High endogenous CCL2 expression promotes the aggressive phenotype of human inflammatory breast cancer

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Inflammatory Breast Cancer (IBC) is a highly aggressive malignancy with distinct clinical and histopathological features whose molecular basis is unresolved. Here we describe a human IBC cell line, A3250, that recapitulates key IBC features in a mouse xenograft model, including skin erythema, diffuse tumor growth, dermal lymphatic invasion, and extensive metastases. A3250 cells express very high levels of the CCL2 chemokine and induce tumors enriched in macrophages. CCL2 knockdown leads to a striking reduction in macrophage densities, tumor proliferation, skin erythema, and metastasis. These results establish IBC-derived CCL2 as a key factor driving macrophage expansion, and indirectly tumor growth, with transcriptomic analysis demonstrating the activation of multiple inflammatory pathways. Finally, primary human IBCs exhibit macrophage infiltration and an enriched macrophage RNA signature. Thus, this human IBC model provides insight into the distinctive biology of IBC, and highlights potential therapeutic approaches to this deadly disease.
Inflammatory Breast Cancer (IBC) is the most aggressive form of breast cancer, with the lowest survival rate compared to all other breast cancers. At diagnosis, these tumors are considered at least stage IIIB, with most patients exhibiting extensive lymph node metastases and nearly 30% distant metastases. Clinical symptoms include skin redness, an orange-peel appearance (peau d’orange) and swelling, making it initially challenging to distinguish from non-cancerous inflammatory diseases of the breast, as these tumors also grow diffusely rather than as palpable nodules. Another prominent feature of IBC is the presence of tumor emboli in dermal lymphatic vessels. Diagnosis of IBC is made based on biopsy confirming the presence of cancer cells either in dermal lymphatics or in the breast parenchyma associated with clinical detection of inflammation.

These distinct clinical and histopathological features suggest unique molecular drivers of the IBC phenotype. However, despite many efforts over the past decade to find unique molecular characteristics, IBC has not been defined as a separate molecular subtype. Genomic studies have revealed that IBC is a heterogeneous entity; however, despite the many triple-negative molecular phenotypes identified in IBC, there are no robust differences in the gene expression signature between IBC and non-IBC.

One of the key barriers in advancing understanding of IBC has been the lack of models which recapitulate all of the unique features of human IBC. To date, there are only a few human IBC-derived cell lines. The paucity of models that reproduce features of human IBC upon xenotransplantation and are amenable to manipulation has been a major impediment to the mechanistic understanding of unique aspects of the IBC phenotype. Here, we describe a triple-negative human IBC cell line that recapitulates all key features of human IBC in a mouse xenotransplant model. We show that IBC tumors mobilize distinct subsets of inflammatory cells locally and systemically, and demonstrate a key role of CCL2 secreted at very high levels in recruiting macrophages into tumors and thereby facilitating tumor growth and metastasis.

**Results**

**Biological characterization of the A3250 human IBC cell line.**

The A3250 breast carcinoma cell line was established from a primary tumor of a patient with triple negative IBC, as previously described for other A series lines. Short Tandem Repeat (STR) analysis confirmed that this line was unique based on its comparison to all human cell lines in ATCC STR and DSMZ STR databases, with less than 55% similarity to any of the existing STR profiles. In tissue culture, confluent A3250 cells exhibited epithelial morphology (Fig. 1a). At lower cell densities, we observed two distinct phenotypes: cell colonies consisting of epithelial-like polygonal cells in close contact, and colonies comprised of scattered cells with more elongated appearance (Fig. 1b). A3250 cells exhibited higher colony-forming ability than MDA-MB-231 cells, a commonly studied triple negative breast cancer (TNBC) line and retained the ability to form colonies even at low serum concentration (1% FBS) and at very low seeding densities (Supplementary Fig. 1a, b). In monolayer culture, A3250 cells exhibited a population-doubling time of 28 h in 10% FBS and proliferation was not significantly reduced upon short-term culture in 1% serum (Supplementary Fig. 1c). They lacked detectable estrogen or progesterone receptors (ER or PR), and expressed HER2 (Human Epidermal Growth Factor Receptor-2, ERBB2) mRNA at levels comparable to other TNBC cell lines (Supplementary Fig. 1d). In comparison, BT474 cells with amplified HER2 expressed more than 70-fold higher levels (Supplementary Fig. 1d, Supplementary Table 2).

A3250 human xenograft model recapitulates key clinical and histopathological features of human IBC. We engineered A3250 cells to express green fluorescent protein (GFP) and firefly luciferase (Fluc) to allow for analyses at single-cell resolution in tissue sections, and for quantitative analyses of tumor burden by bioluminescence imaging, respectively. Orthotopic injection of 10⁶ cells into the mammary fat pad of either SCID or NOD SCID mice led to the formation of primary tumors at 100% incidence, with comparable growth kinetics (Fig. 1c, d). A3250 tumors induced pronounced erythema in the overlying skin (Fig. 1e). Nipple changes observed in IBC patients, such as inversion and protrusion, were also seen early in A3250 tumor development (Supplementary Fig. 2a–c). Tumors did not form typical palpable nodules characteristic of non-IBCs, but were flat in appearance (Fig. 1f, f). Analysis of GFP-tagged A3250 tumors revealed a diffuse distribution of tumor cells as clusters or single cells, throughout the mammary gland and adjacent skin (Fig. 1g, Supplementary Fig. 2d, e). Accordingly, A3250 tumors had abundant stroma and low tumor-stroma ratio (% tumor cell area: 33% ± 10.6; range 18–43%, n = 3). By comparison, both SUM149 IBC and MDA-MB-231 non-IBC tumors formed nodules (Supplementary Fig. 2f–j) and exhibited high tumor-stroma ratio (% tumor cell area: SUM149, 61% ± 3.44, range 55–64%, n = 4; MDA-MB-231, 77% ± 2.0; range 74–79%, n = 4). SUM149 formed many invasive clusters (Supplementary Fig. 2i), but did not show the dispersed growth pattern observed with A3250. Because A3250 tumors exhibited a diffuse pattern of growth, bioluminescence imaging provided more accurate assessment of tumor burden than volume measurements.

Histopathological evaluation of hematoxylin and eosin (H&E) stained tumor sections indicated poorly differentiated carcinoma with pleiotropic atypical nuclei (Fig. 1i), showing extensive invasion into the subcutaneous fat (Fig. 1j), indicating its aggressive nature. A3250 tumors exhibited prominent dermal lymphatic vessel invasion (LVI), one of the key characteristics of IBC. Lympho-vascular emboli were frequently seen in greatly enlarged dermal lymphatic vessels (Fig. 1k). In contrast, we did not observe dermal LVI in SUM149 tumors at four weeks (Supplementary Fig. 2j), despite their highly invasive nature. Immuno-histochemical (IHC) staining revealed keratin expression throughout the A3250 tumors, with higher intensity signal in tumor emboli in lymphatic vessels (Fig. 1l). A3250 tumors also showed high mitotic rates based on Ki67 staining (Fig. 1m), defined as ≥10% of Ki67+ tumor cells. Analysis of tumor cell proliferation by EdU (5-ethynyl-2′-deoxyuridine) pulse independently revealed a high proliferation rate, with an average of 458 Edu+ tumor cells per mm² tumor/6 hr (STD+/-70.2) (Supplementary Fig. 3a–f, Supplementary Table 3).

