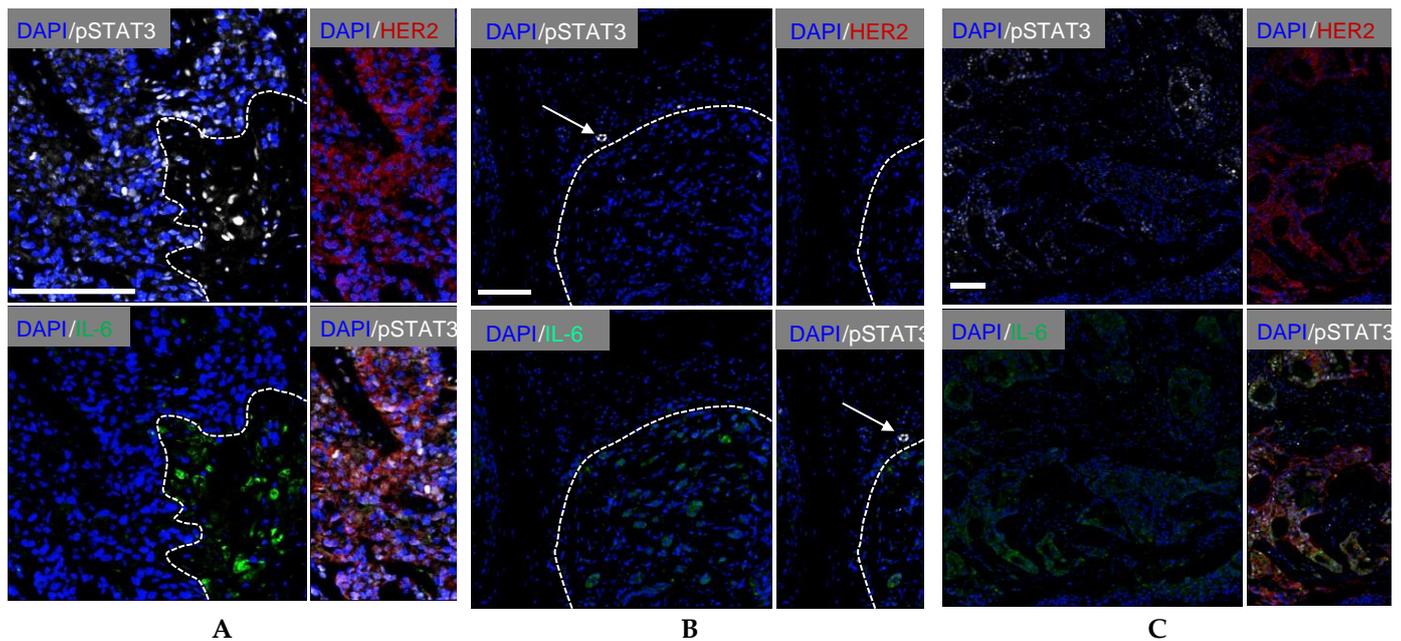
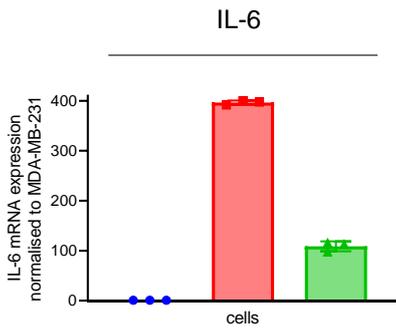


## Supplementary Materials: Paracrine IL-6 Signaling Confers Proliferation between Heterogeneous Inflammatory Breast Cancer Sub-Clones

