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Supplemental Information

CCL8 Promotes Postpartum Breast Cancer by Recruiting M2 Macrophages

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Supplemental Information

Supplemental Figures

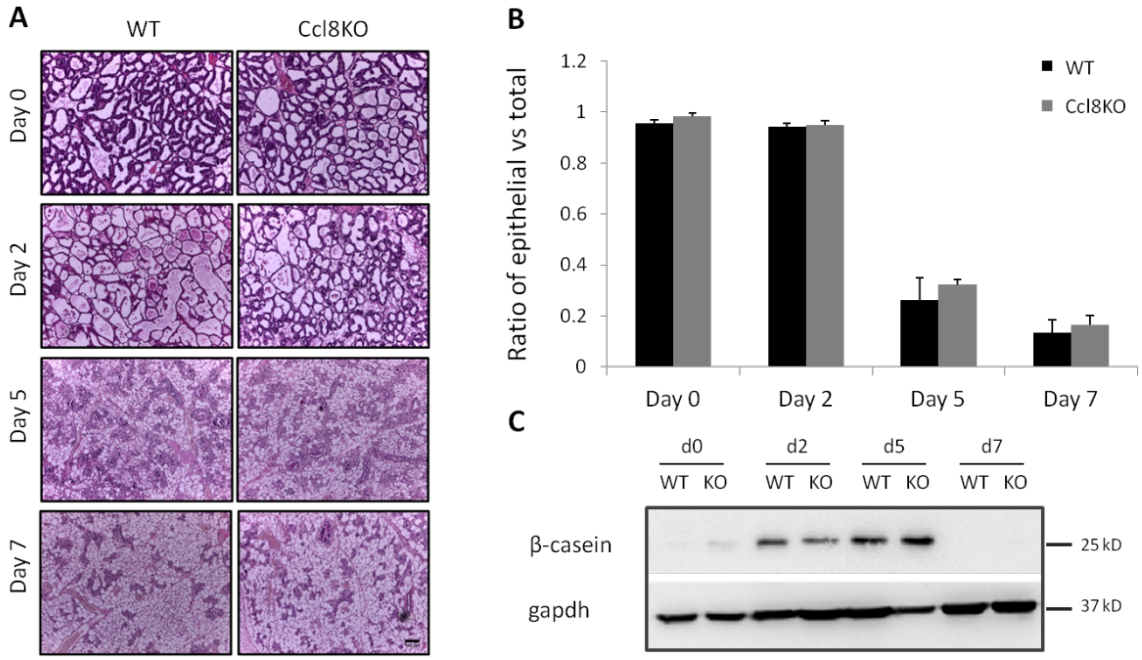

Figure S1. Histological analysis of mammary glands from wt and Ccl8KO mice at different stages of mammary gland development. Related to Figure 1. (A) Hematoxylin and eosin (H&E) staining in the mammary glands from wt or Ccl8KO mice at involution day 0 (lactation day 10), 2, 5 and 7 (n=3). Scale bar 100μm in 10x. (B) Quantification of the epithelial surface by image analysis. Results are shown as average + SD. (C) Representative image of β-casein levels in mammary glands from wt or Ccl8KO mice at involution day 0 (lactation day 10), 2, 5 and 7 assessed by immunoblot.