Ingenuity Pathway Analysis (IPA) of mRNA Sequencing (mRNA-Seq) data revealed that pathways implicated in cell growth and protein synthesis, in particular mTOR signaling and its effectors, and translation initiation factors eIF2, eIF4 and p70S6K signaling were among those most highly activated in A3250 cells. Among the top ten were also pathways related to cell metabolism, such as mitochondrial dysfunction, oxidative phosphorylation and sirtuin signaling, as well as integrin signaling and junction remodeling (Fig. 1n and Supplementary Data 1). The majority of these pathways were conserved in A3250 cells in vitro and tumors in vivo.

A3250 IBC tumors spontaneously metastasize to lymph nodes and distant organs. Rapid and extensive regional and distant...
metastasis is a salient feature of human IBC. In accordance with the notion that A3250 tumors retained key characteristics of human IBC, A3250 tumor xenografts spontaneously metastasized to multiple organs upon orthotopic injection into the mammary fat pad. This was determined by bioluminescence analysis of organs ex vivo and validated by analysis of GFP signal by stereomicroscopy of whole organs and tissue sections (Fig. 2). A3250 cells metastasized to the sentinel (inguinal) lymph nodes (LNs) at 100% incidence and to more distant LNs (axillary and brachial) at 46% incidence within six weeks (16/35 LNs positive) (Fig. 2a). Lymph node metastases commonly presented as large, highly proliferative lesions, and in the majority of the cases almost completely replaced the lymph node (Fig. 2b, d–f).

Fig. 1 Characteristics of A3250 tumor cells in vitro and tumors in vivo. a Bright-field microscopy of confluent A3250 tumor cells in vitro. b Morphology of A3250 tumor cell clones in vitro. Images are representative of three independent experiments. c Tumor growth following orthotopic injection of 10⁶ A3250 tumor cells in SCID or NOD SCID mice measured by caliper weekly. NOD SCID \( n = 8 \) and SCID \( n = 6 \) biologically independent mice. Data represent mean \( \pm \) SEM (Standard Error of the Mean). d Bioluminescence imaging of A3250 tumors in SCID mice at week six \( (n = 12 \) mice). Black line denotes mean value. e Representative picture of an A3250 tumor at 6 weeks showing erythema of the skin and flat tumor appearance. f Fluorescence stereo-microscopy of an A3250 tumor (GFP, green) ex vivo. g A3250/GFP 300 \( \mu m \) tumor section imaged by confocal microscopy and reconstructed in 3D. Note diffuse pattern of growth. (GFP, green) (h). Higher magnification of boxed area in (g). H&E staining of paraffin embedded tumor sections (i, j) and overlying skin (k). Note the presence of lymphovascular emboli in the dermis (k, arrows). IHC staining for cytokeratin (l) and for the proliferation marker Ki-67 (m). Images are representative of three independent experiments. \( n = 4 \) biologically independent mice. n Top ten A3250 canonical pathways based on mRNA-Seq of A3250 tumors, determined by IPA. Top pathways are ranked based on \( p \) values. Scale bars: a, b, 100 \( \mu m \); f, 2 mm; g–m, 100 \( \mu m \). Source data are provided as a Source Data file.
A3250 tumors also showed a high incidence of metastases to the lung and liver (89%) (Fig. 2a). Lung lesions exhibited a unique growth pattern, with cell clusters distributed diffusely through the lung parenchyma (Fig. 2c), closely resembling the diffuse pattern of primary tumor growth. Lung metastases were large (Fig. 2g), frequently replacing an entire part of the lobe, and were highly proliferative (Fig. 2h–j). Proliferation was particularly high in small lesions (Fig. 2h–j). We did not observe solitary disseminated cells or micro-metastases at six weeks post-injection, indicating that upon seeding, A3250 cells proliferated rapidly and colonized the lung. Associated with the high metastatic burden at endpoint, we observed notable weight-loss (Fig. 2k) and muscle-wasting of tumor-bearing animals, consistent with cachexia. Together, these findings indicate that A3250-derived tumors recapitulated the aggressive metastatic phenotype of human IBC.

Transcriptomic profiling reveals activation of multiple pathways associated with inflammation in A3250 cells. To gain insight into potential drivers of its aggressive IBC phenotype, we performed transcriptomic analyses of A3250 cells. We compared the A3250 gene expression profile with that of six human TNBC cell lines; two IBC and four non-IBC. Hierarchical clustering of mRNA-Seq data revealed that the transcriptional profile of A3250 cells was closest to that of SUM149 IBC cells (Fig. 3a). There were 4881 Differentially Expressed Genes (DEGs) in A3250 when compared to MDA-MB-231, 7033 DEGs compared to IBC3, and 4919 DEGs compared to SUM149. There were 1779 DEGs in A3250 when compared to all three cell lines, MDA-MB-231, SUM149 and IBC3 (Fig. 3b).

IPA revealed that in comparison to MDA-MB-231 cells, A3250 displayed remarkable enrichment in pathways associated with inflammation. There were ten canonical pathways activated in A3250 compared to MDA-MB-231, of which eight were associated with inflammation (Fig. 3c, Supplementary Data 2). The wound healing pathway was most significantly upregulated, followed by the tumor microenvironment (-log(p value): 9.99 and 8.12, respectively), whereas TREM1 signaling showed the highest z-score (3.58). The TREM1 pathway triggers and amplifies inflammation through interactions with TLR pathways, and is
upstream from NF-κB. Other top enriched canonical pathways included hypercytokinemia (cytokine storm), IL-6, oncostatin M (member of the IL-6 family), IL-17, acute phase response signaling and pyroptosis, which are all related to inflammation and perturbation of the immune system. Among pathways inactivated in A3250 compared to MDA-MB-231, several were related to neuronal signaling, such as semaphorin, netrin and CREB signaling in neurons (Supplementary Data 2).

In comparison to IBC3, A3250 showed activation of pathways related to fibrosis, EMT and cytoskeleton among others (Fig. 3c, Supplementary Data 3). Activation of PI3/AKT signaling and tumor microenvironment, and inactivation of semaphorin signaling pathway were observed in A3250 in comparison to both, MDA-MB-231 and IBC3. A3250 showed only five pathways enriched in comparison to SUM149, of which LXR/RXR canonical pathway, related to lipid metabolism and inflammation was most significantly upregulated (Fig. 3c, Supplementary Data 4).

We next performed Gene Set Enrichment Analysis (GSEA) by using the hallmark collection of molecular signature databases (MSigDB), which capture and refine information from multiple gene sets and show high degrees of association with the corresponding protein profiles. GSEA showed that from 50 hallmark datasets, 34 gene sets were significantly enriched in A3250 compared to MDA-MB-231, 31 gene sets compared to IBC3, and 22 gene sets compared to SUM149. Among the pathways implicated in inflammation, hallmark “TNFA signaling via NFKB” was significantly enriched in A3250 compared to MDA-MB-231, IBC3 and SUM149 (Fig. 3d).