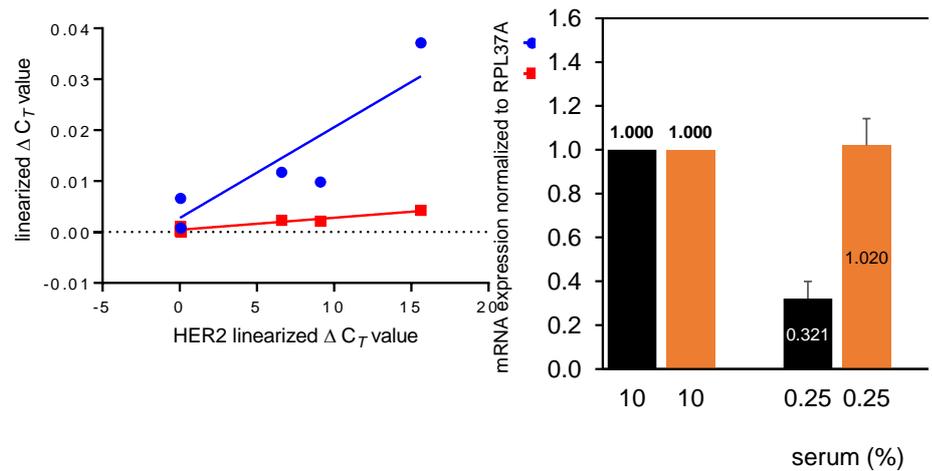
Riley J. Morrow, Amr H. Allam, Belinda Yeo, Siddhartha Deb, Carmel Murone, Elgene Lim, Cameron N. Johnstone and Matthias Ernst



**Figure S1.** **A.** Localization of HER2, pSTAT3 and IL-6 in human inflammatory breast cancers. FFPE sections from human inflammatory breast cancer pre-treatment core biopsies were co-stained with pSTAT3 (white), HER2 (red), and IL-6 (green) antibodies using multiplexed multispectral imaging and counterstained with DAPI (blue) to visualize the nuclei (see Materials and Methods). The specimen shown is a luminal (ER<sup>pos</sup>, PgR<sup>pos</sup>, HER2<sup>neg</sup>) tumor (INFLAM #4, **Table S1**), which displays co-localization of cell surface HER2 expression and nuclear pSTAT3 in a region of tumor cells and diffuse IL-6 staining in an adjacent region of stroma (**Pattern 2**). Scale bar represents 100  $\mu$ m. **B.** Localization of HER2, pSTAT3 and IL-6 in human inflammatory breast cancers. FFPE sections from human inflammatory breast cancer pre-treatment core biopsies were co-stained with pSTAT3 (white), HER2 (red), and IL-6 (green) antibodies using multiplexed multispectral imaging and counterstained with DAPI (blue) to visualize the nuclei (see Materials and Methods). The specimen shown is a luminal (ER<sup>pos</sup>, PgR<sup>pos</sup>, HER2<sup>neg</sup>) tumor (INFLAM #8, **Table S1**), which displays nuclear pSTAT3 positivity in a small cluster of tumor cells (white arrow) adjacent to an IL-6<sup>pos</sup>/pSTAT3<sup>neg</sup> tumor region (**Pattern 3**). Scale bar represents 100  $\mu$ m. **C.** Localization of HER2, pSTAT3 and IL-6 in human inflammatory breast cancers. FFPE sections from human inflammatory breast cancer pre-treatment core biopsies were co-stained with pSTAT3 (white), HER2 (red), and IL-6 (green) antibodies using multiplexed multispectral imaging and counterstained with DAPI (blue) to visualize the nuclei (see Materials and Methods). The specimen shown is a HER2 amplified/enriched (ER<sup>neg</sup>, PgR<sup>neg</sup>, HER2<sup>pos</sup>) tumor (INFLAM #8, **Table S1**), which displays co-localization of each of cell surface HER2 expression, nuclear pSTAT3, and IL-6 in tumor cells (**Pattern 4**). Scale bar represents 100  $\mu$ m.

