**Supplementary data**

**Supplementary Materials and Methods**

**Isolation of human venous neutrophils**

Buffy coat sample from healthy female donor was immediately processed upon receipt. Buffy coat was diluted 1/3 in cold DPBS 1X (Gibco, cat. #14200-067) with 0.1% heat inactivated FBS (Invitrogen, cat. #16140-071) and 2 mM EDTA (Invitrogen, cat. #AM9260G) and washed by centrifugation. Peripheral blood mononuclear cells were removed by density gradient centrifugation with Ficoll-Paque (GE Healthcare, cat. #17-1440-02) and most of the red blood cells were removed by density gradient with sterile 3% dextran T-500 (Amersham Pharmacia Biotech AB, cat. # 17-0320-02) in 0.9 % NaCl. The residual red blood cells were completely eliminated by hypotonic lysis.

**Optimization of neutrophil infiltration assay in mammospheres.**

Optimization for neutrophils/mammospheres concentration was performed ranging from 2x104 to 1x105 per mammosphere. For MCF-7 mammospheres, in E2 group, the various concentration of infiltrating neutrophils per mammosphere was not significantly different. The experiments were set up using 1x105 neutrophils/MCF-7 mammospheres/well since there was a lower variability for this concentration (Supplementary Fig. 2A). However, for MDA-MB-231 mammospheres, 1x105 neutrophils/mammospheres/well resulted in structural damaged and reduced growth (Supplementary Fig. 2B) therefore; a concentration of 1x104 neutrophils/mammospheres/well was used.

**Paraffin embedding of mammospheres**

Fixed mammospheres were stained with Mayer’s hematoxylin (Histolab Products AB cat. #01820) during 5 minutes, washed with PBS and rehydrated in ethanol (70% 2x30 min, 90% 2x30 min, 99.5% 3x30 min) tissue clear 2x30 minutes (Histolab cat. #14250) and liquid paraffin at 60°C 3x30 minutes in an eppendorf lid. Then mammospheres were transferred to a warm metal mold at 60°C for 30 minutes, centered with a warm spoon, and cooled at room temperature for about 15 minutes to keep them in place and then placed at 60°C with more liquid paraffin and a plastic mold for another 30 minutes. After incubation, the mold was carefully placed at room temperature for 10 minutes and then in 4°C to solidify the paraffin.

**Quantification of soluble ICAM-1**

MCF-7 and MDA-MB-231 mammospheres culture medium samples were analyzed for soluble ICAM-1 using the ELISA kit (BioVision cat. #K7161-100) according to the manufacturer’s instructions.

**Giemsa staining, immunocytochemistry, immunohistochemistry and immunofluorescence**

Cytospins of freshly isolated neutrophils were dried and stained with SNABB-DIFF kit (LABEX Instrument AB cat. #2115) following manufacturer’s instructions. For immunocytochemistry, cytospins of freshly isolated neutrophils were dried, fixed with cold acetone 10 minutes at -20°C and incubated with ~~an~~ anti-human LFA-1 antibody 1:100 (Biolegend cat. #301213) at 4°C overnight. The MACH Univ HRP-polymer detection system (Histolab cat. #BC-BRI4012L) and the betazoid DAB kit (Histolab cat. #BC-BDB2004H) were used. Slides were mounted with Glycergel (Dako cat. #C0563), negative control did not show stain. For immunohistochemistry, MCF-7 and MDA-MB-231 mammospheres were stained with mouse anti-human ICAM-1/CD54 1:50 (Biolegend cat. #353101), mouse anti-human ICAM-2/CD102 1:100 (Novus Biological cat. #NBP2-00320), rabbit anti-human ICAM-3/CD50 1:100 (Sino Biological cat. #10333-R002-50) and counterstained with Mayer’s hematoxylin (Histolab). For immunofluorescence, sections of PyMT mouse tumors were double stained with rat anti-mouse Ly6G 1:400 (BD Pharmingen cat. #551459) and rabbit anti-mouse F4/80 1:25 (Abcam cat. #ab111101) at 4°C overnight. Anti-rabbit Alexa 488 1:200 (Abcam cat. #ab150077) and anti-rat Alexa 546 1:200 (Invitrogen cat. #A11081) were used as secondary antibodies. Slides were mounted using SlowFade Gold antifade reagent with DAPI (Life Technologies cat. #S36938). Images were acquired by Olympus BX43 microscope light/fluorescence microscope with excitation filters BP460-495 and BP530-550, using an Olympus DP72 CCD camera and analyzed using Olympus CellSens Imaging software.