Hallmark “IL6 JAK STAT3 signaling” was significantly enriched in A3250 compared to MDA-MB-231 and IBC3 (Fig. 3d) and only marginally compared to SUM149 (p = 0.098, FDR = 0.12), indicating that JAK/STAT3 pathway is activated in A3250 and in agreement with previous studies implicating the JAK/STAT3 pathway in IBC5.

Consistent with our evidence indicating activation of multiple inflammatory pathways in A3250 by IPA and GSEA, analysis of activated upstream regulators showed a striking association with inflammation in comparison to MDA-MB-231 (Fig. 3e, Supplementary Data 5). The top differentially activated upstream regulator was TNF (z-score 8.0; p value of overlap 1.86E–50), followed by other upstream regulators driving inflammation and regulating innate immunity. Prominent categories included interferons (INFG, INFNA, INFNA2, INFNL1), interferon regulatory factors (IRF7, IRF3, STING), interleukins (IL1A, IL1B) and TLRs (TLR4, TLR7) (Fig. 3e, Supplementary Data 5). IRF7 for example is a key transcriptional regulator of type 1 INF-dependent immune responses and plays a critical role in innate immune responses. Among the top inactivated upstream regulators was RC3H1 that modulates activity of the NF-κB pathway and restrains inflammation by suppressing TNF expression18. TNF was also among the top ten upstream regulators activated in A3250 compared to IBC3 (Fig. 3e, Supplementary Data 6), but not to SUM149 (Fig. 3e, Supplementary Data 7). Taken together, these data demonstrate prominent activation of pathways associated with inflammation and innate immunity in A3250 IBC compared to MDA-MB-231 non-IBC cells, whereas differences in inflammatory pathways between A3250 and IBC3 or SUM149 IBC were less pronounced.

A3250 IBC tumors induce prominent regional and systemic inflammation. Pathologists have divergent views about the extent and significance of inflammatory infiltrate in IBC19, and the prevailing view is that inflammation is a consequence of the blockage of dermal lymphatic vessels by cancer cells. Thus, we sought to characterize the kinetics of inflammatory cell accumulation in A3250 tumors in comparison to LVI. Immunostaining demonstrated that A3250 tumors were heavily infiltrated with CD45+ cells four weeks post-injection, in comparison to MDA-MB-231 tumors (Fig. 4a, b). In the dermis, CD45+ cells were already abundantly present at three weeks post-injection (Fig. 4c). Dermal lymphatics were numerous and enlarged at that same time-point, but did not yet contain tumor emboli (Fig. 4d). A high
density of CD45+ cells was also observed in the dermis of the skin overlying A3250 tumors, even when the tumor was distant from the skin, separated by a layer of muscle and fat (Supplementary Fig. 4a–c). Striking accumulation of CD45+ cells in A3250 tumors was seen as early as a week post-inoculation (Fig. 4e, f), concomitant with lymphatic vessel dilation in the dermis, but prior to detectable tumor invasion of lymphatic vessels. These results demonstrate that inflammatory cells accumulated prior to tumor invasion of dermal lymphatics. Thus, the abundance of inflammatory cells in A3250 xenografts likely resulted from their active recruitment by tumor-derived factors, rather than as a consequence of the blockage of lymphatic vessels by tumor emboli.

Immunostaining further revealed a very high density of F4/80+ macrophages throughout the viable A3250 tumor and in the adjacent dermis (Fig. 4g–i, Supplementary Fig. 4d–f). In comparison, both SUM149 (Fig. 4j–m, Supplementary Fig. 4g–i) and MDA-MB-231 tumors (Supplementary Fig. 4j–l) had much lower macrophage content. Quantification of F4/80+ macrophages in tumor sections revealed ~3-fold increase in A3250 tumors compared to either SUM149 or MDA-MB-231 (Supplementary Fig. 4m). Very few Ly6G+ neutrophils were detected in viable areas of A3250 or SUM149 tumors, in contrast to MDA-MB-231 tumors which were uniformly infiltrated (Supplementary Fig. 4n–p). Immunostaining with F4/80 also revealed high density of macrophages in the skin overlying A3250 tumors in comparison to MDA-MB-231 (Supplementary Fig. 4q–s).

Flow cytometry demonstrated that A3250 tumors contained two major populations of CD11b+ myeloid cells at four weeks post-injection (Fig. 5a–c, Supplementary Fig. 5a, b), based on Ly6C and Ly6G in combination with other markers to define the myeloid lineage subsets.20–22 Tumor-associated macrophages (TAMs, defined as CD45+CD11b+ Ly6C−Ly6G−) were most abundant (avg 32.2%, median 31.7%), followed by Ly6Chi inflammatory monocytes (avg 19.5%, median 19.6%) (CD45+CD11b+Ly6ChIF4/80lo/Ly6G−). In contrast, tumor-associated neutrophils (TANs, defined as CD45+CD11b+Ly6G+Ly6CloF4/80−) were the least represented CD11b+ subset (avg 17.6%, median 9.6%) (Fig. 5c). A3250 tumors had ~3-fold higher proportion of TAMs and ~3-fold lower proportion of TANs compared to MDA-MB-231. In contrast, TANs were the most highly represented subset in MDA-MB-231 tumors, and the proportion of TAMs was the lowest (Fig. 5d–f).

We next examined lungs from mice bearing A3250 tumors at four weeks post-injection to gain insights into the
microenvironment of metastases. H&E staining revealed abnormal lung architecture characterized by areas of dense inflammatory infiltrate and loss of alveolar structures (Fig. 6a–d). Immunostaining showed that CD45+ inflammatory cells were associated with disseminated A3250 tumor cells, and were more prominent than in the lungs of mice bearing MDA-MB-231 tumors of comparable size (Fig. 6e–h). Flow cytometry demonstrated a trend towards an increase of Ly6Chi myeloid cells and a ~40% increase in Ly6Ghi neutrophils compared to the lungs of tumor-naive mice (Fig. 6i). Fractions of total CD11b+ cells, Ly6Chi inflammatory monocytes and Ly6Ghi neutrophils were significantly increased in blood in comparison to tumor-free mice (Fig. 6j), and this correlated with the increase of these cell subsets in the lung. In comparison to MDA-MB-231, Ly6G+ cells were significantly increased and there was a trend towards increase of Ly6C+ cells in blood. The fraction of Ly6G+ cells relative to F4/80+ and Ly6C+ cells was lower in lungs with A3250 metastases than in the lungs with MDA-MB-231 metastases (Fig. 6k), because the total number of Ly6G+ cells was 2.8-fold lower (Avg. number of lung Ly6G+ cells/g tissue: A3250, 3.7 × 10^6 vs. MDA-MB-231, 14.2 × 10^6). Of note, despite an increase of Ly6G+ neutrophils in the lungs and blood, there were very few Ly6G+ cells within A3250 primary tumors, suggesting a mechanism for selective exclusion of neutrophils. In contrast, in the MDA-MB-231 tumor model Ly6G+ neutrophils were the main cell subset recruited to both, primary tumors (Fig. 5c, e, f) and to the lungs (Fig. 6k).

CCL2 is highly expressed in A3250 cells and in human IBCs.

