CHARACTERIZING NEUTROPHIL RESPONSE TO ESTROGEN DURING MAMMARY INVOLUTION

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A thesis submitted to Nanyang Technological University in partial fulfillment of the requirement for the degree of Master of Science

2016

Acknowledgements

I would like to thank my supervisor, Associate Professor Valerie Lin, for her patient guidance throughout this research journey. I would also like to thank my lab members, especially Ms. Or Yu Zuan and Mr. Lim Chew Leng, for their encouragement and valuable support for the completion of this thesis. I extend my gratitude too to other colleagues in the School of Biological Sciences for their friendly help in times of need. Last but not least, I am also thankful for the constant support from my loved ones, especially my late grandmother, who has always been one of my greatest cheerleaders.

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Abbreviations

36B4	Acidic ribosomal phosphoprotein P0
AREG	Amphiregulin
BN	Banded neutrophils
cDNA	Complementary deoxyribonucleic acid
COX2	Cyclooxygenase-2
C_t	Comparative threshold cycle
CTS B	Cathepsin B
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCR2	Chemokine (C-X-C motif) receptor 2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPN	Diarylpropionitrile
DPX	Distyrene plasticizer xylene
E2B	17β-estradiol-3-benzoate
ERs	Estrogen receptors
FACS	Fluorescence-activated cell sorting
FSC-A	Forward scatter (area)
FSC-H	Forward scatter (height)
FSC-W	Forward scatter (width)
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
InvD	Involution day
LacD	Lactation day
LSD	Least significant difference
MM	Myelocytes and metamyelocytes
MMPs	Matrix metalloproteinases

MN	Mature neutrophils
NH_4Cl	Ammonium chloride
PABC	Parity-associated breast cancer
PBS	Phosphate-buffered saline
PPT	Propyl pyrazole triol
PRs	Progesterone receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real-time quantitative polymerase chain reaction
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
SSC-A	Side scatter (area)
Stat3	Signal transducer and activator of transcription 3
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TEBs	Terminal end buds
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

Abstract

Women, especially those who experienced first full-term pregnancy at late age, face increased risk of pregnancy-associated breast cancer (PABC) in 10 years following childbirth. Mammary involution has been implicated as a key risk factor due to its inflammatory nature. Estrogen exposure is also another risk factor for breast cancer, but its specific effect during mammary involution has yet to be elucidated. Here, we showed that estrogen exposure during mammary involution locally amplifies the inflammatory response by enhancing neutrophil chemotaxis in a CXCR2-dependent manner, and differentiated estrogen-regulated functions of cell growth and death from neutrophil-regulated functions of neutrophil recruitment and adipogenesis. These findings highlight a novel mechanism of estrogenic action on neutrophils during mammary involution. This could be useful for development of novel therapeutics for PABC that are specific to the context of estrogen exposure during mammary involution.

INTRODUCTION

Breast Cancer

Breast cancer is the most frequent cause of cancer for women worldwide. According to the International Agency for Research on Cancer (2016), the incidence of breast cancer in females stands at a high rate of approximately 1.67 million new cases in 2012, which accounts for 25% of all cancers diagnosed in that year. This high incidence rate of breast cancer is compounded by a high mortality rate, with breast cancer ranking amongst the top three leading causes of cancer death for women living in both developed and underdeveloped regions (International Agency for Research on Cancer, 2016). Likewise, in Singapore, a study of cancer incidence by the Singapore Cancer Registry found that breast cancer was the most frequent cancer in females at 29.2% between 2010 and 2014, with a mortality rate of 95.7% (National Registry of Diseases Office, 2016). These figures highlight the significance of studying breast cancer in both a global and local context.

In recent years, traditionally lower-risk countries such as those in Asia are witnessing a rise in breast cancer cases, and this has been attributed to changes in a number of overlapping risk factors, including hormonal exposure, use of contraceptives, and later childbirth (American Cancer Society, 2015). A common denominator of these risk factors is estrogen – one of the main hormones involved in the female reproductive system. Even though dysregulation of estrogen has long been known to promote tumorigenesis of breast cancer (Clemons & Goss, 2001), it is still commonly used in contraceptives to prevent pregnancy nowadays.

