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Host Cxcr2-dependent regulation of mammary tumor growth and metastasis

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Abstract

Host-derived angiogenic and inflammatory tumor supportive microenvironment regulates progression and metastasis, but the molecular mechanism(s) underlying host-tumor interactions remains unclear. Tumor expression of CXCR2 and its ligands have been shown to regulate angiogenesis, invasion, tumor growth, and metastasis. In this report, we hypothesized that host-derived Cxcr2-dependent signaling plays an important role in breast cancer growth and metastasis. Two mammary tumor cell lines Cl66 and 4T1 cells were orthotopically implanted into the mammary fat pad of wild-type and Cxcr2^{-/-} female BALB/c mice. Tumor growth and spontaneous lung metastasis were monitored. Immunohistochemical analyses of the tumor tissues were performed to analyze proliferation, angiogenesis, apoptosis and immune cell infiltration. Our results demonstrated that knock-down of host Cxcr2 decreases tumor growth and metastasis by reducing angiogenesis, proliferation and enhancing apoptosis. Host Cxcr2 plays an important role in governing the pro-inflammatory response in mammary tumors as evaluated by decreased Gr1⁺ tumor-associated granulocytes, F4/80⁺ tumor associated macrophages, and CD11b⁺Gr1⁺ myeloid derived suppressor cells in Cxcr2^{-/-} mice as compared to control wild-type mice. Together, these results demonstrate that host Cxcr2-dependent signaling regulates mammary tumor growth and metastasis by promoting angiogenesis and pro-inflammatory responses.

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Conflict of interest Authors disclose no conflict of interest.

Keywords

CXCR2; Angiogenesis; Metastasis; Inflammatory response; Chemokines

Introduction

Despite improvement in current therapeutic regimens, breast cancer still remains the second most common cause of cancer death among women [1]. The vast majority of these deaths are due to therapy resistance, disease progression and metastasis [2]. The molecular mechanism(s) underlying breast cancer growth and invasion have been extensively examined; however, most of these studies are focused on malignant cells. The outcome of tumor progression and metastasis depends on both intrinsic properties of tumors and responses of the host [3–5].

Recent reports from our laboratory and others demonstrated increased expression of pro-inflammatory chemokines in various cancers and documented that they have an important role in the tumor microenvironment [6–8]. Chemokines have been shown to regulate the inflammatory response in multiple tumor types [9, 10]. The host immune response regulates tumor growth and progression through favorable host homeostatic mechanisms stimulating migration and interrupting these mechanisms may inhibit cancer metastasis [4, 5, 10]. CXCR2 and its ligands are known to be pro-inflammatory and angiogenic, supporting tumor growth and metastasis in an autocrine and paracrine manner [9, 11–15]. Importantly the ligands, CXCL8 and CXCL1, have been observed to influence breast tumor growth, chemoresistance and metastasis [6–8, 16, 17]. In addition, CXCR2 expressed by endothelial cells binds to its angiogenic ELR⁺ (Glu-Leu-Arg) ligands secreted by tumor cells and facilitates angiogenesis in breast tumors [11, 12]. Similarly, neutrophils, bone marrow-derived myeloid cells (BMDCs) and myeloid suppressor cells (MDSC) express CXCR2 and aid in tumor growth [17–19]. Neutrophils once recruited to the tumor site help establish a niche for inflammatory cells via production of cytokines [15, 20]. BMDCs on the other hand mature to M2 type macrophages and instead of eradicating cancer cells provide growth benefits to cancer cells [9, 21]. Our lab has shown that inhibiting CXCR2 expression in tumor cells decreases metastasis, angiogenesis, proliferation and increases apoptosis of mammary tumor cells. Moreover, the functional role of tumor CXCR2 and its ligands in the regulation of the malignant phenotype is well established [13, 22], however, the role of host CXCR2 dependent signaling in breast cancer remains unclear. In this part of the project, we demonstrate that host *Cxcr2* dependent signaling plays an important role in mammary tumor growth, angiogenesis and metastasis.