To identify factors that might mediate the florid recruitment of myeloid cells by A3250 tumors, we analyzed chemokine expression in a mRNA-Seq data set generated from A3250 tumors and cells in vitro. Analysis of human chemokines expressed by the A3250 tumors revealed expression of 10 out of 44 chemokines (FPKM ≥ 1 in each sample; Fragments Per Kilobase of exon per Million mapped reads), of which only three, CCL2, CXCL5, and CCL20, were expressed at high levels both in vivo and in vitro (FPKM > 10) (Fig. 7a, Supplementary Data 8). By comparison of their expression levels in tumors and cell culture in the same experiment, CCL2 was the highest expressed chemokine by mRNA-Seq (388 and 467 FPKM, respectively) and by quantitative Polymerase Chain Reaction (qPCR) (Fig. 7a, b; Supplementary Data 8). SUM149 cells expressed several chemokines from the CXCL family that were also expressed by A3250, including CXCL1, 2, 3, 5, and 16, but did not express CCL2 (Fig. 7b). CCL2 mRNA was also highly upregulated in A3250 cells not only in comparison to SUM149, but also to IBC3 and to a panel of non-IBC breast cancer cell lines (Fig. 7c, Supplementary Table 4). High levels of CCL2, CXCL5 and CCL20 proteins were confirmed in supernatants of A3250 cells by Olink multiplex immunoassay. SUM149 IBC cells also secreted CXCL5 and CCL20, but not CCL2 protein, whereas MDA-MB-231 expressed either no detectable or very low levels of this chemokine (Fig. 7d, Supplementary Table 5). While these results correlated relatively well with qPCR or mRNA-Seq, any differences could reflect cell specific variations in RNA translational efficiency or protein secretion.

To investigate how the chemokine expression profile of A3250 cells related to that of human breast cancers, we analyzed expression levels of the top three most highly expressed chemokines, CCL2, CXCL5, and CCL20, in the TCGA-BRCA database of human invasive breast cancer samples of which none were annotated as IBC (n = 1222). All three chemokines were expressed at much higher levels in A3250 IBC compared to their median expression levels in TCGA breast cancer samples (Fig. 7e). To interrogate expression of CCL2 in IBC patient samples, we analyzed a Van Laere microarray data set consisting of 41 human IBCs and 55 non-IBCs, and an Iwamoto data set consisting of 25 IBCs and 57 non-IBCs. These data sets were chosen because they had most IBC samples and because mRNA-Seq data for human IBCs have
CCL2 median expression levels were ranked significantly higher in IBCs than in non-IBCs in both datasets (Fig. 7f, g). Taken together, these data indicate that human IBCs, like A3250, commonly exhibit high CCL2 expression levels.

Chemokine receptor CCR2 is highly expressed by myeloid cells in the A3250 IBC tumor model. CCL2 exerts its effects through CCR2, which is the main chemokine receptor mediating monocyte and macrophage recruitment to sites of inflammation.27,28. We investigated the expression of CCR2 in A3250 tumors, lungs and on leukocytes in the blood. In the blood, CCR2 was expressed by ~30% of total CD11b+ cells, and mainly by Ly6Chi inflammatory monocytes (76% CCR2+ of Ly6Chi on average), suggesting increased mobilization of these cells from the bone marrow. A small fraction of Ly6Clo monocytes (7% CCR2+ of Ly6Clo on average) also expressed CCR2, whereas CD11b+ cells that were Ly6C− were also negative for CCR2 (Fig. 7h–j, Supplementary Fig. 5c, d). In A3250 tumors, CCR2 was expressed by a majority of CD11b+ cells (~90%), and in lungs by ~45% of all CD11b+ cells. In tumors, CCR2 was expressed by Ly6Clo monocytes and by Ly6CloF4/80+ macrophages to a comparable extent (Fig. 7h–j and Supplementary Fig. 5e). In the lung, Ly6Clo cells were the main subtype that expressed CCR2, with some expression also by Ly6Clo macrophages (Fig. 7h–j, Supplementary Fig. 5f). CCR2 was not expressed by CD11b+F4/80+ Ly6C− cells in the blood, tumors or in the lungs (Supplementary Fig. 5c–f). Together, these results demonstrated high levels of CCR2+ inflammatory monocytes in the blood and high CCR2 expression by myeloid cells associated with A3250 tumors and lung metastases. While CCL2 was highly expressed by A3250 cells, other human chemokines that activate CCR2 (CCL7, CCL8, CCL13, and CCL16), were not expressed (Supplementary Fig. 5g, Supplementary Table 6). These findings implicated CCL2 as the key tumor cell-derived agonist of CCR2 in the A3250 model and strongly suggested an important role for CCL2 in monocyte and macrophage recruitment to both A3250 primary and metastatic sites.

CCL2 knockdown markedly reduces A3250 tumor growth, lung metastasis, and inflammatory phenotype. To directly investigate the function of CCL2 in the malignant phenotype of A3250 tumors, we performed shRNA knockdown (KD) for CCL2. shCCL2 resulted in a ~63% reduction in CCL2 mRNA, as determined by qPCR, and in a decrease of secreted CCL2 protein in vitro (Fig. 8a, b). CCL2 protein showed multiple bands on Western; including a 11 kDa native form and 13 kDa and 15 kDa glycosylated forms.29. In tumors, CCL2 transcripts were reduced not yet been reported. CCL2 median expression levels were ranked significantly higher in IBCs than in non-IBCs in both datasets (Fig. 7f, g). Taken together, these data indicate that human IBCs, like A3250, commonly exhibit high CCL2 expression levels.

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by 76% on average, as determined by mRNA-Seq (Fig. 8c; Supplementary Table 6). Downregulation of CCL2 in A3250 cells did not result in an altered expression of other human CCR2 ligands, which remained undetectable (Supplementary Table 6).

CCL2 KD led to a striking inhibition of tumor growth, as determined by bioluminescent imaging (Fig. 8d). A3250 CCL2 KD tumors also exhibited a striking reduction in clinical signs of skin inflammation characteristic of IBC (Supplementary Fig. 6a–c). Erythema in particular was drastically diminished upon CCL2 KD, and this effect was independent of tumor size. By comparison, SUM149 IBC tumors did not show pronounced erythema (Supplementary Fig. 6a, b). Furthermore, CCL2 KD tumors also exhibited a striking reduction in clinical signs of skin inflammation characteristic of IBC (Supplementary Fig. 6a–c). Erythema in particular was drastically diminished upon CCL2 KD, and this effect was independent of tumor size. By comparison, SUM149 IBC tumors did not show pronounced erythema (Supplementary Fig. 6a, b). Furthermore, CCL2 KD
decreased both the incidence and the overall metastatic burden of lung metastases (Fig. 8e, f). The majority of mice bearing CCL2 KD tumors lacked macro-metastases in the lungs, and mainly presented with disseminated tumor cells, whereas mice with control A3250 tumors mainly presented with macro-metastases. Histopathological analysis of cytokeratin-stained tumor sections revealed that the invasiveness of A3250 tumors was not decreased upon CCL2 KD (Supplementary Fig. 7a–f), suggesting that CCL2 KD primarily decreased metastatic colonization rather than dissemination.