Estrogen is produced mainly by the ovaries and other extragonadal tissues like adipose. However, it can be supplemented through dietary phytoestrogens and

oral contraceptives (Clemons & Goss, 2001). Estrogen carries out its functions by binding to estrogen receptors (ER), ERα and ERβ. In normal physiological setting, estrogen has a key role in the female reproductive system, with pleiotropic effects on mammary glands, uterus, ovaries, and hypothalamic-pituitary axis (Hennighausen & Robinson, 2005). However, excessive hormonal stimulation can result in tumor formation when the organ's response progresses from normal proliferation to hyperplasia and then to neoplasia. This theory is supported indirectly by epidemiological evidence of increased breast cancer risk in women who experienced early menarche, late first full-term pregnancy and late menopause, all of which reflect greater total exposure of the mammary gland to estrogen (Clemons & Goss, 2001).

Another source of estrogen is through the use of contraceptives, and the modern trend of delayed childbirth through the use of contraceptives is a major concern at two levels. Firstly, such exogenous estrogen exposure has been found to increase breast cancer risk by 10-30% up to ten years even after cessation of use (American Cancer Society, 2015; Clemons & Goss, 2001). Secondly, delayed childbirth, especially when first full-term pregnancy occurs after the age of 30, is associated with an increase in short-term risk of post-parturition breast cancer and slower crossover to long-term protection against breast cancer (Figure 1) (American Cancer Society, 2015; Schedin, 2006).

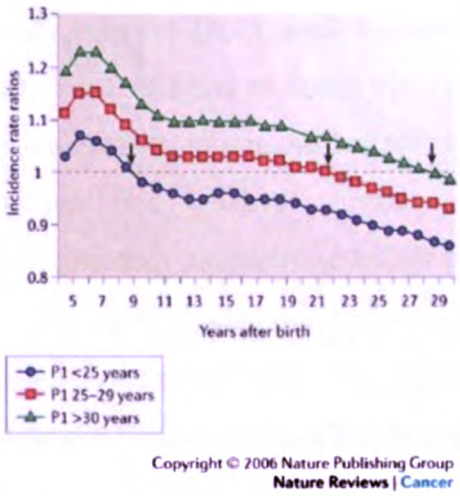

Figure 1. Later first full-term pregnancy is associated with higher breast cancer risk and slower crossover to protection. Predicted incidence rate ratio of women of parity according to time since first birth (P1) are compared, with relative rate ratio of nulliparous women set at 1.0. Arrows indicate crossover to protection. Women with later first full-term pregnancy not only have higher incidence rate ratio than women with earlier first full term pregnancy, but also a later crossover to protection i.e. crossover at approximately 10 years (PI < 25 years) versus 22 years (25 years < PI <29 years) versus 30 years ((PI > 30 years). (Republished with permission of Nature Reviews Cancer, from Pregnancy-associated breast cancer and metastasis, Pepper Schedin, 6, 4, 2006; permission conveyed through Copyright Clearance Center, Inc.)

Breast cancer diagnosed within five to ten years post-parturition is known as parity-associated breast cancer (PABC). As critically reviewed by Schedin (2006) and reinforced by Baumgarten and Frasor (2012), PABC is known to have a poorer prognosis and survival rate compared to breast cancer in both women who have not recently given birth, and age-matched nulliparous women. Specifically, an inverse relationship has been identified between survival rate, and the length of time between childbirth and diagnosis of breast cancer. Factors contributing to poor prognosis include (i) higher incidence of metastasis, (ii) difficulty in timely diagnosis due to pregnancy- and breastfeeding-induced increase in breast density, and (iii) production of gestational hormones like estrogen that can not only stimulate breast cancer progression, but may also select for tumors with poor prognostic characteristics. PABC tumors have also been characterized to have a higher tendency for estrogen and progesterone receptor negativity (Borges & Schedin, 2012). The obstacles to effectively prevent PABC is aggravated by the high

possibility for poor outcomes, thus making treatments of PABC so difficult that more research efforts have to be made in order to shed light on improved treatments. So far, epidemiology-based studies have picked up on a close correlation between PABC and a recently completed pregnancy to suggest that mammary involution plays a key role in the development of PABC.