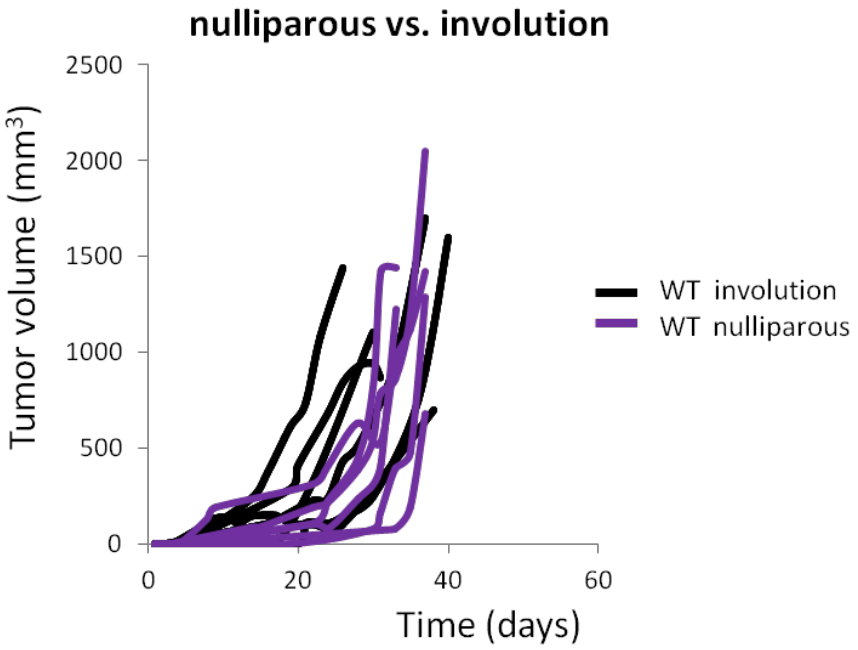

Figure S2. Tumor growth in nulliparous SCID mice and SCID mice during involution, Related to Figures 3A and 3B. MCF10.DCIS.com ($2x10^5$ cells) were implanted in about 3 month SCID nulliparous mice (n=6, purple lines) or in SCID mice at day 2 of forced involution that followed 10 days of lactation (n=6, black lines).

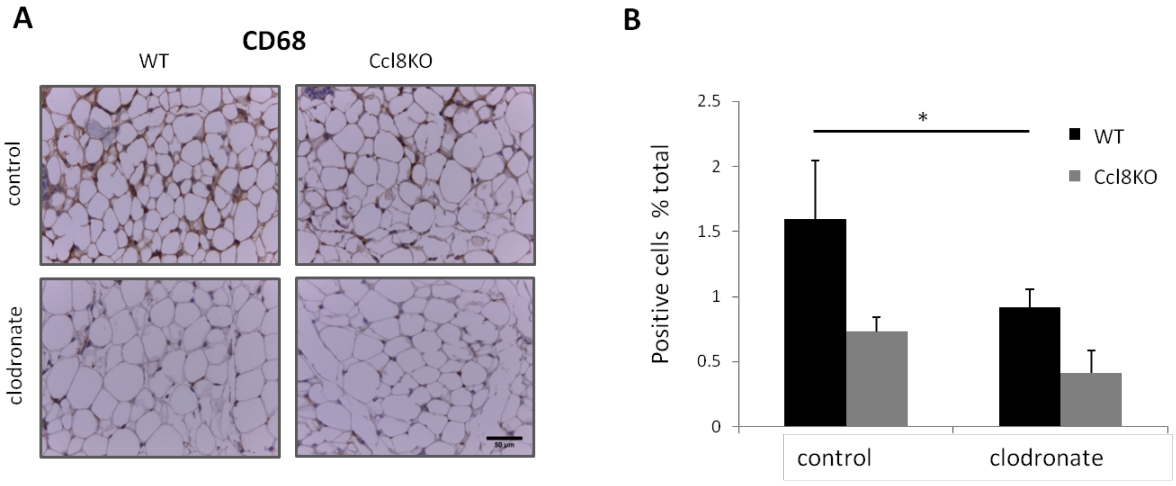

Figure S3. Macrophage depletion in the mammary glands from wt and Ccl8KO, Related to Figure 3C. (A) Representative images (40x) of mammary glands from wt or Ccl8KO CB7B6 mice stained for the macrophage marker CD68 after 5 daily ip injections of 1mg clodronate liposomes. Scale bar 50µm. (B) Quantification of cells per optic field positive for CD68 of the results described above. Results are shown as average + SEM. (*, $P<0.05$, Student's t-test).