Primary tumors exhibited an early onset of tumor necrosis upon CCL2 KD. Analysis of endogenous expression of GFP in tumor sections revealed that CCL2 KD tumors, only the peripheral tumor area was viable (Fig. 8g, h), and H&E staining showed large necrotic areas in comparison to control tumors at the four-week time-point (Fig. 8i–k). This was not due to the inhibition of angiogenesis, since blood vessel densities in tumors were not changed upon CCL2 KD (Supplementary Fig. 7g–i). CCL2 KD also reduced tumor proliferation by as much as 83%, with an average number of EdU+ cells/mm² tumor decreased from 458 to 78 per 6 h (Fig. 8l–m). This was not due to the decrease in proliferating cells in vitro (Fig. 8o, p), arguing that CCL2 did not stimulate A3250 growth in an autocrine manner. Similar in vitro and in vivo data were obtained using A3250 cells transduced with an independent shCCL2 construct (Supplementary Fig. 8). In accordance, A3250 tumor cells lacked detectable levels of the CCR2 receptor by mRNA-Seq (Supplementary Table 7). In contrast, CCR2 was highly expressed by mouse cells in the microenvironment of A3250 tumors (Supplementary Table 7).
experiments. Black lines on the plots indicate mean values ± SEM. (provided as a Source Data Table 7). These results indicated that CCL2 did not promote A3250 tumor growth through autocrine stimulation and supported the concept that macrophages recruited by CCL2 promoted A3250 tumor cell proliferation and viability in vivo.

CCL2 downregulation in tumors decreases macrophage infiltration and systemic inflammation. Immunostaining of immune cells in CCL2 KD tumors revealed a striking reduction of CD45+ cells in viable tumor areas, most of which were F4/80+ macrophages (Fig. 9a, b), thus correlating the loss of tumor macrophages with inhibition of tumor growth. To examine the systemic effects of CCL2 downregulation, we examined the lungs of A3250 tumor-bearing mice during the early stages of tumor dissemination by immunostaining (3–4 weeks post-injection). In the lungs of mice bearing A3250 control tumors, hotspots of dense inflammatory infiltrates associated with disseminated tumor cells were observed (Fig. 9c–e). These inflammatory hotspots were not present in the lungs of mice bearing A3250/CCL2 KD tumors, and CCL2 KD led to a striking normalization of the lung architecture (Fig. 9c–e). Flow cytometry revealed a reduction of Ly6C\textsuperscript{hi} cells in the lungs by 75%, to the normal level, and of total CD11b+ cells by 58% (Fig. 9f). These results correlated with a decrease of Ly6C\textsuperscript{hi} inflammatory monocytes in the blood to the normal level of naïve age-matched mice (88% reduction) (Fig. 9g). In contrast, the number of Ly6C\textsuperscript{hi} cells in the spleen was unchanged upon CCL2 KD and was not correlated with the levels of Ly6C\textsuperscript{hi} cells in the blood or in the lungs (Fig. 9g). This decrease in inflammatory cell accumulation in the lungs correlated with the decrease in A3250 lung metastases, consistent with a metastasis-promoting role of Ly6C\textsuperscript{hi} cells in the lung.

Human IBCs are enriched in macrophages. A recent study identified distinct clusters of human TNBCs based on immune infiltrates that ranged from macrophage-enriched to neutrophil-enriched\textsuperscript{21}. To examine whether human IBCs are macrophage-enriched, we analyzed microarray data sets of 41 human IBCs and 55 non-IBCs\textsuperscript{24,25}. We applied a 37-gene signature specific for TAMs in breast cancer\textsuperscript{30}. Hierarchical clustering revealed two groups of breast cancers, enriched or not enriched in TAM genes. Among breast cancers, there were significantly more IBC samples enriched in TAM genes (60% IBCs, 40% non-IBCs), than not enriched in TAM genes (24.5% IBCs, 75.5% non-IBCs) (p value of the chi-square test of 0.007) (Fig. 10a).

We extended this analysis by immunostaining an independent cohort of nine human IBCs for the presence of macrophages (CD68\textsuperscript{+}) and neutrophils (CD66b\textsuperscript{+}) (Fig. 10b and Supplementary Fig. 9). High macrophage content was seen throughout the tumors, both in the stroma and/or within tumor cell clusters, in tight apposition to tumor cells. In IBC cases presenting as tumor cell clusters in dermal lymphatic vessels, we observed a dense infiltration of CD45\textsupersupport{+} cells in the blood or in the lungs (Fig. 9g). This decrease in inflammatory cell accumulation in the lungs correlated with the decrease in A3250 lung metastases, consistent with a metastasis-promoting role of Ly6C\textsuperscript{hi} cells in the lung.
accumulation of CD68+ macrophages in the dermis. In stark contrast, neutrophils were rare in all IBC tumors analyzed (Fig. 10b, Supplementary Fig. 8). These results were consistent with the gene expression analysis indicating that human IBCs are macrophage enriched, and in agreement with our findings with A3250 tumors, further underscoring the relevance of the A3250 tumor model to human IBC.

Discussion

IBC remains a major clinical challenge, as this highly aggressive form of breast cancer is the most difficult to diagnose and treat, yet has no specific targeted therapies. Understanding of the cellular and molecular determinants of IBC has lagged far behind that of other breast cancers, largely due to the lack of adequate models for this disease and the relative rarity of this tumor phenotype. Although several human IBC-derived cell lines have been reported, none recapitulates the full spectrum of IBC features in a mouse model. The human triple-negative IBC cell line A3250, described here, closely recapitulates the key features of human IBC in a mouse xenograft model, including skin erythema, flat tumor appearance, diffuse tumor growth pattern, high stroma to tumor ratio, lympho-vascular tumor emboli in the skin, and extensive lymph node and distant metastases. The fact that the SCID mouse strain utilized is deficient in adaptive immunity indicates that T and B lymphocytes are not necessary for the aggressive IBC phenotypic manifestations.

It has been generally assumed that the inflammatory phenotype of IBC, which includes erythema and edema of the breast and overlying skin, is a consequence of tumor emboli in dermal lymphatics, which are believed to impede egress of inflammatory cells from the tissue. While dermal lymphatic vessel invasion and clinical symptoms of inflammation coincide in IBC, there have been no studies to date concerning their causal relationship. We demonstrated in the A3250 IBC tumor model that inflammation is driven directly by the tumor, and is not secondary to dermal lymphatic vessel invasion. This conclusion is based on our observations from the longitudinal analysis of tumor development that demonstrated vigorous recruitment of inflammatory cells into the tumor and adjacent skin early in tumor development, prior to dermal vessel invasion and formation of lympho-vascular emboli. This does not preclude, however, that at the later stages of tumor development potential changes in lymphatic vessel functions may further impact tissue inflammation.

