Figures and figure supplements

CCL5 promotes breast cancer recurrence through macrophage recruitment in residual tumors

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Figure 1. Her2 downregulation induces an inflammatory gene expression program driven by the TNFα/IKK pathway. (a) RNA-seq analysis of two independent primary Her2-driven tumor cell lines in the presence of Her2 expression (+dox) or 2 days following Her2 downregulation (-dox). The Figure 1 continued on next page.
heatmap shows the top 100 differentially expressed genes between +dox and -dox conditions. R1 and R2 are biological replicates. (b) Gene set enrichment analysis (GSEA) of RNA-seq data showing enrichment of an inflammatory response signature and a TNFα/NF-κB signature in cells following Her2 downregulation. p-Values and normalized enrichment scores (NES) are shown. (c) Heatmap showing expression of select genes from the TNFα/ NF-κB signature in the presence of Her2 expression (+dox) or following Her2 deinduction (-dox). (d) qRT-PCR analysis of CCL5 expression following 1- or 2-day treatment with conditioned media harvested from primary cells following Her2 downregulation. Dox was added to conditioned media prior to treatment to maintain Her2 expression in target cells. Results shown are representative of two independent experiments. (e) qRT-PCR of TNFα expression in primary cells in the presence of Her2 expression (+dox) or 2 and 4 days following Her2 downregulation. Results shown are representative of two independent experiments. (f) Primary tumor cells were treated with conditioned media as described in (d), and activation of the NF-κB pathway was assessed by Western blot analysis of total and phospho-p65. Results show three biological replicates per time point. (g) qRT-PCR analysis of the indicated genes in primary tumor cells in the presence of Her2 expression (+dox) or 1 and 2 days following Her2 downregulation (-dox). At the time of Her2 downregulation, cells were treated with the pan-IKK inhibitor IKK16 (100 nM) or vehicle control. Results show the average of 3 biological replicates per condition. Error bars denote mean ± SEM. Significance was determined using a two-tailed Student’s t-test. DOI: https://doi.org/10.7554/eLife.43653.003
Figure 1—figure supplement 1. Gene expression changes following Her2 inhibition. (a) qRT-PCR analysis of Erbb2 expression in primary cells with Her2 on (+dox) or Her2 off (-dox). (b) Gene set enrichment analysis (GSEA) of RNA-seq data showing an E2F gene signature is enriched in cells with Figure 1—figure supplement 1 continued on next page.
Figure 1—figure supplement 1 continued

Her2 signaling on. p-Values and normalized enrichment scores (NES) are shown. (c) Western blot showing p65 phosphorylation in primary tumor cells treated with the indicated concentration of Neratinib for 24 hr, or 24 hr following dox withdrawal. (d–f) qRT-PCR analysis of TNFα, CCL5, and CXCL5 expression 24 hr after treatment with 0.1 μM Neratinib. (g) qRT-PCR analysis of CCL2, CCL5, and CXCL5 expression in NIH-3T3 treated with 2 μg/mL dox, 10 ng/mL TNFα, or both for 24 hr. (h) qRT-PCR analysis of Erbb2 expression of cells treated with -dox conditioned media with dox supplementation. (i) Primary tumor cells were treated with +dox conditioned media and activation of the NF-κB pathway was assessed by Western blot analysis of total and phospho-p65. Results show two biological replicates per time point.

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Figure 2. Immune cell infiltration during tumor regression and residual disease. (a) H and E-stained section of a representative residual tumor from a previously tumor-bearing MTB/TAN mouse. Insets show higher magnification view of residual tumor cells (left) and staining for CK8 (right). (b–d) Figure 2 continued on next page.
Figure 2 continued

Representative images of a primary tumor (b), regressing tumor (5 days -dox) (c), and residual tumor (d), stained with H and E, Masson’s Trichome (MT), CD45, or F4/80. Primary tumors show little collagen deposition and only modest leukocyte infiltration. Her2 downregulation leads to infiltration of CD45+ cells, predominantly F4/80+ macrophages. Residual tumors have abundant collagen deposition and leukocyte infiltration.