Materials and methods

Animals

BALB/c mice heterozygous for *Cxcr2* (*Cxcr2*^{+/-}) were obtained from Jackson Laboratory (Bar Harbor, ME). Mice that lack an intact *mIL-8Rh* (mouse homologue of human IL-8 receptor/*Cxcr2*) gene, were originally developed by gene targeting with a vector constructed by deleting the single exon containing the 350-amino acid open reading frame of the murine

IL-8 receptor [which has 68 and 71 % amino acid identity with human IL-8 receptors A (CXCR1) and B (CXCR2)] [23]. We generated *Cxcr2*^{-/-} mice following crosses between BALB/c mice *Cxcr2* heterozygous female and *Cxcr2* homozygous male. Mice were housed and handled according to protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Mice were genotyped using DNA from their tail and amplifying it for *Cxcr2*^{tm1Mwm} using the primers 5'-GGT CGT ACT GCG TAT CCT GCC TCA G-3' and 5'-TAG CCA TGA TCT TGA GAAGTC CAT G-3' which amplified a 360 bp fragment of the wild-type allele and the primers 5'-CTT GGG TGG AGA GGC TAT TC-3' and 5'-AGG TGA GAT GAC AGG AGA TC-3' which amplified a 280 bp fragments of the inserted neomycin gene (Fig. 1a, b).

Cell lines

Two murine mammary adenocarcinoma cell lines differing in their metastatic potential, 4T1 (highly metastatic) and Cl66 (moderately metastatic) [24, 25] were used. Cell lines were maintained in Dulbecco's Modified Eagle Media (DMEM) (Mediatech, Hendon, VA) with 5 % serum supreme (Biowhitaker, Walkersville, MD), 1 % vitamins, 1 % L-glutamine (Mediatech Inc. Manassas, VA) and 0.08 % gentamycin (Invitrogen, Carlsbad, CA). All cultures were free of mycoplasma and pathogenic murine viruses. Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

Tumor growth and metastasis

Cl66 and 4T1 cells (50,000 in 50 μ l of Hank's Balanced Salt Solution) were orthotopically implanted in mammary fat pad (MFP) to study tumor growth and spontaneous metastasis in wild-type or *Cxcr2*^{-/-} female mice. Tumor growth was measured twice a week. Tumor volume was calculated using the formula $\pi/6 \times (\text{smaller diameter})^2 \times (\text{larger diameter})$. Tumors resected from mice were fixed in formalin, embedded in paraffin and processed for histopathological evaluation and immunohistochemistry.

For spontaneous metastasis, primary tumors were surgically removed when the tumors reached 0.5 cm³ in size and mice were monitored for metastases. Mice were sacrificed at 35 days after implantation. Lungs were collected and fixed in Bouin's fixative. Metastatic lung nodules were then manually counted using a dissecting microscope.

Immunohistochemistry and immunofluorescence staining

Immunohistochemical analysis was performed to determine proliferation, micro-vessel density, apoptosis and immune infiltration as previously described [26]. In brief, 6- μ m thick tumor sections were de-paraffinized by xylenes and ethanol and blocked for 30 min. Tumor sections were incubated overnight in a humid chamber with the following primary antibodies: monoclonal mouse anti-PCNA (1:40; Santa Cruz Biotechnology) or monoclonal rabbit antibody against cleaved caspase-3 (Cell Signaling, 1:200) or monoclonal biotin-conjugated rat anti-mouse Ly-6G and Ly-6C (Gr-1) (BD Biosciences, 1:50) or rabbit anti-CD31 (Abcam, 1:50) or monoclonal rat anti-F4/80 (Abcam, 1:100). Corresponding biotinylated secondary antibody was used (except for Gr-1) at room temperature.

Immunoreactivity was detected using the ABC Elite kit and DAB substrate (Vector Laboratories) as per the manufacturer's instructions. A reddish brown precipitate indicated a positive reaction. Negative controls had all reagents except antibody. The number of positive cells was quantitated microscopically with a 5×5 reticle grid (Klarmann Rulings, Litchfield, NH) using 400 \times objective (250 μm total area).

For flow cytometric analysis to determine MDSC recruitment, single cell suspension of tumor associated leukocytes from Cl66 tumors derived from $Cxcr2^{-/-}$ mice and wild type mice were prepared as described earlier [27] and stained for CD11b and Gr1 antibodies (BD Bioscience). Cells were analyzed using FACScan Plus (BD Biosciences).

Enzyme linked immunosorbant assay

Serum from $Cxcr2^{-/-}$ and wild type tumor bearing mice were collected and analyzed for CXCL-1, -2, -5, and -7 using methods described earlier [8]. Standards (recombinant proteins) and samples were added 100 μl /well in duplicate. After incubation, plates were washed and then incubated with biotinylated secondary antibody 100 μl /well (at concentrations recommended by manufacturers; R&D Systems Inc). After washing streptavidin-horseradish peroxidase (1:20000) was added and 3,3',5,5'-tetramethyl-benzidine substrate (100 μl /well) was used. Reactions were stopped and plates were read at 450 nm using an ELx800 (Bio-Teck) plate reader. Concentrations were normalized to total protein concentrations.