IBC is still primarily a clinical diagnosis, and despite the inflamed clinical appearance of the skin, very few studies have investigated inflammatory cells in IBC, and to what extent they contribute to IBC progression. We observed exceptionally high macrophage content in A3250 tumors and in adjacent skin, and this was confirmed in human IBC tissues. Macrophages were frequently seen in direct contact with IBC tumor cells, whereas in most breast cancers macrophages are typically localized at the tumor periphery or in the stroma. High macrophage densities were also observed in uninvolved skin, in line with a recent report that in patients, breast tissue not involved with IBC was enriched in macrophages. Another study found a significant increase of CD14+ monocytes in blood and in tumors of patients with IBC compared to non-IBC, consistent with our findings. Taken together, our data reveal that the prominent presence of macrophages in close contact with tumor cells is a common characteristic of IBC. Finally, in most human breast cancers and in mouse models, neutrophils are the most abundant subset of myeloid cells, while in A3250 tumors and in human IBCs neutrophils were sparse. A recent study found that in breast cancer categorized TNBCs based on TAN and TAM frequency. Accordingly, IBC can be classified as a macrophage-enriched subtype with low TAN to TAM ratio.

CCL2 is a well-known mediator of the innate response and inflammation that supports breast cancer progression through several mechanisms. TAMs recruited by CCL2 facilitate early steps of metastases by promoting tumor cell migration and intravasation. CCL2 enhances recruitment and retention of metastasis-associated macrophages, and has been shown to promote metastatic recurrence after chemotherapy. We demonstrated that the high level of endogenous CCL2 expression by A3250 tumor cells was a key factor in driving the inflammatory cell influx into IBC and identified the important function of CCL2 in promoting IBC tumor growth. CCL2 KD, and consequently reduction in TAM densities, led to a striking reduction of tumor cell proliferation and a concomitant increase in tumor growth.

Hierarchical clustering of human IBCs and non-IBCs based on expression of genes characteristic for tumor-associat...
necrosis. Thus, A3250 cells were highly dependent on TAMs for primary tumor growth, while exaggerated systemic inflammation driven by exceptionally high levels of CCL2 promoted expansion of metastases. These results make it tempting to speculate that macrophages may directly support tumor cell metabolic needs critical for proliferation and survival. Of note, SUM149, an IBC line, which does not express CCL2 at the RNA and protein level, showed a much less aggressive IBC phenotype, lacking erythema or dermal lymphatic invasion and with significantly less inflammatory infiltrate as compared to A3250. These findings further support the important role of high level endogenous CCL2 production in promoting the florid IBC phenotype. Finally, our evidence that macrophages are a dominant subset in clinical IBCs suggests that immunotherapeutic strategies that deplete or otherwise target macrophages may be promising therapeutic approaches for IBC. Thus, this IBC tumor model provides a discovery tool with which to facilitate further understanding of IBC biology and potentially foster the development of much needed IBC-targeted therapeutics.

Methods

Cells and culture conditions. The A3250 breast carcinoma line was isolated from a primary tumor of a 50-year-old anonymized patient with IBC, provided to the Aaronson lab from a tissue biorepository directed by Dr. Dirk Iglehart, Duke University. We have compiled with all relevant ethical regulations. Duke IRB Pro00012025 “Breast Cancer Blood/Tissue Repository and Database” is a continuance of Dr. Iglehart’s IRB protocol permitting them to provide anonymized human tumor tissues as byproducts of standard clinical procedures to other investigators for cancer research. The Mount Sinai IRB considers research performed with anonymized human samples as non-human subjects research. The A3250 IBC cell line was established in culture by the Aaronson lab as performed for other A series tumor lines45,46. Briefly, the tumor was cut into 2 mm pieces in high-glucose DMEM supplemented with 4 mM L-glutamine (Sigma, Cat. # D6429), 10% FBS (Corning, Cat. # 35-010-CV) and 1X antibiotics (100 U/ml penicillin, 100 U/ml streptomycin) (Gibco, Cat. # 15140-122), and pieces were evenly dispersed within several T25 tissue culture flasks (~12 pieces/flask). Cultures were incubated at 37 °C and medium was replaced only if pH changes occurred or when significant debris was present. Once confluent, cells were split at a 1:2 ratio. The SUM149 IBC and HCC3153 TNBC cell lines were generously provided by Dr. Stephen Ethier (Medical University of South Carolina), and Dr. John Minna, respectively. MDA-MB-157, MDA-MB-231, SKBR3 and MCF7 breast cancer cell lines were obtained from ATCC. A3250 and all other tumor cells except MDA-MB-231 and SUM149 were cultured in high-glucose DMEM supplemented with 4 mM L-glutamine (Sigma, Cat. # D6429), 10% FBS (Corning, Cat. # 35-010-CV) and 1X antibiotics (100 U/ml penicillin, 100 U/ml streptomycin) (Gibco, Cat. # 15140-122), and pieces were evenly dispersed within several T25 tissue culture flasks (~12 pieces/flask). Cultures were incubated at 37 °C and medium was replaced only if pH changes occurred or when significant debris was present. Once confluent, cells were split at a 1:2 ratio. The SUM149 IBC and HCC3153 TNBC cell lines were generously provided by Dr. Stephen Ethier (Medical University of South Carolina), and Dr. John Minna, respectively. MDA-MB-157, MDA-MB-231, SKBR3 and MCF7 breast cancer cell lines were obtained from ATCC.

Retrovirus and lentivirus generation. To generate retroviruses for constitutive gene expression, the expression plasmid of interest was co-transfected with the pCL-ampho packaging plasmid in HEK293T cells. For lentivirus production46, HEK293T cells were co-transfected with expression plasmid of interest, pCMV–D8.91 packaging and pMD VSV-G envelope plasmids using Lipofectamine 2000 according to manufacturer’s instructions. Conditioned medium containing viral particles was harvested two, three and four days after transfection and virus stock was pooled and aliquots were stored at −80 °C until use.

Generation of stable cell lines. To generate A3250 and MDA-MB-231 cells expressing eGFP (enhanced Green Fluorescent Protein) and firefly luciferase (Fluc) (A3250-eGFP-Fluc and MDA-MB-231-eGFP-Fluc), parental cells were transduced with the pCCBS-EGFP retrovirus, followed by puromycin selection46. The cells were then transduced with the NSNI-luc lentivirus45 and selected with neomycin and puromycin. A3250 Ctrl#2 and A3250-shCCL2#2 cells were generated by transducing A3250-eGFP-Fluc cells with either pLRKO.1 scramble or pLRKO.1 vector with CCL2 targeting sequence GATGTGAAACATTATGCCTTA from Sigma MISSION shRNA (Drug Discovery Institute, Mount Sinai). Cells were maintained in the presence of puromycin and neomycin. All transductions were performed in presence of 5 µg/ml polybrene.

Cell line authentication. Short tandem repeat (STR) data were generated on the SeqStudio Genetic Analyzer, at the Dept. of Oncological Sciences Sequencing core. We confirmed that the STR signatures of A3250 cells did not match STRs of known tumor cell lines in the ATCC or DMSZ STR database. SUM149, MDA-MB-157, MDA-MB-231, HCC3153, SKBR3, and MCF7 cell lines were also authenticated using karyotype analysis. Cells were propagated per the manufacturer’s recommendations (FTA Sample Collection Kit, cat. no. 135-XV) and processed as described46.

Incucyte proliferation assay. A3250 cells were seeded to 40% confluence in 6-well plates in complete media with 10% FBS, allowed to adhere for 6 hours, and subsequently changed to media containing 10% or 1% FBS. Cells were imaged every four hours for four days using the Incucyte® ZOOM (Essen BioScience Inc, GV UI Version 2018), for a total of 16 10X images per well for each time-point. Media was changed once, 48 hr after the start of imaging. Average percent confluence was calculated for each well at every time point (n = 2 wells per condition).