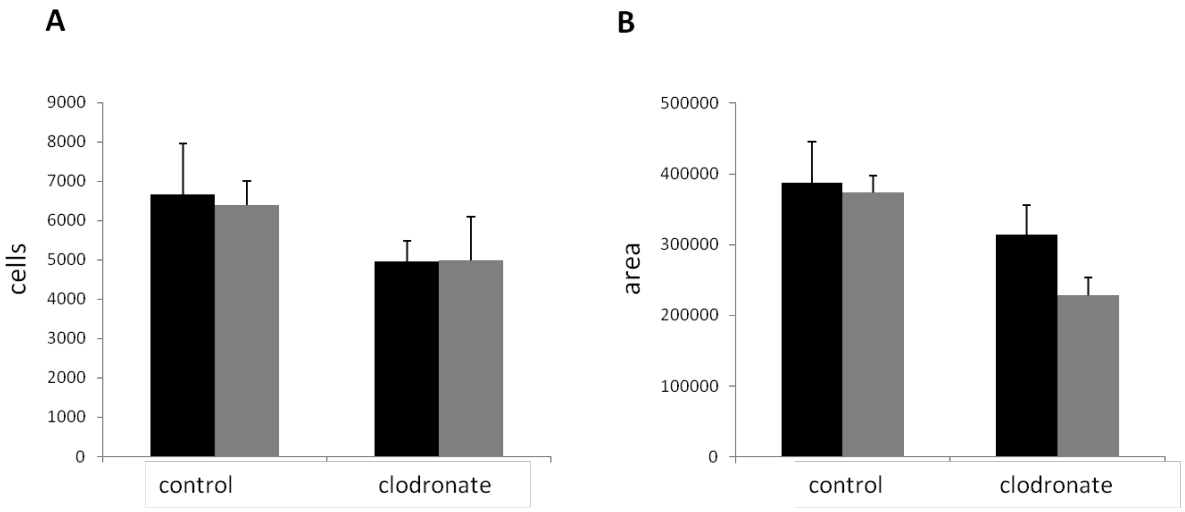

Figure S4. Effect of clodronate liposomes on involution, Related to Figure 3C. Quantification of the epithelial surface by image analysis of mammary glands from wt or Ccl8KO CB7B6 mice at involution day 2, after 5 daily ip injections of 1mg clodronate liposomes. Results are shown as average + SEM.

Transparent Methods

Animal studies

Ccl8KO mice in C57B6 background were obtained from KOMP Repository (University of California, Davis, CA, USA) and subsequently bred and maintained at the USC. SCID (C.B-17 scid) mice were obtained from Taconic (Hudson, NY, USA) and bred with Ccl8KO mice. Littermates were used for all experiments. C57B6 mice were obtained from Jackson lab and maintained at the USC. Animal studies complied with Institutional guidelines. Animal numbers for the experiments were determined based on pilot studies and depended on the number of litters for the Ccl8KO mice. The assignment to groups was performed by a different investigator from the one that acquired the measurements.

For involution studies, the number of litters of the female mice was equalized to 6 pups. For mammary gland involution initiation, females were force weaned at day 10 of lactation post-parturition. Same age nulliparous littermates were used as controls. For the qPCR

confirmation of the microarray results tissues from involution day 4 were used, similarly to the microarray study. For normal development analysis tissues from involution days 2, 5 and 7 were used to cover both the reversible and irreversible phases of involution (Lund et al., 1996, O'Brien et al., 2012).

For tumor reconstitution experiments, 2×10^5 MCF10.DCIS.com cells were resuspended in 0.2 mL of PBS and then injected subcutaneously in the mammary gland fat pad of mice (n=6), either nulliparous or at day 2 of forced weaning that followed 10 days of lactation. Animals were observed daily for tumor development. Tumor volume was calculated based on the formula (LxW2)/2. Results are shown as average + SEM (*, $P<0.05$ Student's t-test).

For macrophage staining during tumorigenesis, mice (n=5) were injected with 1×10^6 MCF10.DCIS.com cells resuspended in 0.2 mL of matrigel (Corning) at day 2 of forced involution that followed 10 days of lactation. Animals were sacrificed 48h later and the matrigel nodules were removed and fixed in formalin. Results are shown as average + SEM (*, $P<0.05$ Student's t-test). Representative images (20x) are shown.

For macrophage depletion, mice (n=5) received 5 daily intraperitoneal injections of 1mg clodronate liposomes in PBS (Liposoma B.V., Amsterdam, Netherlands) starting from lactation day 7 (van Rooijen et al., 2010). Results are shown as average + SEM.

Macrophage depletion was confirmed by immunohistochemistry for CD68 in mammary glands from wt or Ccl8KO mice (Figures S3A and S3B).

Histology

For histological analyses, mammary glands (n=3) or tumors (n=6) tumors were fixed in 10% formalin, paraffin-embedded, serially sectioned, and stained with hematoxylin/eosin. Representative images (20x or 10x) are shown.