Statistical analysis

In vivo analysis was performed using the Mann-Whitney U-test and paired t test using Sigma Plot 11. All the values are expressed, as mean \pm SEM. $p < 0.05$ was considered statistically significant.

Results

Host $Cxcr2$ knockout inhibits tumor growth and metastasis

To assess the effect of host $Cxcr2$, we used an orthotopic syngenic model for breast cancer. We injected Cl66 and 4T1 murine mammary tumor cells in the MFP of wild type and $Cxcr2^{-/-}$ female mice (Fig. 1). Tumor growth was examined by measuring the tumor size twice a week for 3 weeks (Fig. 1). Tumor incidence was lower in $Cxcr2^{-/-}$ mice when compared to wild type mice implanted with Cl66 tumors. Furthermore, we observed a decrease in tumor growth in $Cxcr2^{-/-}$ mice when compared to wild type mice suggesting that host $Cxcr2$ has a crucial role in suppressing the tumor growth of Cl66 tumors. However, in mice with 4T1 tumor cells, we did not observe any change in the in vivo tumor growth kinetics in $Cxcr2$ knockout ($Cxcr2^{-/-}$) mice as compared to wild-type mice (Figs. 2a and 3a).

We next investigated the role of host $Cxcr2$ on spontaneous lung metastasis of murine mammary tumor. Primary tumors were surgically removed when reached to 0.5 cm^3 in size and animals were monitored for spontaneous metastasis (Fig. 1c). Mice were sacrificed and metastatic lung nodules were counted in all groups. We found a significant reduction in

metastatic lung nodules in $Cxcr2^{-/-}$ tumor-bearing mice as compared to wild-type tumor-bearing mice (Figs. 2b and 3b). Although, 4T1 tumor bearing mice had no significant difference in tumor growth, there was a significant difference in metastatic lung nodules with wild-type mice having more nodules than $Cxcr2^{-/-}$ mice (Fig. 3b).

Host Cxcr2 knockdown decreased in situ cell proliferation, angiogenesis and increased apoptosis

To evaluate the mechanism of tumor regression in $Cxcr2^{-/-}$ mice, we immunostained tumor tissues using PCNA, cleaved caspase-3 and CD31 antibodies. We observed decreased in situ tumor cell proliferation in $Cxcr2^{-/-}$ compared to wild-type mice ($p = 0.01$) (Fig. 4a, c). To further investigate the role of host Cxcr2 in tumor progression, we analyzed microvessel density and detected a significant reduction in the number of blood vessels in tumor tissues obtained from $Cxcr2^{-/-}$ mice ($p = 0.001$) as compared to wild-type mice (Fig. 4b, d). Similarly, there was a significant increase in cleaved caspase-3 staining in tumor tissues from $Cxcr2^{-/-}$ mice ($p = 0.001$) in comparison to wild-type mice (Fig. 5a, b). Taken together, our cell proliferation and neo-vascularization data along with the apoptosis results demonstrate that host Cxcr2-dependent signaling in the tumor microenvironment is an important regulator of progression and metastasis.

Diminished pro-inflammatory response in Cxcr2 knockout mice

Immune inflammatory cells in the tumor microenvironment have been linked with tumor growth, angiogenesis and metastasis [5, 28–30]. Tumor-associated neutrophils and macrophages have also been documented to express CXCR2 [10, 15]. To analyze the inflammatory response (neutrophils) in wild-type and $Cxcr2^{-/-}$ mice, tumor tissue sections were stained using an anti-Gr-1 (Ly-6G/C) antibody. Tumors from $Cxcr2^{-/-}$ mice contained a lower frequency of Gr-1⁺ cells as compared to tumors obtained from wild type mice (Fig. 6a, b) suggesting that Cxcr2 receptor status of the host influences neutrophil infiltration in mammary tumors. In addition, we analyzed the frequency of CD11b⁺Gr1⁺ MDSCs in primary tumors. We observed a significant difference in frequency of MDSCs in tumors derived from $Cxcr2^{-/-}$ mice as compared to wild type mice (Fig. 6c).

