^+ cells - bone marrow**
  - CD45^+ cells (%)
  - Parental vs. FIGF: p=0.03
  - Parental vs. IL11: p=0.009
  - Parental vs. Polyclonal: p=3x10^-8

**d**

- **Neutrophils - bone marrow**
  - CD45^+IL11RA^+ cells (%)
  - Parental vs. FIGF: p=0.001
  - Parental vs. IL11: p=0.04
  - Parental vs. Polyclonal: p=0.03
Fig. 4
Ubiquitin mediated proteolysis

Mesenchymal stromal cell markers

IL11RA expression

Upregulated in -DOX lung MSCs
Lung mesenchymal stromal cells: genes encoding secreted factors

Upregulated in +DOX lung MSCs

Mesenchymal stromal cells

Neutrophil chemoattractants

Activated pro-metastatic neutrophils

Lymphangiogenesis

Polyclonal metastasis