Colonies formation assay. A3250 and MDA-MB-231 tumor cells were seeded in triplicate into 6-well plates at a density of 500 or 250 cells per well (52 or 26 cells/cm²). Cells were seeded in complete media with either 10% or 1% FBS and imaged every 11 days, with medium changes every four days. Cells were then fixed with ice-cold methanol (100%) for 10 min and stained with a 0.2 % crystal violet in methanol for 30 min at room temperature. Images of each well were taken and colony numbers were quantified using ImageJ v. 1.52.

Mouse experiments and tissue collection. SCID mice were used in all experiments unless indicated otherwise. Animals were housed in climate-controlled barrier conditions with automated light/dark cycles. A minimum of 100 µl PBS, Phosphate-Buffered Saline solution), SUM149 (2 × 10⁶ cells in 100 µl PBS) or MDA-MB-231 (2 × 10⁶ cells in 100 µl PBS) tumor cells were injected orthotopically into the fourth mammary fat pads on both sides of six to eight-week-old SCID or NOD SCID mice (Charles River Laboratories). For most experiments, SCID mice were bred inhouse. Mouse weights and tumor sizes were measured weekly. Mice were sacrificed one to eight weeks post-injection, and primary tumors and organs were collected. Six hours before sacrifice, mice were injected with 100 µg of 5-ethyl-2’-deoxyuridine (EdU). For each mouse, tumors, sentinel lymph nodes (inguinal), distant lymph nodes (axillary and brachial), lungs and liver were collected. Tumors and organs were fresh-frozen in OCT, or fixed for 1 hr in formalin and embedded in paraffin. Organs collected from mice with GFP-labeled tumors were fixed for 1 hr in formalin and frozen in OCT, to preserve GFP and allow the evaluation of metastases at a cellular resolution. The percent change in body weight was determined through the difference between the body weights measured during week 6 and week 6 (i.e. the endpoint) of the experiment. Mouse experiments were approved by Icahn School of Medicine at Mount Sinai Animal Care and Use Committee (IACUC) and in compliance with all relevant ethical regulations for animal testing and research. Maximal tumor burden permitted by Mount Sinai’s IACUC is 10% of mouse body weight or tumor diameter of 10 mm in any dimension. We confirm that tumors did not exceed maximum approved tumor size or burden. In most experiments tumors were collected when 5–7 mm in diameter.

Bioluminescent imaging of tumors and metastases. Mice were anesthetized and background luminescence signals were acquired. Mice were then received an intraperitoneal injection of D-luciferin dissolved in sterile PBS (150 mg/kg), and images were acquired every two minutes until peak luciferin signal was achieved for each mouse. Subsequent to in vivo imaging, mice were sacrificed and organs were quickly harvested. D-luciferin was added again to excise organs and metastases in organs imaged ex vivo. Organs from a mouse injected with the parental tumor cell line not labeled with Fluc were imaged ex vivo and used to determine background values for each tissue. Bioluminescent imaging was performed on the IVIS® Spectrum (PerkinElmer) and analyzed using LivingImage software, version 4.5 (PerkinElmer).

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macrometastases (clusters of >10 tumor cells). The biomimetic value for each tissue was then compared to the metastasis evaluation by GFP, and the following classification emerged: tissues with Fluc values ≥10^5 to <10^6 were detectable by GFP analysis; Fluc values ranging from ≥10^4 to <10^5 represented few isolated tumor cells and some micrometastases; Fluc values from ≥10^3 to <10^4 reflected mainly micrometastases and some isolated tumor cells; and Fluc values ≥10^2 reflected presence of macrometastases, in addition to many micrometastases and isolated tumor cells.

**Immunofluorescent staining and quantification.** The following antibodies were used for immunostaining of frozen tissue: LYVE-1 (Abcam ab14917; 1:600), CD34 (Invitrogen 14-0341-82; 1:100), CD45 (Abcam ab10558; 1:100), F4/80 (Bio-Rad MCA497; 1:100), CD11b (Biolgend 101201; 1:200), Ly6G (Biolgend 127601; 1:100) and pan-lymphocytes (Abcam ab3977; 1:100). Tissues were incubated in 100% (pH 10.5) and 80% methanol (pH 3, 5 min), primary antibodies were diluted in 12% BSA/PBS, and incubated for 1 hr (LYVE-1, CD45, Ly6G) or 2 hr (F4/80, CD11b, pan-lymphocyte) in a humidity chamber at room temperature. Corresponding secondary antibodies labeled with Alexa Fluor 488 (Invitrogen cat. no. A-11070), 555 (Invitrogen cat. no. A-21434) or 594 (Invitrogen cat. no. A-11037) were incubated for 1:300 and incubated at room temperature for 30–45 min. Nuclei were counterstained with DAPI (10 μg/ml). Slides were coverslipped using fluorescein mounting medium (Dako cat. no. S3023). Images were captured using the Nikon Eclipse E600 microscope and DS-Qi1 monochrome camera. Whole-slide scans were acquired with the NanoZoomer S60 scanner C13210-01 (Hamamatsu). Analyses were performed using IMaris software (Oxford Instruments) to determine tumor boundaries and exclude necrotic areas. Viable tumor area was quantified as above and data expressed as GFP+ tumor area/total tumor area analyzed (% GFP area).

**Macrophage in tissues.** EdU was visualized in formalin-fixed OCT-embedded frozen tumor sections using the Click-it® EdU Imaging Kit with Alexa Fluor 594 (red) (Invitrogen cat. no. A-11037, 1:300). EdU proliferation and GFP quantification in tumors. EdU was visualized in formalin-fixed OCT-embedded frozen tumor sections using the Click-it® EdU Imaging Kit with Alexa Fluor 594 (red) (Invitrogen cat. no. A-11037, 1:300). Following EdU staining, sections were incubated in 0.5% TritonX/PBS at room temperature for 20 min, and immunostained with anti-GFP antibody (Abcam ab26556; 1:100) to enhance the GFP signal. Images were captured with the Nikon Eclipse E600 microscope and DS-Qi1 monochrome camera. Tumor cell proliferation was determined by quantifying EdU+ signal in the GFP-positive tumor areas using NIS Elements 3.22 (Nikon). Three 20X images per tumor were quantified (total area of 2.5 × 10^5 μm² per tumor, n = 3 tumors per experimental condition) and data expressed as viable tumor area (%). EdU proliferation was measured as % positive cell area/ viable tumor area analyzed.