To further evaluate whether host Cxcr2 receptor modification modulates the inflammatory macrophage response in mammary tumors, we examined tumors for F4/80⁺ cells by immunohistochemistry. Tumors from $Cxcr2^{-/-}$ mice showed a significantly ($p = 0.001$) lower frequency of F4/80⁺ cells than tumors from wild-type mice (Fig. 6b, d), indicating that knockdown of host Cxcr2 affects macrophage recruitment to tumors. These findings suggest that infiltration of pro-inflammatory immune cells play a role in mammary tumor growth.

Higher expression of Cxcr2 ligands in Cxcr2^{-/-} tumor bearing mice

We examined the levels of circulating Cxcr2 ligands in $Cxcr2^{-/-}$ and wild type tumor bearing mice. We observed undetectable levels of CXCL-1 and CXCL-3 in both wild type and $Cxcr2^{-/-}$ mice (data not shown). Interestingly, the serum levels of CXCL-2 and CXCL-5 was significantly higher in $Cxcr2^{-/-}$ tumor bearing mice as compared to wild type tumor bearing mice (Fig. 7). The level of CXCL-7 was high in wild type and $Cxcr2^{-/-}$ tumor

bearing mice. These data suggest an elevation of Cxcr2 ligands in Cxcr2^{-/-} mammary tumor bearing mice.

Discussion

We investigated the role of host Cxcr2 in mammary tumor progression and metastasis. Our data demonstrated that knockdown of host Cxcr2 reduces tumor metastasis. Host Cxcr2 influenced angiogenesis, spontaneous metastasis, proliferation, apoptosis and the inflammatory response in mammary tumor models. Our earlier reports demonstrate that CXCR2 knockdown in malignant tumor cells significantly decreases spontaneous lung metastasis both in breast cancer and melanoma tumor models [13, 31]. Similar observations were made in our earlier report demonstrating that host Cxcr2 regulates melanoma growth and experimental lung metastasis [31]. Our present data show that mammary tumors from Cxcr2^{-/-} mice were significantly smaller compared to tumors in wild type mice using Cl66 cells. Although we didn't observe any difference in tumor growth with 4T1 cells there was a significant decrease in the spontaneous lung metastasis. An earlier report has demonstrated that CXCR2 antagonist treatment reduced the number of PyVMT/TGFBR2MGKO metastases to the lung and this was coincident with the inhibition of recruitment of MDSCs to the lung [17]. However there was little effect of the CXCR2 antagonist on the growth of the very aggressive primary tumor [17]. In addition, 4T1 cells are more aggressive than Cl66 based on their 6-thioguanine resistance [32] which might explain the difference in primary tumor growth. Also, injecting less cell number for 4T1 may better evaluate the difference in tumor growth with respect to host CXCR2 expression. Furthermore, we have observed similar tumor growth kinetics and metastasis pattern with Cl66 cells selected for Doxyrubicine (Dox) resistance (personal communication). We are currently investigating the the significance of cancer stem cell like cells and/or modulation of metastatic niche in Cxcr2 deficient mice using 4T1 and Dox resistant Cl66 tumor cells.

CXC chemokines having ELR motifs, particularly CXCL1 in the mice have been documented to recruit macrophages and neutrophils during inflammation and cancer [15, 20, 33, 34]. CXCL1 binds to its receptor CXCR2, which is also present on the surface of endothelial cells and has been shown to enhance angiogenesis [33]. We observed higher serum levels of CXCL-2, -5 and -7 in Cxcr2^{-/-} mice as compared to wild type mice. This observation is similar to earlier finding that Cxcr2 acts as scavenger to its ligands and lack of Cxcr2 leads to increased ligand accumulation [35]. Despite higher levels of the ligands, we observed a significant decrease in microvessel density in tumors from Cxcr2^{-/-} mice as compared to wild type mice.

This study also evaluated the significance of host CXCR2 in modulating the host inflammatory response to mammary tumor growth. We observed that absence of Cxcr2 in mice reduced macrophage, neutrophil, and MDSC recruitment to the tumor site. These observations are in accordance with an earlier report where they observed a decrease in inflammation-driven and spontaneous tumorigenesis in both benign and malignant intestinal adenomas [36] and promote colitis-associated tumorigenesis [19].