Overview of the Mammary Gland

As reviewed by Richert et al. (2000), mice have five pairs of mammary glands (1^{st} and 6^{th}, 2^{nd} and 7^{th}, 3^{rd} and 8^{th}, 4^{th} and 9^{th}, 5^{th} and 10^{th}), three of which are in the thoracic region, while the other two pairs are in the inguinal region. Each mammary gland can be divided into two compartments – epithelium and mammary fat pad. The epithelium consists of ducts and milk-producing alveoli that together make up the parenchymal structures of the mammary gland. On the other hand, the mammary fat pad is the stromal compartment where parenchymal structures are embedded (Figure 2). The mammary fat pad is predominantly made up of adipocytes, but also comprises lymphatics, blood vessels, fibroblasts and neurons. Each mammary gland also contains a lymph node, as well as an exterior nipple that links the connected central lumen of alveoli and ducts to the external environment for the release of milk during lactation. While the development of the mammary gland begins embryonically, most of the mammary gland development takes place postnatally with the onset puberty at about 3 weeks of age (Hennighausen & Robinson, 2005; Richert, et al., 2000).

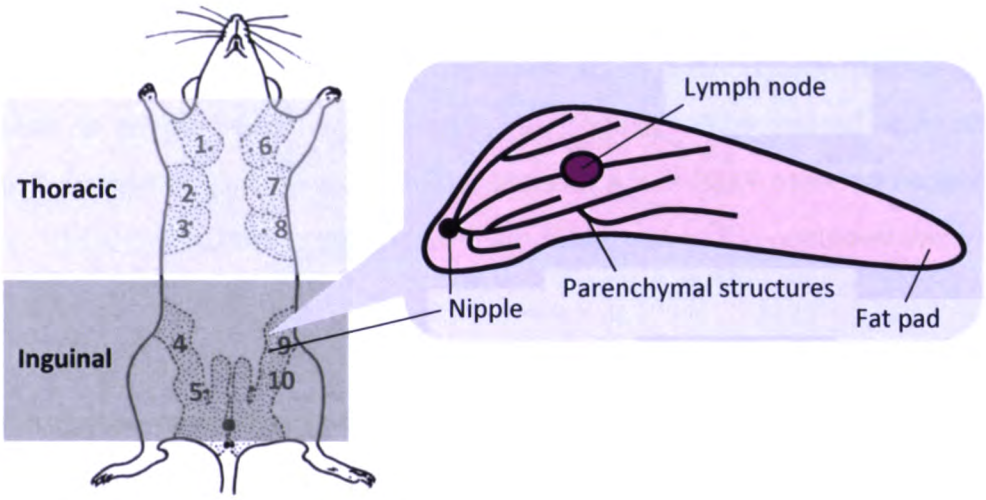

Figure 2. Anatomy of the mouse mammary gland. The mouse has five pairs of mammary glands in the thoracic and inguinal region. Each dot represents a nipple and the stippled area the maximum area of the mammary gland. Each mammary gland consists of parenchymal structures embedded in the mammary fat pad, a lymph node, and a nipple. (Adopted with permission from the online version of *Biology of the Laboratory Mouse (2nd ed.)* by The Staff of The Jackson Laboratory, Earl L. Green, Editor, Dover Publications, Inc., New York 1966, http://www.informatics.jax.org/greenbook/)

Puberty initiates the first stage of postnatal mammary growth – ductal growth. Secretion of hormones by the ovaries and the consequent binding of estrogen to ERα on stromal and epithelial cells causes the rudimentary ductal structure of the mammary gland to exit quiescence, and triggers the amphiregulin (AREG)-mediated process of ductal elongation and the appearance of terminal end buds (TEBs) (Hennighausen & Robinson, 2005; LaMarca & Rosen, 2007; Richert, et al., 2000). Consequently, the developed non-pregnant, non-lactating mammary gland consists of ducts that branch extensively throughout the mammary fat pad and end in rudimentary alveolar buds. Each ductal branch comprises of a central lumen surrounded by a single layer of luminal epithelial cells, which is in turn encompassed by myoepithelial cells (Figure 3) (Richert, et al., 2000).