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Figure 2—figure supplement 1. Immune cell infiltration in autochthonous and orthotopic tumors following Her2 downregulation. (a) CD3 staining of representative MTB;TAN primary, 5 days -dox, and residual tumors. (b) Bright-field and fluorescent images of a representative GFP-labeled orthotopic tumor.

Figure 2—figure supplement 1 continued on next page
residual tumor in the context of a non-fluorescent mammary gland. (c) Quantification of IHC and MT staining of primary, regressing, and residual tumors from the MTB;TAN model. (d–f) F4/80 staining of representative orthotopic primary, 5 days -dox, and residual tumors showing macrophage infiltration.

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Figure 3. Differential cytokine expression in residual tumors. (a) Volcano plot showing differential cytokine expression between primary and residual tumors. Antibody-based cytokine arrays were used to measure cytokine expression in orthotopic primary tumors or microdissected residual tumors. Cytokines that are upregulated (fold change >2, p-value < 0.1) in dormant tumors are in red, and downregulated cytokines (fold change < -2, p-value < 0.1) are in blue. Significance was determined using a two-tailed Student’s t-test. (b) Quantification of CCL5, IL-13, IGFBP6, VCAM-1, OPG, HGF, Resistin, and P-Selectin expression in primary tumors and residual tumors. Values were derived from the cytokine arrays shown in (a). Significance was determined using a two-tailed Student’s t-test. (c) CCL5 expression in 18 matched pre- and post-treatment samples from GSE10281. Red lines show tumors in which CCL5 expression increased following treatment (>1.5 fold change), and blue lines show tumors with decreased CCL5 expression (<1.5 fold change). (d) Average CCL5 expression in pre- and post-treatment samples from (e). Significance was determined using a two-tailed paired Student’s t-test. Error bars denote mean ± SEM.

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Figure 3—figure supplement 1. Cytokine gene expression in human breast cancers following neoadjuvant therapy. (a) Heatmap showing expression of selected cytokine and chemokine genes from 18 matched human breast tumors prior to treatment, or in residual tumors following neoadjuvant therapy.
Letrozole treatment (GSE10281). Gene expression values were log2 transformed and median centered. (b–m) Average expression of CCL2, CXCL1, CXCL2, CXCL5, SELE, HGF, IGFBP6, IL-13, TNFRSF11B, SELP, RETN, and VCAM-1 in 18 matched pre- and post-treatment samples following neoadjuvant Letrozole treatment (GSE10281). Two-tailed paired t-test was performed between pre- and post-treatment samples. (n) Average CCL5 expression in 25 matched pre- and post-treatment samples from human breast tumors treated with neoadjuvant chemotherapy (GSE21974). Two-tailed paired t-test was performed between pre- and post-treatment samples.

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**Figure 4.** CCL5 expression promotes tumor recurrence following Her2 downregulation. (a) CCL5 protein levels in orthotopic primary (n = 4), residual (n = 3), and recurrent (n = 2) tumors as determined by ELISA. (b) CCL5 protein levels in primary tumor cells engineered to express CCL5. Results show the mean ± SEM for two independent experiments. Significance was determined using a two-tailed Student’s t-test. (c) Recurrence-free survival for mice with control tumors or tumors expressing CCL5. CCL5 expression significantly accelerated recurrence (Hazards Ratio (HR) = 2.1, p=0.02). Results are from a single experiment with 20 control tumors and 21 CCL5 tumors. p-Values and hazards ratios are indicated. Statistical significance was determined by Mantel-Cox log rank test. (d) CCL5 expression as determined by ELISA in primary tumor cells expressing a control sgRNA or a sgRNA targeting CCL5. Results show the mean ± SEM for a single representative experiment. (e) Recurrence-free survival of mice with control tumors or CCL5 knockout tumors. CCL5 knockout in tumor cells did not significantly delay tumor recurrence (HR = 0.76, p = 0.46). Results are from a single experiment with 26 control tumors (sgControl) and 24 sgCCL5 tumors. Statistical significance was determined by Mantel-Cox log rank test. Error bars denote mean ± SEM.