**Migration, survival and retention assays**

MCF-7 cells were cultivated during 3 days in DMEM (Gibco cat. #11880) with 10% charcoal filtered FBS (Gibco cat. #12676-029), 50 IU/ml Penicillin-G, 50 mg/ml streptomycin (Gibco cat. #15070) and 2 mM glutamine (Gibco cat. #25030) ± E2 10-9 M (Sigma cat. #2758). Conditioned media from MCF-7 cells was used for migration and survival assays. Human neutrophils were freshly isolated as described above and re-suspended in DMEM/F12 with 0.02% of bovine serum albumin (Merck cat. #1.12018.0025), 10 µg/ml apo-transferrin (Sigma cat. #T2036) and 1g/ml insulin (Sigma cat. #I5500). Migration assay was performed with CytoSelect™ 96-well cell migration assay kit (Cell Biolabs cat. #CBA-105) according to the manufacturer´s instructions. 5x104 neutrophils/well were placed in the upper chamber ± E2 10-9 M and conditioned media in the lower chamber, migrated cells were quantified after 24 h incubation at 37°C by using the Spark™ 10M multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). For survival assay, 5x105 neutrophils/well were cultured in MCF-7 cells conditioned media ± E2 in a 96-well plate. Living cells were quantified at 24 and 48h of culture with trypan blue viability exclusion and reported as percentage of survival. For retention study, 7x105 neutrophils/mL were added to a monolayer culture of MCF-7 cells grown in DMEM (Gibco) with 10% charcoal filtered FBS (Gibco), 50 IU/ml Penicillin-G, 50 mg/ml streptomycin (Gibco) and 2 mM glutamine (Gibco) ± E2 10-9 M (Sigma). Co-cultures were incubated at 37°C and concentration of neutrophils in culture supernatant was quantified at 24 and 48 h of culture in a Bürker chamber. Number of retained neutrophils was calculated by subtracting the concentration of neutrophils in culture supernatant to the initial concentration of neutrophils added to MCF-7 cells cultures.

**Supplementary Figure legends**

**Supplementary Figure 1.**

**No expression of Ly6G in murine macrophages in breast cancer xenographs**

**A.** Immunostaining of PyMT tumors sections with the neutrophil marker Ly6G (red) and the macrophage marker F4/80 (green). Scale bar 20 µm.

**Infiltrated neutrophil in mammospheres with multi-lobulated nucleus**

**B.** Mammosphere stained with the neutrophil marker CD45 (green) as specified in materials and methods. A 60X magnification shows a multi-lobulated nucleus of the neutrophil. Scale bar 20 µm.

**Morphology of freshly isolated neutrophils**

**C.** Freshly isolated neutrophils stained with Giemsa or anti-human LFA-1 with typical multi-lobulated nuclei. Scale bar 10 µm.

**Supplementary Figure 2.**

**A. E2 increased infiltration of neutrophils into mammospheres in ER+ breast cancer**

Addition of 2x104 to 1x105 neutrophils/mammosphere in presence of E2 increased the infiltration of neutrophils, which was inhibited by fulvestrant (Fulv). \* *p <* 0.05, \*\* *p <* 0.01 compared to control groups; + *p <* 0.05, ++ *p <* 0.01, +++ *p <* 0.001, compared to E2 groups. Scale bar 200m.

**B.** **High concentration of neutrophil infiltration affected mammosphere integrity**

1x105 neutrophils/mammosphere were added to MDA-MB-231 mammospheres resulting in partial destruction of the mammosphere.

**Supplementary Figure 3.**

**ICAM expression in MCF-7 and MDA-MB-231 mammospheres.**

**A.** Immunohistochemistry for ICAM-1, ICAM-2, and ICAM-3 of paraffin embedded mammospheres of MCF-7 and MDA-MB-231 cells. Scale bar 100µm.

**B.** Culture media from MCF-7 and MDA-MB-231 mammospheres was analyzed for soluble ICAM-1, n=8 in each group, \*\*\*\* *p <* 0.0001

**Supplementary video 1 and 2.** MCF-7 breast cancer cells (red) co-injected with human neutrophils (blue) and E2. Co-migration of neutrophils and cancer cells (pink or violet). Neutrophils aid the intravasation and invasion of breast cancer cells. Blood vessels are shown in green. Time-lapse imaging was performed from 12-48 hours post injection.

**Supplementary video 3.** ZR-75-1 breast cancer cells (red) co-injected with human neutrophils (blue) and E2. It shows how neutrophils closely interact with breast cancer cells in the metastatic niche and participate in breast cancer cells transendothelial migration. Blood vessels are shown in green. Time-lapse imaging was performed from 12-48 hours post injection

**Supplementary video 4.** MCF-7 breast cancer cells (red) co-injected with human neutrophils (blue), E2, and anti-human LFA-1 antibody. It shows how breast cancer cells invasion and movement is impaired due to blocking of cell-interactions in presence of antibody. Blood vessels are shown in green. Time-lapse imaging was performed from 12-48 hours post injection.

**Supplementary Table 1.** Characteristics of breast

cancers subjected to microdialysis. One catheter was

inserted within the breast cancer and another in

adjacent normal breast tissue the day before surgery.

All cancers were HER-2 negative.

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| --- | --- | --- | --- | --- |
| Patient | Tumor Size (mm) | Grade (NHG) | ER (%) | PR (%) |
| 1 | 28 | 2 | >50 | >50 |
| 2 | 25 | 3 | >50 | 10-50 |
| 3 | 24 | 2 | >50 | >50 |
| 4 | 22 | 3 | >50 | >50 |
| 5 | 21 | 2 | >50 | 10-50 |
| 6 | 30 | 3 | >50 | <5 |
| 7 | 30 | 2 | >50 | >50 |
| 8 | 25 | 3 | >50 | >50 |
| 9 | 19 | 2 | >50 | >50 |
| 10 | 25 | 2 | >50 | 10-50 |
| 11 | 27 | 2 | >50 | >50 |
| 12 | 40 | 2 | >50 | >50 |

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ER=estrogen receptor, PR=progesterone receptor.