The second stage of mammary growth, alveolar differentiation, mainly occurs during pregnancy. The initial stage of pregnancy-induced mammary growth

is similar to that triggered by puberty, with massive proliferation of ductal branches and formation of alveolar buds. The second stage of pregnancy-induced mammary growth is characterized by the lobuloalveolar phase of mammary growth, whereby alveolar buds cleave and differentiate into individual alveoli that eventually become milk-secreting lobules (Figure 3) (Richert, et al., 2000). Unlike in puberty-mediated ductal elongation and outgrowth, ERα is dispensable for pregnancy-mediated alveolar expansion. Instead, estrogen exerts its effects through progesterone receptors (PRs) to stimulate proliferation of mammary epithelial cells in both an autocrine and paracrine manner (Hennighausen & Robinson, 2005). In each alveolus, luminal epithelial cells produce and secrete milk proteins and lipids into the lumen, while contractile myoepithelial cells push milk along the ducts out of alveolus during lactation (Figure 3). In both the late stage of pregnancy and lactation, the stromal compartment is reduced, in part due to the expansion of alveoli, and also the metabolism of fats in the adipocytes (Richert, et al., 2000).

Lactation continues until weaning which can occur either naturally or forcedly by preventing breastfeeding. In experimental studies, pups are often weaned forcedly by separating pups and their mother into different cages for more controlled conditions. Weaning results in milk stasis, which in turn triggers the onset of involution, a process of cell death and tissue remodeling that returns the mammary gland to resemble its pre-pregnant state (Richert, et al., 2000).

Involution can be divided into two stages. The first stage of involution is reversible and can be reinitiated by suckling. It presents no major morphological changes from the lactating mammary gland, and does not seem to be highly susceptible to influences by hormones (Figure 3). In contrast, the second stage of involution that occurs 48 hours after initiation of involution (involution day 3 (InvD3) onwards) is irreversible and accompanied by major remodeling of the epithelium and stroma. Secretory epithelial cells undergo apoptosis causing the collapse of

alveolar structures, while adipocytes begin to fill up the mammary gland once more (Richert, et al., 2000). These structural remodeling are accompanied by changes in the expression of proteinases and proteinase inhibitors, such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which are found to be involved in both degradation of extracellular matrix components and adipogenesis (Figure 3) (Alexander, et al., 2001). On the other hand, apoptosis of epithelial cells in the involuting mammary gland has been found to be controlled by a lysosomal-mediated pathway under the action of signal transducer and activator of transcription 3 (Stat3) and cathepsins, including cathepsin B (CTS B) (Kreuzaler, et al., 2011). Once involution is completed, the mammary gland is ready to go through another cycle of pregnancy, lactation and involution (Figure 3).