**Flow cytometry.** Xenograft mouse tumors (~5 mm in diameter, spleens and lungs were harvested into RPMI 1640). Age- and sex-matched normal tissue mice were used as controls. Tissues were digested in collagenase D (Sigma 11088858001) using the MACSmiX tube rotor (Miltenyi) at 37 °C, and pressed through a 70 μm filter. Lymph nodes were dissociated by mechanical disruption. Blood was collected by cardiac puncture. Erythrocytes in blood, spleen and lung were lysed using RBC Lysis Buffer (Bio-Rad 127608). As per the manufacturer’s protocol, Cells were counted using the Countess II Automated Cell Counter (Invitrogen). Samples were stained with antibodies on ice for 30 minutes in FBS Stain Buffer (BD Pharmingen 554656), and run on the BD LSRSorter X-20 cell analyzer. The antibodies used were all from Biolegend: anti-mouse: CD45- BV785 (Biolegend 103149), CD11b-APC (Biolegend 101212), Ly6G-PE (Biolegend 127608), Ly6C-PE-Cy5.5 (BD Biosciences 655723; 1:600) and anti-CD68 antibodies (Dako M0814; 1:100) on the same IBC tumor samples, assessed as highly invasive ductal carcinomas, whereas three samples presented exclusively as tumor emboli in dermal lymphatics. Human IBC tumor samples were immunostained with anti-CDC66 (BD Biosciences 555723; 1:600) and anti-CD68 antibodies (Dako M0814; 1:100) on the same sections, using Leica Bond RX immunostainer and according to the protocol for Multiplexed Immunohistochemical Consecutive Staining in Single Slide (MCCISS)[1,2]. Whole-slide scans were acquired with the NanoZoomer S60 scanner C13210-01 (Hamamatsu). Alternatively, images were captured with the Nikon Eclipse E600 microscope and DS-Ri1 color camera.

**Western Blotting.** For CCL2 detection, A3250-control or A3250-shCCL2 tumor cells (~7 × 10^6 cells) were cultured in 5 ml serum-free media for 24 hr. Conditioned media were concentrated 23-fold using Amicon Ultra Centrifugal Filter Units (Ultracel-3K, Millipore) and 20 μl was loaded on a 12% SDS-PAGE gel. Western blots were performed using FluoBlot Source Data is provided as a Data Source file.

**Oink multiplex immunoassay.** Supernatants of A3250, SUM149 and MDA-MB-231 cultures were collected at 24h and 48h after seeding 1 × 10^6 cells per 10 cm plate in 8 ml of standard media for each cell line. The conditioned media was harvested and analyzed for chemokines within the Oink inflammation panel (Oink Bioscience, Sweden) by the Human Immune Monitoring Center, Mount Sinai. The assay uses proximity extension assay (PEA) technology wherein oligonucleotide labeled antibody pairs allow pair wise binding to target proteins. The antibody bound target antigens which correspond to oligonucleotides from an amplicon are then quantified by high throughput qPCR and provided as relative quantification of protein concentration denoted as Oink proteomics arbitrary unit (NPX (normalized protein expression) on a log2 scale.

**RNA isolation and qPCR.** Total RNA was isolated from subconfluent cultures after 24 hr of plating. RNA was isolated using the RNeasy kit (Qiagen). RNA concentration and purity were measured using a NanoDrop spectrophotometer (ThermoFisher) and cDNA was generated from 1 μg RNA using SuperScript II reverse transcriptase (Invitrogen). qPCR was performed with Viia Real Time PCR System (Life Technologies, Carlsbad) using Fast SYBR Green Master mix (Applied Biosystems, Thermofisher). Transcript expression was normalized to the GAPDH housekeeping gene and miR21. The relative expression was calculated using the 2^(-ΔΔCT) method for qPCR analysis. For comparison of human chemokine expression in A3250 and MDA-MB-231 cell lines, sequences of the PCR primers were as follows: CCL2: 5′-ATCAC...
CAGCAGCAAGTGT-C, 5'-AGGTGCTGATGAGATGTGTC-3'; CCLX5: 5'-GAGAAGCTCTGTGATATTGAC-3'; 5'-TTTCTTGTTTGCTGACGCTCGA-3'; CCL20: 5'-TTTCTTGTTTGCTGACGCTCGA-3'; 5'-GATCCGCTAGGTGGTCCATGGAATCCTG-3'; A3250-shCCL2 (5'-AAGCCAAAGTACCCGCACTG-3'; 5'-AGGTGGTCCATGGAATCCTG-3'; 5'-TAAAAGCAGCCCTGGTGAC-3'; 5'-AAGTGTGAATGTAAGGTCCCC-3') and A3250-shCCL4 (5'-AAGGCAAAACCGAAATCTGG-3'; 5'-ATGGGGGATGAGAATGGTAGG-3'; GAPDH: 5'-CTC TGCTCCCTGGTGCC-3'; TFA: 5'-AAAAAGCAGCCCTGGTGAC-3'. For the heatmaps and trees, mRNA-Seq data was downloaded from Gene Expression Omnibus database GSE180788. For the Venn diagrams, we used the R package VennDiagram. We then calculated the percentile rank of gcrma normalized signal intensity for each of the analysed genes in each of the samples. Percentile ranks of CCL2 were calculated as described above and the significance of the difference in distributions of percentile ranks in IBC vs non-IBC samples was assessed using Wilcoxon rank sum test. We calculated the percentile rank of normalized signal intensity in IBC and non-IBC tumor samples for 37 tam-specified genes. For each gene, the percentile ranks were z-score transformed. The samples were hierarchically clustered using Ward's clustering criterion and Euclidean distance. Sample sets were divided into two clusters and the difference of frequencies of IBC samples in the two clusters compared using chi-square test.

Statistical analyses. Statistical differences in proliferation (EdU) and F4/80+ cell densities in tissue sections were determined through an unpaired parametric two-tailed t test. Differences in necrosis were determined using a one-sample two-tailed t test. Statistical difference in weight loss between tumor-naïve and tumor-bearing mice was determined through a one-sample t test. For flow cytometry analysis, statistical differences between cell subset fractions were determined using a goodness of fit linear regression model. Kruskal-Wallis non-parametric rank sum test was carried out to determine significance between absolute values of cell subsets. In the case of tumors and lymph nodes, where multiple tissues originated from the same mouse, a linear mixed model fit by REML and t test using Satterthwaite's method was used to control for repeated measures and determine significant differences between groups. Differences between absolute values of cell subsets were determined with a Wilcoxon rank sum test (Mann-Whitney non-parametric test). Tumor bioluminescence data was first log transformed to create symmetry, followed by a linear mixed model fit by REML and t test using Satterthwaite's method to test differences between the groups. Bioluminescence in the organs was first tested for normality, and significant differences were determined by a Mann-Whitney two-tailed t test.
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Author contributions
A.R. and S.D. participated in the experimental design, performed animal experiments, Fluc imaging, histopathology, immunostaining, image acquisition and analyzed data; A.R. also helped write the manuscript; I.P. and L.G. participated in the experimental design, performed cell and molecular biology experiments, and analyzed data; L.G. also generated the expression constructs and transduced cell lines utilized; R.F.R. performed animal experiments, immunostaining, whole-mount tissue imaging, image acquisition, and analyzed data; A.K.E. performed FACS experiments, immunostaining, and analyzed data; A.S., R.K. and R.S. performed bioinformatic analyses of mRNA-Seq data; R.S. developed the software for STR analysis, and. R.K. also performed microarray gene expression analyses; S. Y. and R.Q. performed animal experiments and histology; S.Y. also performed image acquisition (slide scanning); G.A. and S.J. evaluated pathology of human IBC specimens; M. S. designed and supervised the study, analyzed data and wrote the manuscript; S.A.A. initiated, designed and supervised the study, revised and edited the manuscript.

Competing interests
The authors declare no competing interests.

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