Reduced tumor angiogenesis and in situ cell proliferation in *Cxcr2*^{-/-} mice provide a plausible explanation for the smaller tumor size observed in these mice. Moreover, increased apoptosis in tumor tissues from *Cxcr2*^{-/-} mice also favors the smaller tumor size. Data from our previous studies with CXCR2 knockdown tumor cells also demonstrated increased apoptosis, reduced angiogenesis and proliferation in tumors [13]. These studies together suggest that the absence of CXCR2 expressing host-derived cells (neutrophils, macrophages and endothelial cells) in the tumor microenvironment might prevent tumor cells from responding to the chemokine storm, preventing their migration to various organs resulting in decreased metastasis.

Together our data suggest that host *Cxcr2* expression plays a critical role in mammary tumor growth, proliferation and angiogenesis. Although we observed that *Cxcr2* signaling has a significant role in mammary tumors, *Cxcr1* another CXC receptor, which binds some of the same *Cxcr2* ligands, may also affect tumor growth or metastasis. Our present study does not evaluate the status of *Cxcr1*. However, previous studies from our lab have shown that in melanoma both CXCR1/2 are expressed by tumor cells but expression of CXCR2 increases with aggressiveness of tumor cells and is involved during metastasis [26]. Moreover, data from other laboratories has shown that the CXCR1/CXCL8 axis plays an important role in selecting cancer stem cells in breast tumors [37] and that CXCR1/2 mediated signaling can be targeted to significantly reduce breast cancer stem cell activity [38]. Furthermore, our lab has reported previously that both tumor and host expression of CXCR2 modulate melanoma growth, angiogenesis, proliferation, apoptosis and metastasis [31, 39, 40]. Also, CXCR2 knockdown in tumor cells alone or in combination with chemotherapeutic drugs reduces angiogenesis and enhances apoptosis in mammary tumors [8, 13]. These studies along with our current data substantiate the role of CXCR2 in mammary tumor growth and metastasis.

In summary, our data suggest that targeting CXCR2 in breast cancer may provide a novel therapeutic alternative for treating breast cancer. Preclinical data in prostate cancer showed that blocking this signaling using CXCR1/2 antagonist inhibits prostate cancer indicating the possibility of blocking this signaling in various cancers [41]. Our lab has also shown in melanoma that when given orally CXCR1/2 antagonists inhibit tumor growth and metastasis in preclinical settings [40]. The CXCR2 antagonists are in phase II clinical trials for chronic obstructive pulmonary disease (COPD) [42] and there are no side effects of CXCR2 antagonists in patients with severe asthma [43] suggesting its safe usage. Ongoing developments of these agents for other diseases provide essential data regarding the efficacy and safety profile, which provide an important basis for accelerated development as a novel therapeutic modality for breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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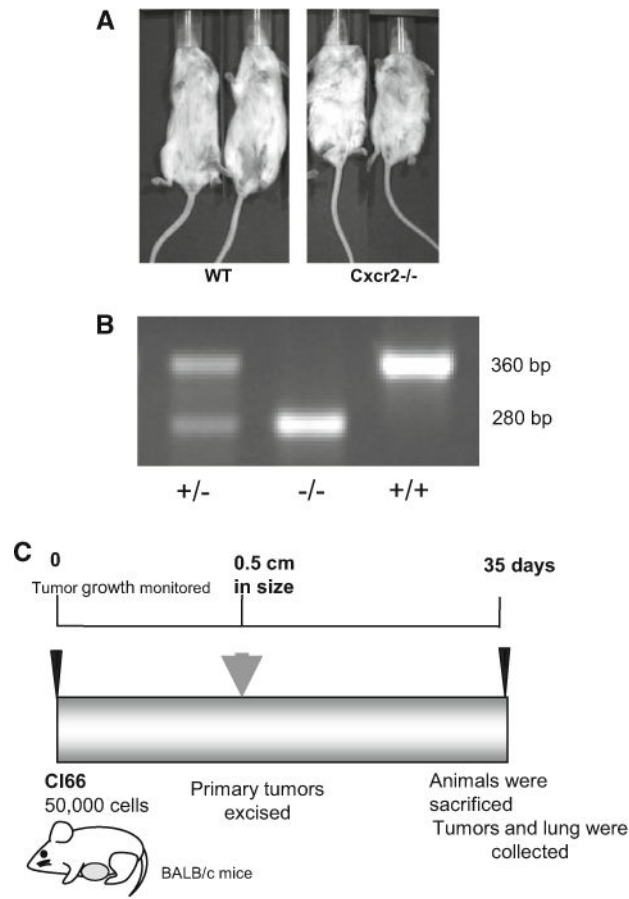