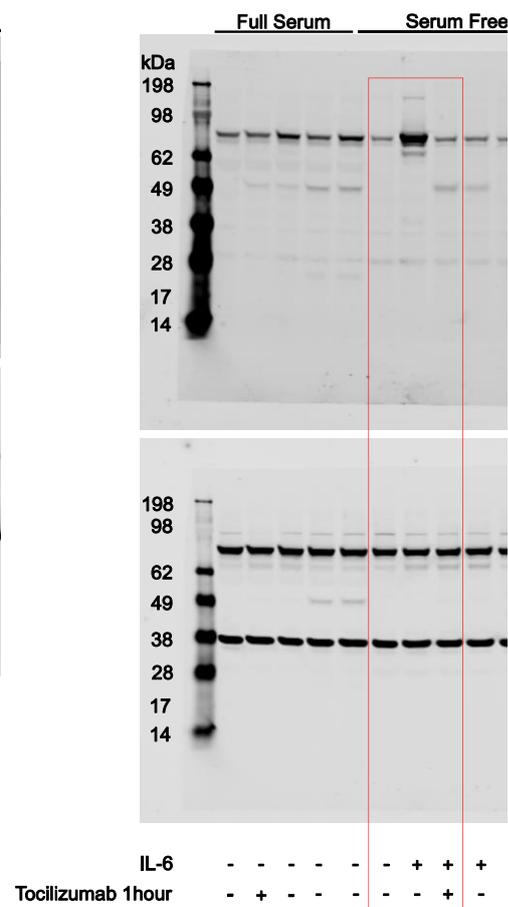
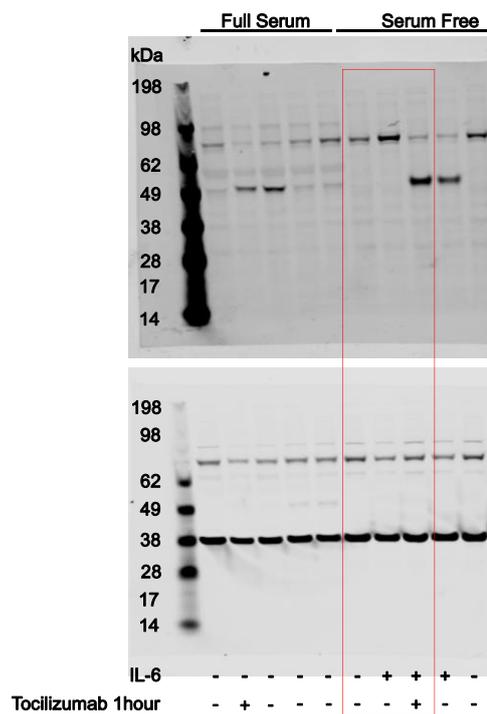
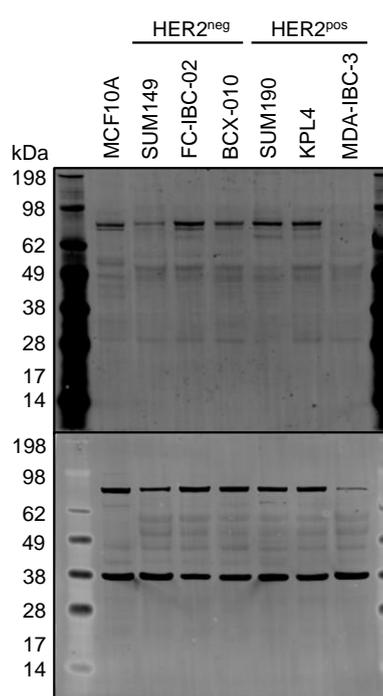


**Figure S2.** RNA-Seq analysis of IL-6 mRNA expression in cultured TNBC cell lines. RNA-Seq data (Gene Expression Omnibus (GEO) Accession # GSE180788) was interrogated for IL-6 mRNA levels. Expression in MDA-MB-231 non-IBC TNBC cells was compared to A3250 and SUM149 IBC TNBC cells cultured *in vitro* [11]. Expression in MDA-MB-231 was set to 1. Mean  $\pm$  SD ( $n = 3$ ). IL-6 mRNA levels in A3250 are approximately four fold higher than in SUM149. Data taken from Rogic *et al.*, 2021 [11].