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Figure 5. CCL5 promotes macrophage infiltration in residual tumors. (a–d) Flow cytometry of immune cells in primary (n = 6), regressing (5 days -dox; n = 3), residual (n = 3), and recurrent (n = 3) tumors from autochthonous MTB;TAN mice. Immune cell populations analyzed include CD11b+/F4/80+ macrophages (a), CD4+ T cells (b), CD8+ T cells (c), PDGFRα fibroblasts (d), and tumor cells (e). Each immune cell population was divided into CCR5- or CCR5+ cells, and the median fluorescence intensity (MFI) of the CCR5+ population was calculated. (f) Flow cytometry of CD45-/PDGFRα+ fibroblasts in control residual tumors (n = 4) or residual tumors expressing CCL5 (n = 4). (g) Flow cytometry of CD11b+/F4/80+ macrophages in control residual tumors (n = 4) or residual tumors expressing CCL5 (n = 4). Error bars denote mean ± SEM. Significance was determined using a two-tailed Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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Figure S—figure supplement 1. CCL5 recruits CCR5+ macrophages to residual tumors. (a) qRT-PCR analysis of Erbb2 in primary, 5 days – dox, residual, and recurrent tumors from the MTB;TAN model cohort used for flow cytometry analysis of CCR5 expression. (b) qRT-PCR analysis of CCR5 on sorted tumor cells and macrophages from primary, 5 days -dox, residual, and recurrent tumors from the MTB;TAN model. (c) Flow plots of CD45+/PDGFRα+ fibroblasts in control (n = 4) and CCL5-expressing (n = 4) residual tumors (d) Flow plots of CD11b+/F4/80+ macrophages in control (n = 4) and CCL5-expressing (n = 4) residual tumors.

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Figure 5—figure supplement 2. CCR5 staining on immune cell populations in primary, regressing, residual, and recurrent tumors. Histograms showing CCR5 staining in macrophages, PDGFRα fibroblasts, CD4+ T cells, CD8+ T cells, and tumor cells from primary tumors (n = 6), regressing tumors (5 days -dox; n = 3), residual tumors (n = 3), and recurrent tumors (n = 3).

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Figure 6. Macrophages express collagen and collagen deposition factors. (a) RNA-seq analysis of tumor-associated macrophages from primary (n = 3), regressing (5 days -dox; n = 3), and recurrent (n = 3) tumors. The heatmap shows differentially expressed genes (p<0.01, Student’s t-test) between primary and recurrent TAMs. (b) Heatmap showing expression of specific collagen genes from RNA-seq analysis in (a). (c) qRT-PCR analysis of COL5A1, ASPN, COL24A1, and PCOLCE expression in the cohort in (a) along with sorted macrophages from residual tumors. ND = not detected (d) qRT-PCR analysis of COL5A1, ASPN, COL24A1, and PCOLCE expression in unsorted MTB;TAN primary (n = 5) and recurrent (n = 5) tumors. (e) Masson’s trichrome staining showing collagen deposition in primary (n = 3), residual (n = 3), and recurrent (n = 3) tumors from the MTB;TAN model. Collagen is stained in blue, and higher collagen staining is present in residual and recurrent tumors. (f) Masson’s trichrome staining in a subset of control and CCL5-expressing orthotopic recurrent tumors. The entire cohort of tumors is shown in Figure 6—figure supplement 1. Error bars denote mean ± SEM. Significance was determined using a two-tailed Student’s t-test. *p < 0.05, ***p < 0.001.

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Figure 6—figure supplement 1. Collagen gene expression and deposition in residual and recurrent tumors. (a) Average expression of ASPN, COL5A1, COL24A1, and PCOLCE in 18 matched pre- and post-treatment samples from human breast tumors treated with neoadjuvant Letrozole.

Figure 6—figure supplement 1 continued on next page.
(GSE10281). Two-tailed paired t-test was performed between pre- and post-treatment samples. (b) Masson’s trichrome staining showing collagen deposition in control (n = 4) and CCL5-expressing (n = 4) recurrent tumors. (c) Quantification of (b).

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