Fig. 1. Genotyping of mice: **a** Representative images of wild type and Cxcr2^{-/-} mice depicting the phenotype differences. **b** Genotyping of genomic DNA by PCR to determine Cxcr2 status of the mice. **c** Schematic diagram representing experimental strategy for tumor implantation and end point analysis for metastasis

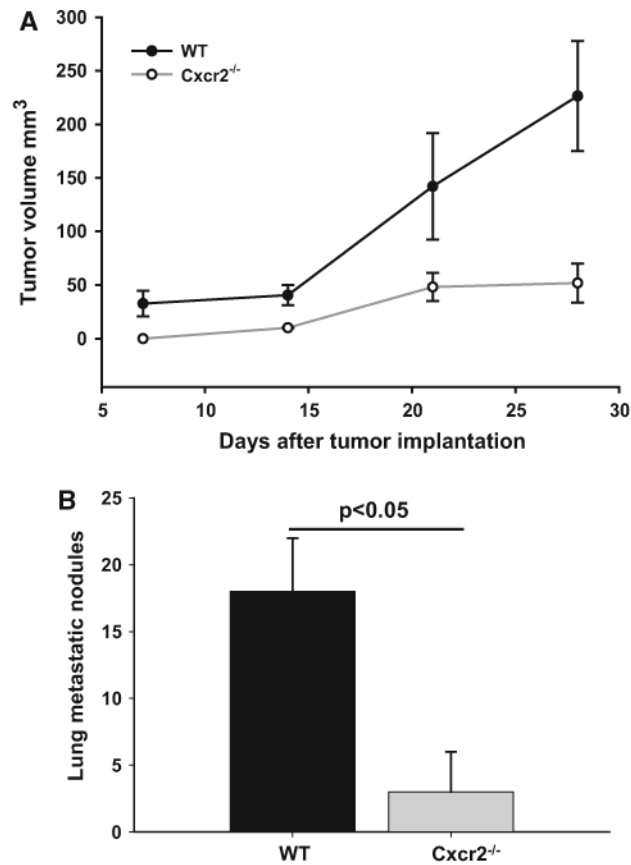


Fig. 2. Tumor growth in wild type and Cxcr2 null mice: **a** Growth kinetics of tumors formed by Cl66 cells in wild type and Cxcr2^{-/-} mice (n = 5, p = 0.036). **b** Spontaneous lung metastasis in wild type and Cxcr2^{-/-} mice. Lung metastatic nodules were counted manually for both groups (n = 5) and the mean was calculated. Mean ± SEM was graphed for each group and p < 0.05 was considered significant

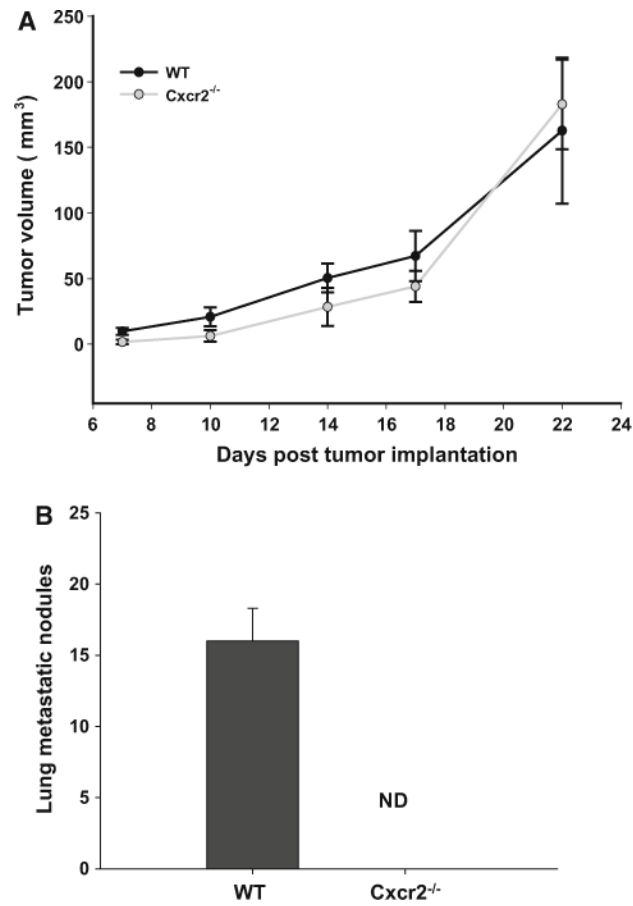


Fig. 3. Tumor growth by 4T1 cells in mice with different Cxcr2 status: **a** Growth kinetics of tumors formed by 4T1 cells in wild type and Cxcr2^{-/-} mice (n = 5). **b** Spontaneous lung metastasis in wild type and Cxcr2^{-/-} mice. Lung nodules were counted for both groups (n = 5) and mean \pm SEM was plotted p < 0.05