**Figure S3.** Correlation between IL-6R and HER2 mRNA expression in IBC cell lines. Linearized  $\Delta C_T$  values from TaqMan qRT-PCR analyses of IL-6R or sIL-6R (y axis) and HER2 (x axis) mRNA expression were taken from Figure 2 and plotted. Simple linear regression analysis carried out using Prism v8 (GraphPad Software, San Diego, CA, USA) showed significant correlation of IL-6R and HER2 mRNA expression ( $r^2 = 0.80$ ; \*,  $p < 0.05$ ), and of sIL-6R and HER2 mRNA expression ( $r^2 = 0.93$ ; \*\*,  $p < 0.01$ ).

**Figure S4.** TaqMan qRT-PCR analysis of IL-6 (black) and IL-6R (orange) mRNA expression in SUM190 cells in full serum (10%) and low serum conditions. SUM190 cells were cultured in 10, 0.25, or 0% serum for 48h prior to RNA isolation. Expression was set to 1 in 10% serum condition. Mean  $\pm$  SD ( $n = 3$ ). IL-6,  $p = ***$ . IL-6R,  $p = **$ , by one-way ANOVA.



**Figure S5A.** Original Western blot corresponding to Figure 3A.

**Figure S5B.** Original Western blot corresponding to Figure 3C (SUM149).

**Figure S5C.** Original Western blot corresponding to Figure 3C (FC-IBC-02).

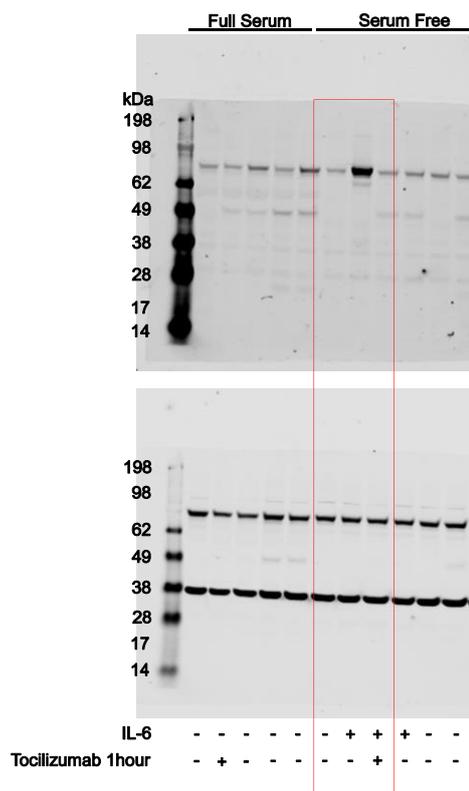


Figure S5D. Original Western blot corresponding to Figure 3C (BCX-010).

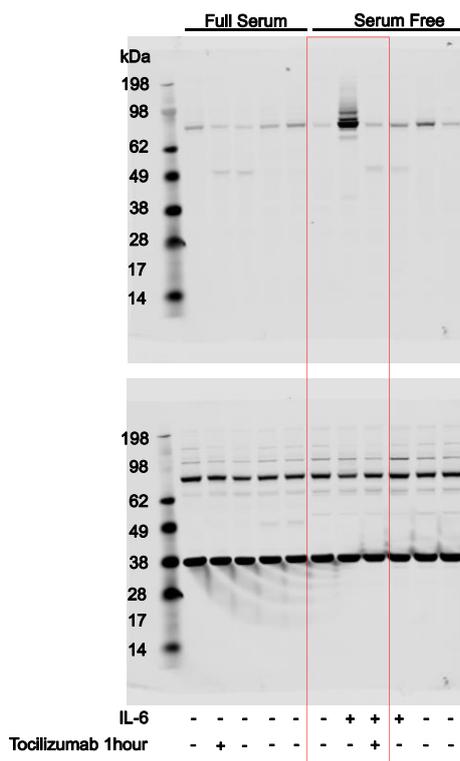


Figure S5E. Original Western blot corresponding to Figure 3C (SUM190).