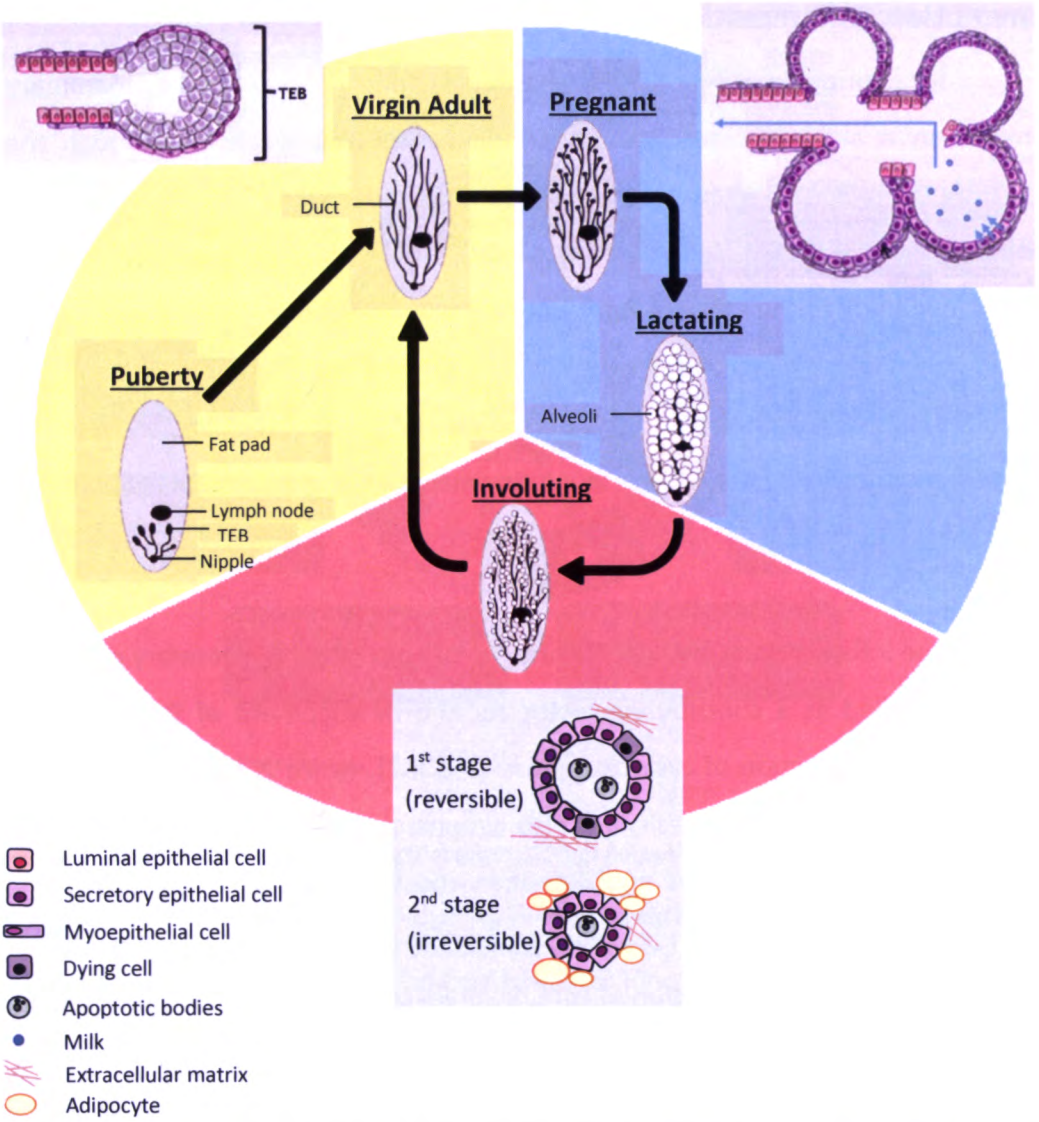

Figure 3. Cycle of the mammary gland development. The bulk of mammary gland development occurs postnatally with the start of puberty. During puberty, ductal elongation and branching occurs to form an extensive network of ducts that end in rudimentary alveolar buds known as terminal end buds (TEBs) in the adult virgin mammary gland. Pregnancy is accompanied by massive ductal proliferation and formation of alveolar buds that cleave and differentiate into milk-secreting lobules. During lactation, secretory epithelial cells in the alveoli produce milk while contractile myoepithelial cells help to deliver milk out of the gland. With milk stasis, involution occurs. Involution takes place in two stages, the first being a reversible phase without much morphological changes, while the second is an irreversible phase with major morphological changes including degradation of the extracellular matrix, adipogenesis, and collapse of the alveolar structures. Upon completion of involution, the mammary gland resembles its pre-pregnant state and is ready for another round of pregnancy, lactation and involution. (Adopted with permission from (i) *Autophagy in Development and Remodelling of Mammary Gland, Autophagy – A Double-Edged Sword – Cell Survival or Death?* © 2013 Gajewska M et al., published in InTech under CC BY 3.0 license, available from http://dx.doi.org/10.5772/54558 and (ii) "Patterns of cell signaling pathway activation that characterize mammary development." by E. R. Andrechek et al., 2008, *Development*, Vol.135, Issue 14.)

Involution, Inflammation and PABC

In addition to the structural changes in the mammary gland, mammary involution is also characterized by an inflammatory component. Although the tightly controlled mammary gland involution has caused it to be considered non-inflammatory by some (Monks, et al., 2002), others have compared mammary gland involution to the inflammatory wound healing response. This is supported by recent histological and microarray studies, which revealed extensive infiltration of immune cells such as macrophages, neutrophils and lymphocytes, and activation of pro-inflammatory pathways in the involuting mammary glands (Atabai, et al., 2007; Schedin, 2006).

The inflammatory microenvironment of the involuting mammary gland has been identified as a contributing factor for the development of PABC, in part through the production of cyclooxygenase-2 (COX2), which is known to facilitate not only collagen fibrillogenesis, but also lymphangiogenesis (Lyons, et al., 2011; Schedin, 2006). This is in line with Hanahan and Weinburg's studies (2011) that identified the inflammatory microenvironment as an enabling characteristic that helps to facilitate the acquisition of core hallmark capabilities by the tumor (Figure 4). This is supported by observations of immune cells in almost all neoplastic lesions and poorer prognoses of cancers with an inflammatory component as compared to cancers without an inflammatory component (Hanahan & Weinburg, 2011; Schedin, 2006). Immune cells in neoplastic lesions contribute to cancer through the secretion of signaling and effector molecules to stimulate cellular survival and proliferation, promote angiogenesis, invasion and metastasis, amplify inflammatory response, and facilitate evasion of the immune system (Hanahan & Weinburg, 2011).