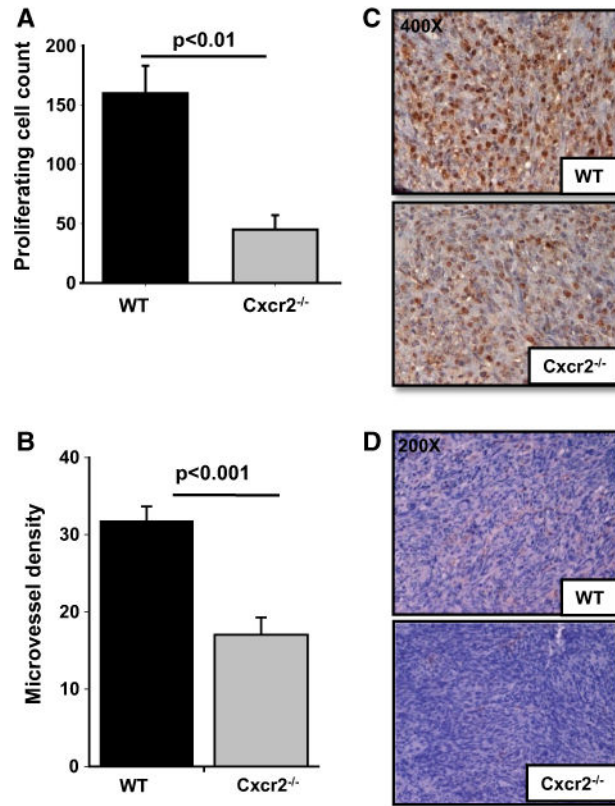


Fig. 4. Immunohistochemical analysis of tumors formed by Cl66 cells in wild type and Cxcr2^{-/-} mice for proliferation and angiogenesis: **a** and **b** Quantification of PCNA (**a**) and CD31 (**b**) staining. Five fields were counted from each tumor for PCNA and CD31 positive cells (n = 5). The mean was calculated and mean \pm SEM was graphed for each group. Significant differences are represented with p values. **c** and **d** Microscopic images of representative tumor sections from wild type or Cxcr2^{-/-} mice showing PCNA and CD31 staining

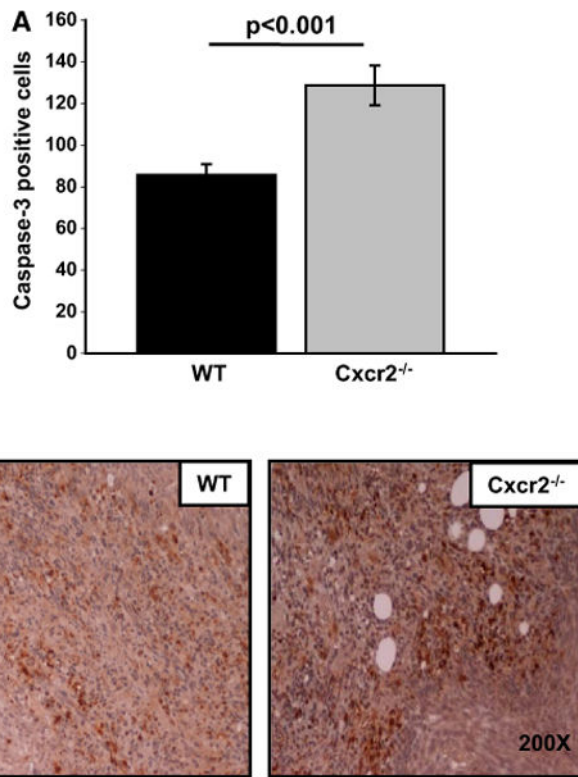


Fig. 5. Apoptosis in C166 tumors formed in wild type and Cxcr2 null mice: **a** Quantification of cleaved caspase-3 staining. Five fields were counted from each tumor for caspase positive cells (n = 5). The mean was calculated and mean ± SEM was graphed for each group. **b** Microscopic image of a representative section of tumor showing caspase-3 staining in tumors from wild type or Cxcr2^{-/-} mice