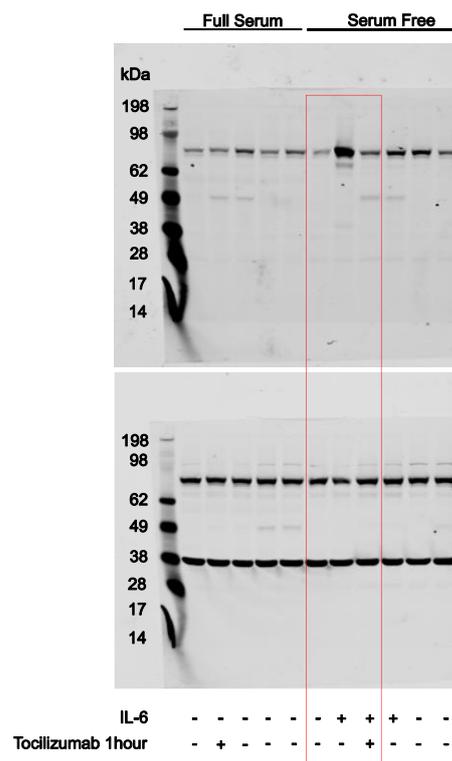
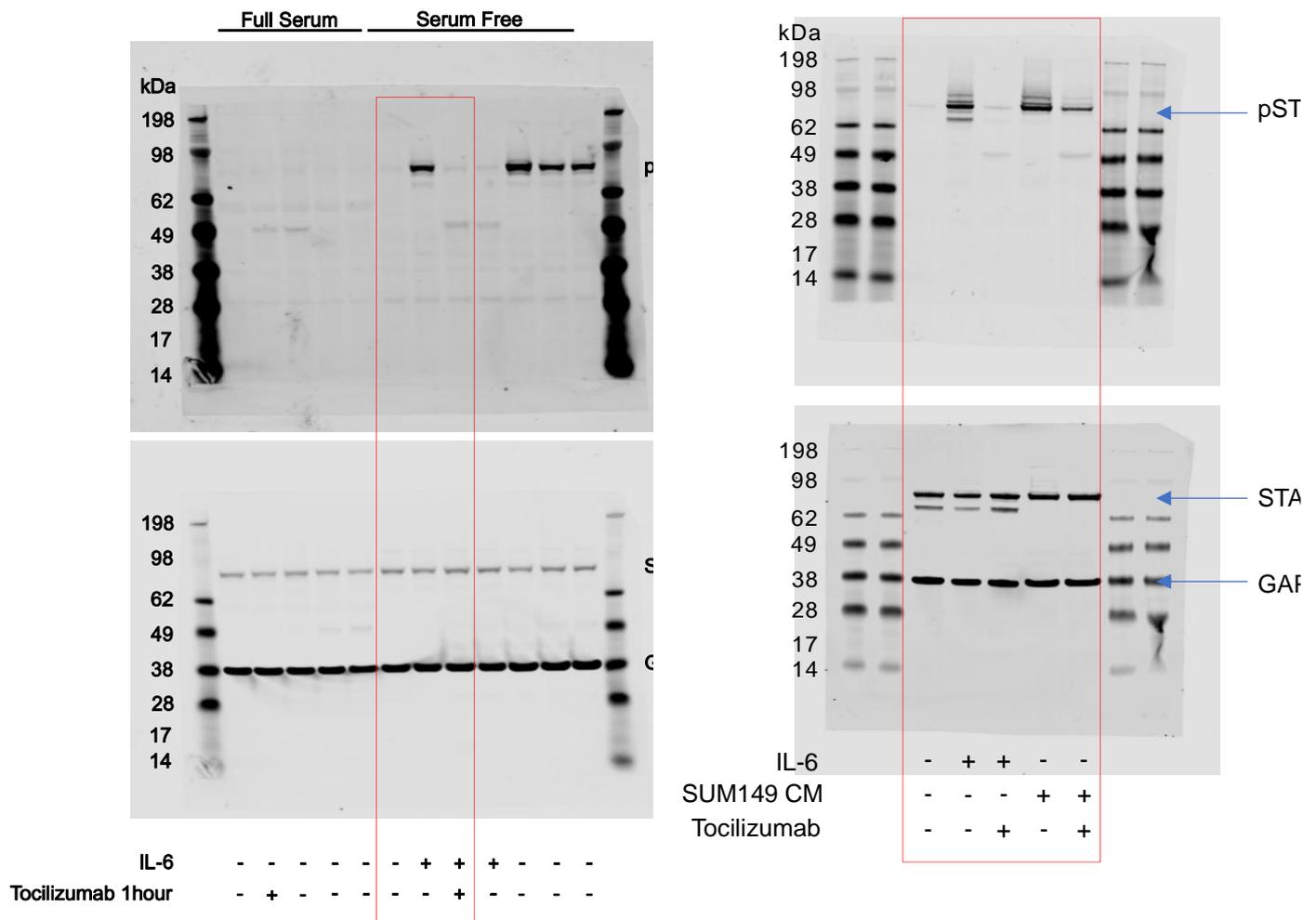