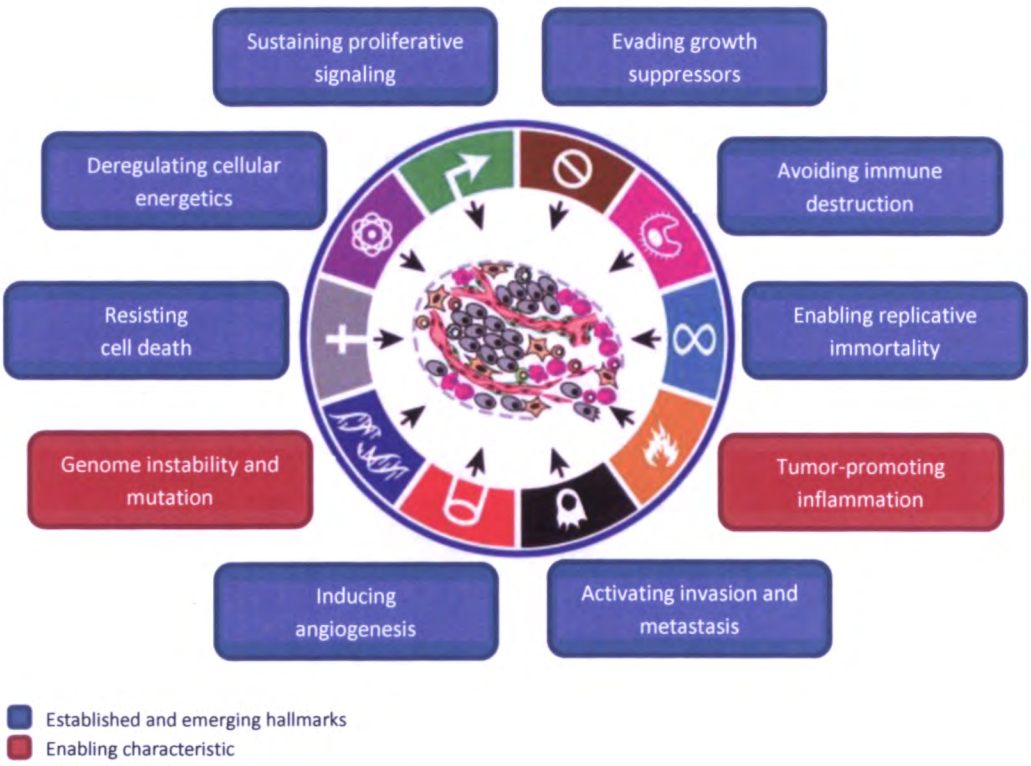

Figure 4. Inflammation is an enabling characteristic that helps the tumor to acquire core hallmark capabilities. Almost all neoplastic lesions are infiltrated by immune cells. These tumor-infiltrating immune cells secrete signaling and effector molecules that help the tumor to acquire core hallmark capabilities that help the tumor to survive and proliferate. These established and emerging hallmarks include activating invasion and metastasis, inducing angiogenesis, resisting cell death, deregulating cellular energetics, sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction and enabling replicative immortality. Genome instability and mutation is another enabling characteristic. (Adopted with permission of Cell, from Hallmarks of Cancer: The Next Generation, Douglas Hanahan and Robert A. Weinburg, 144, 5, 2011; permission conveyed through Copyright Clearance Center, Inc.)

In recent decades, the pro-tumorigenic effect of tumor-infiltrating immune cells, particularly those of the innate immune system, has drawn a lot of attention. The role of macrophages in cancer has well been characterized. A subset of macrophages known as tumor-associated macrophages (TAMs) can be polarized to the activated, tumor-killing M1 phenotype or the alternatively activated, tumor-promoting M2 phenotype (Noy & Pollard, 2014; Solinas, et al., 2009). However, the role of neutrophils in breast cancer, more specifically PABC, has yet to be elucidated.