Cell Culture

The MCF10.DCIS.com breast cancer cell line was kindly provided by Dr. Fariba Behbod (University of Kansas School of Medicine, KS) and was maintained in DMEM/F-12 supplemented with 5% Horse Serum (Corning). Cells were regularly tested for mycoplasma contamination using commercially available Mycoplasma detection kit (Myco Alert kit; Lonza).

RNA and Protein assays

Total RNA extraction from tissues was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was prepared with a total of 1 ug RNA using iScript™ cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's protocol.

For real-time quantitative PCR, cDNA was amplified using the iTaq Universal SYBR Green Supermix (Bio-Rad) in a total reaction volume of 10 µL, using an Applied Biosystems 7300 real time instrument (Applied Biosystems) as described (Farmaki et al., 2016). *Ccl8* gene expression was assessed in the mammary glands from nulliparous mice or mice at involution day 4 (n=4,) and experiments were performed in duplicates. Results were normalized to GAPDH and are shown as average fold expression compared to nulliparous mice + SEM (*, $P<0.05$ Student's t-test).

For immunoblot analysis and Elisa CCL8 detection, mammary gland tissues from wt mice at involution day 0 (lactation day 10) (n=2), 2 (n=2), 5 (n=4), and 7 (n=4) were solubilized with ice-cold RIPA buffer (Thermo Scientific) supplemented with protease inhibitor cocktail (Thermo Scientific). CCL8 protein levels were assessed by Mouse CCL8/MCP-2 DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN, USA) according to

manufacturer's protocol. Experiments were performed in duplicates and results are shown as average + SEM (*, P<0.05 Student's t-test). The protein concentration in the lysates was determined by using Bradford assay (Bio-Rad). Equal amounts of total protein were resolved by SDS–PAGE and immunoblotted for rabbit polyclonal (251309) anti-casein by Abbiotec and rabbit polyclonal anti-Gapdh 18 (5174S) by Cell Signaling. Experiments were performed thrice and representative image is shown.

Immunostaining was performed in formalin fixed, paraffin embedded sections of tissues from mice at involution day 0 (lactation day 10) (n=2-3), day 2 (n=2-3), day 5 (n=3-5), and day 7 (n=3-5) by using the Dako EnVision+ System-HRP (DAB) (K4041), following the manufacturer's instructions and counterstained with hematoxylin. The antibodies used were rat monoclonal Neutrophil Marker (NIMP-R14) sc-59338 (Santa Cruz Biotechnology) 1:50, rabbit monoclonal [SP115] anti-F/480 (ab111101), by Abcam; 1:250, mouse monoclonal Arginase I sc-271430 (Santa Cruz Biotechnology), mouse monoclonal NOS2 sc-7271 (Santa Cruz Biotechnology), mouse monoclonal CD68 sc-20060 (Santa Cruz Biotechnology). Negative controls included non-immune serum instead of antibody. The number of stained cells (5 random optic fields per sample) as well as the number of total cells were quantified using ImageJ software (National Institutes of Health, USA). Evaluation of samples was performed blindly. Images shown were obtained by a Leica ICC50 HD (Buffalo Grove, IL, USA). Results are shown as average + SEM (*, P<0.05, **, P<0.001 Student's t-test) and representative images (40x) are shown.

Quantification and Statistical Analysis

Quantification of the bands and pictures was performed by ImageJ. All data are presented as average values of samples, error bars correspond to standard error of the mean (SEM) unless

otherwise stated. Statistical analysis of the results was performed using Student's two tailed *t* test. The results were considered statistically significant when P-value <0.05.

Oligonucleotides

Mouse Ccl8 primers	Mouse GAPDH primers
5′-TTCCAGCTTTGGCTGTCTCT-3′ 5′-GGGTGCTGAAAAGCTACGAG-3′	5′-ACCCAGAAGACTGTGGATGG-3′ 5′-CACATTGGGGGTAGGAACAC-3′

Supplemental References

Farmaki, E., Chatzistamou, I., Kaza, V. and Kiaris, H. (2016). A CCL8 gradient drives breast cancer cell dissemination. *Oncogene* **35**, 6309-6318.

Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181-193.

O'Brien, J., Martinson, H., Durand-Rougely, C. and Schedin, P. (2012). Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development* **139**, 269-275.

van Rooijen, N. and Hendrikx, E. (2010). Liposomes for specific depletion of macrophages from organs and tissues. *Methods Mol. Biol.* **605**, 189-203.