Figure S5F. Original Western blot corresponding to Figure 3C (KPL4).



**Figure S5G.** Original Western blot corresponding to Figure 3C (MDA-IBC-3). **Figure S5H.** Original Western blot corresponding to Figure 4A.

**Table S1. Clinical variables of the human inflammatory breast cancer cohort.** A collection of inflammatory breast cancer specimens was assembled from 18 different patients divided into pre-surgical biopsies (left side) and surgical specimens obtained following neo-adjuvant therapy (right side). Clinical and histopathological variables of both pre-surgical and surgical specimens are indicated. Pre-treatment core biopsies were obtained all 18 patients, 17 of whom proceeded to neo-adjuvant therapy with the treatments indicated. Thirteen of the 17 patients who received neo-adjuvant therapy (76%) proceeded to surgery. Two of 18 (11%) patients achieved a complete pathological response (pCR) to neo-adjuvant therapy. Six of 18 tumors were HER2 gene amplified (total HER2 positive rate of 6/18 = 33%) and four tumors were classified as triple-negative (TNBC, total TNBC rate of 4/18 = 22%). One patients' tumor (INFLAM #26) switched pathological subtype following neo-adjuvant therapy. Fifteen of 18 pre-treatment biopsies (tissue ID's INFLAM #2 - #17) were available for study and were analyzed for HER2, pSTAT3, and IL-6 protein expression (see Figure 1, Figure S1A-C). Patterns of expression that include a pSTAT3<sup>pos</sup>/IL-6<sup>neg</sup> tumor region adjacent to an inversely stained pSTAT3<sup>neg</sup>/IL-6<sup>pos</sup> region (Pattern 1, Pattern 2, Pattern 3) are highlighted in red font.

**Legend:** AC, Doxorubicin and Cyclophosphamide. AI, Aromatase Inhibitor. Caelyx, liposomal doxorubicin. ER, Estrogen Receptor. FEC-D, Fluorouracil, Epirubicin, Cyclophosphamide, Docetaxel. HER2, Human Epidermal Growth Factor Receptor 2. IDC, Invasive Ductal Carcinoma. ILC, Invasive Lobular Carcinoma. ISH, *In Situ* Hybridization. LTFU, lost to follow up. M, Metastasis. MBC, Metastatic Breast Cancer. N/A, Not Applicable. N, Node. Neg, Negative. pCR, Pathological Complete Response. PgR, Progesterone Receptor. Pos, Positive. T, Tumor. TNBC, Triple Negative Breast Cancer. X, Undetermined.