Neutrophils in Inflammation and Cancer

Neutrophils are short-lived myeloid cells of granulocytic lineage. They can be identified through morphological examination for their characteristic banded (doughnut- or ring-shaped) or (hyper)segmented (lobes linked by thin threads of chromatin) nucleus coupled with a granulocytic cytoplasm that stains faintly pink/purple with Giemsa (Figure 5). Neutrophils can also be identified by their surface markers as $CD11b^{+}$ $Gr\text{-}1^{hi}$ $Ly6G^{+}$ cells (Hestdal, et al., 1991; Pillay, et al., 2013).

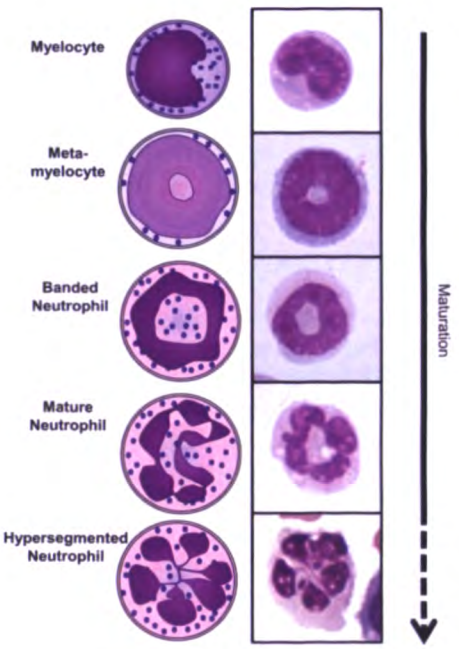

Figure 5. Schematic representations and images of murine neutrophil morphology at different stages of development. As neutrophils mature, their nucleus develops from doughnut or ring shape to take on a (hyper)segmented morphology, where lobes are linked by thin threads of chromatin. ("Republished with permission of Cellular and Molecular Life Sciences, from Immune Suppression by Neutrophils and Granulocytic Myeloid-derived Suppressor Cells: Similarities and Differences, Janesh Pillay et al., 70, 20, 2013; permission conveyed through Copyright Clearance Center, Inc.")

Upon stimulation by neutrophil chemoattractants, neutrophils egress from their site of production in bone marrow, and subsequently transmigrate into the site of inflammation in peripheral tissues where they perform their functions

before programmed cell death. Known as the most effective phagocytes, neutrophils engulf and eliminate their targets through releasing reactive oxygen species and proteases (Nathan, 2006). Their potent weaponry and potential to cause tissue damage calls for tight regulation of their recruitment processes, but this regulation must also be balanced against their importance as the first line of defense against pathogens. Precise control of neutrophil migration occurs through a plethora of non-redundant chemoattractants that are specific to each stage of neutrophil migration, and expressed in a temporal and/or spatial pattern that is unique to each stimulus. This includes chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, S100 calcium binding protein A8 (S100A8) and S100A9, all of which are responsible for the recruitment of neutrophils from the bone marrow to the target sites (Ryckman, et al., 2003; Sadik, et al., 2011).

In the context of cancer, neutrophils are known to be recruited by chemokines and cytokines in the tumor microenvironment. Before the concept of tumor-associated neutrophils (TANs) was developed, the role of neutrophils within the tumor microenvironment was found to be controversial – while some studies have found neutrophils to be pro-tumoral, for example through the production of MMP9 that in turn facilitates angiogenesis, tumor progression and metastasis, other studies have suggested an opposite role of neutrophils which activate cytotoxic T cells to mediate tumor cell clearance (Powell & Huttenlocher, 2016). The model of TANs proposed by Fridlender and colleagues (2009) based on studies on TAMs served to reconcile these seemingly controversial observations.

In their proposed model, TANs can be polarized into anti-tumoral (N1) and pro-tumoral (N2) subtypes by factors present in the tumor microenvironment, such as transforming growth factor beta (TGF-β) and interferon gamma (IFN-γ). Thus far, it is known that polarization into the N1 subtype is stimulated by TGF-β and inhibited by IFN-γ whilst polarization into the N2 subtype is stimulated by IFN-γ and

inhibited by TGF-β (Piccard, et al., 2012). These 2 subtypes of TANs also differ in morphology and array of cytokines produced. The N1 phenotype is characterized by a hypersegmented nucleus and exerts a protective effect on the host against cancer by producing pro-inflammatory chemokines and cytokines and suppressing production of arginase to increase the recruitment and activation of CD8+ cytotoxic T cells. In contrast, the pro-tumorigenic N2 phenotype has a circular nucleus, produces higher amounts of angiogenic substances and MMPs, as well as suppresses the anti-tumor immune response by producing higher amounts of arginase (Fridlender, et al., 2009; Piccard, et al., 2012). Such polarization of neutrophils appears to be context-dependent, calling for a need to study the behavior of neutrophils specifically in the estrogen-stimulated involuting mammary gland.