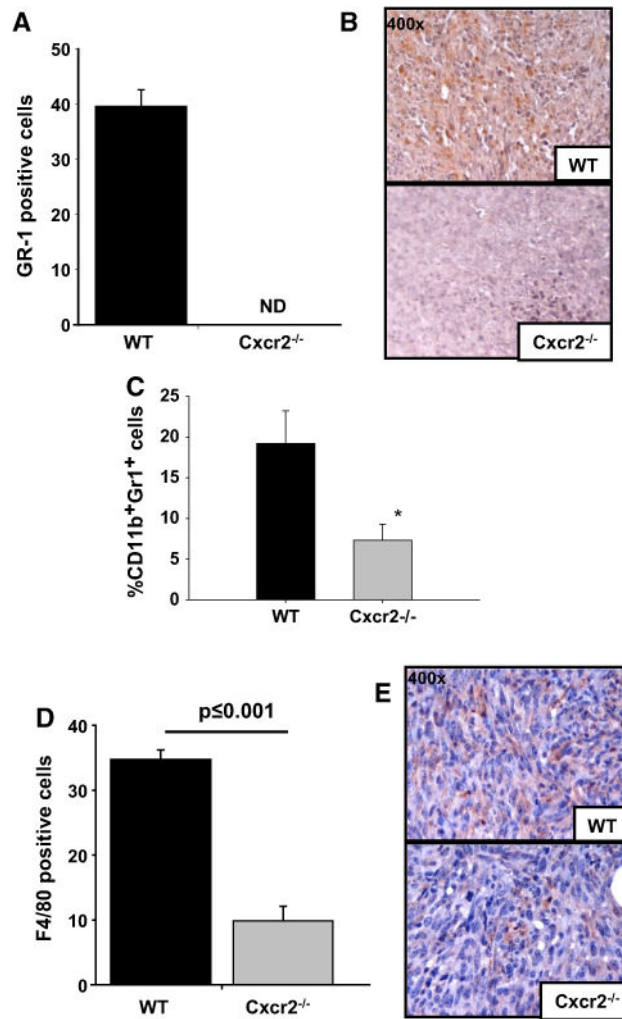


Fig. 6.

Tumor infiltration of inflammatory cells: **a & b** Quantification of Gr-1 (**a**), CD11b⁺Gr1⁺ MDSc (**c**) and F4/80 (**d**) staining to evaluate neutrophil and macrophage infiltration in tumors. Five fields were counted from each tumor for Gr-1 or F/480 positive cells (n = 5). Flow cytometric analysis was performed using CD11b and Gr1 antibodies (n = 5). The mean was calculated and mean ± SEM was graphed for each group. **b** and **e** Microscopic image of a representative section of tumor showing Gr-1 (**b**) or F4/80 (**e**) staining in tumors formed in wild-type or Cxcr2^{-/-} mice

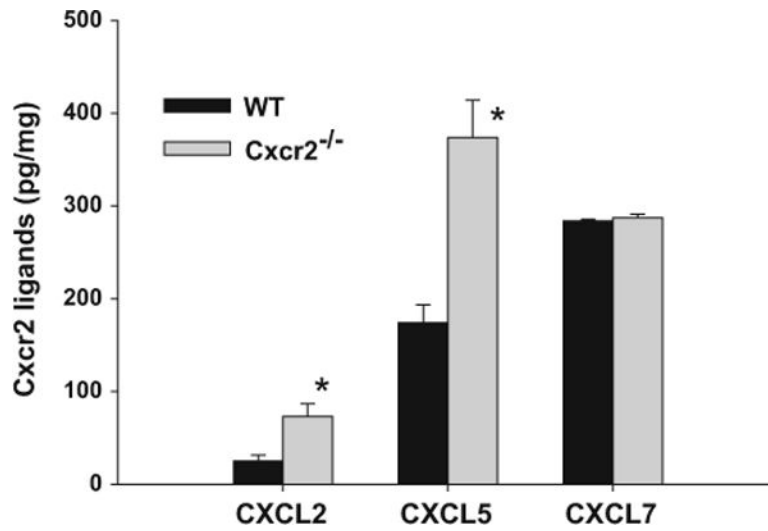


Fig. 7. Cxcr2 ligands expression in wild type and Cxcr2^{-/-} tumor bearing mice. The serum levels of CXCL-2, -5 and -7 was analyzed using ELISA. The levels of ligands were normalized to total serum protein levels. The values are mean \pm SEM of triplicate samples (n = 4)