Estrogen as a Modulator of Neutrophil action

While estrogen is most commonly associated with its effects on the female reproductive organs, it has also been reported to affect many other non-classical target organs and tissues, including the adipose tissue, bone, brain, and cardiovascular system (Mueller & Korach, 2001). Interest in the role of estrogen in regulating host responses was first piqued through observations of differential susceptibility to disease or tissue stress between men and women, as well as between pre- and post-menopausal women (Kassi, et al., 2015; Mendelsohn & Karas, 1999; Reckelhoff, 2001). However, the effect of estrogen is complex, and can be either pro-inflammatory or anti-inflammatory in different diseases. This depends on a range of diverse factors such as the immune stimulus and the consequent immune responses, target cell type, target organ with its specific microenvironment, timing and concentration of estrogen administered, as well as variability in ER subtype expression (Straub, 2007).

The effect of estrogen on neutrophils specifically have been studied in several models, including humans, rats and mice, with varying observations. On one hand, estrogen has been found to attenuate the early vascular injury response, wound healing response, gastric or colonic damage in experimental ulcer or colitis and carrageenan-induced pleurisy by inhibiting expression of pro-inflammatory mediators and adhesion molecules that are involved in the chemotaxis of neutrophils (Ashcroft, et al., 1999; Cuzzocrea, et al., 2000; Kumral, et al., 2014; Miller, et al., 2004). On the other hand, estrogen has been found to be pro-inflammatory in models of influenza A virus infection where it stimulates an increase in neutrophil numbers (Robinson, et al., 2014). Given an important role of neutrophils in the immune system and the diversity of neutrophilic responses to estrogen exposure, there is thus a need to characterize specifically the responses of neutrophils to estrogen in the involuting mammary gland, a hitherto less well understood context.

Scope of Study

During mammary gland involution, the effect of estrogen exposure on neutrophils has yet to be fully elucidated. Our previous data has shown that estrogen exposure during involution promotes a pro-inflammatory response as estrogen treatment for 48 hours at 24 hours post-weaning (InvD1) significantly upregulated not only the expression of estrogen target genes, AREG and PR, but also significantly increased neutrophil percentage and expression of neutrophil chemoattractants, including CXCL1, CXCL2, S100A8 and S100A9, in the involuting mammary gland. However, because of the presence of interspersing blood vessels in the mammary glands, it is unclear if the increased neutrophil percentage was due to an increase in circulating neutrophils or a tissue-specific accumulation of neutrophils in mammary tissues. Furthermore, it is uncertain whether upregulation of chemoattractants have unique roles to play in regulating neutrophil migration during estrogen-stimulated mammary involution.

CXCL1 and CXCL2 are cognate ligands of chemokine (C-X-C motif) receptor 2 (CXCR2), and are well-characterized to be associated with inflammation. Interestingly, Elazar and colleagues (2010) demonstrated that CXCR2 knockout mice had reduced neutrophil infiltration within the alveolar and ductal lumens. This is a phenotype opposite to what we have observed in the estrogen-treated involuting mammary glands, in which there was a significant increase in the number of neutrophils in the alveolar and ductal lumens. This inspired us to look at the effect of CXCR2 inhibition on neutrophil infiltration during mammary involution. Targeting CXCR2 instead of its ligands CXCL1 and CXCL2 has the added advantage of eliminating redundant signaling by other CXC chemokines (Waugh & Wilson, 2008; Yano, et al., 2003).

Hereby, we proposed that estrogen-stimulated upregulation of CXCL1 and CXCL2 are predominantly responsible for the accumulation and function of neutrophils within the alveolar and ductal lumen, and that inhibition of CXCR2 would abolish the estrogen-induced effects of increased neutrophil influx and accelerated mammary involution. To validate our hypothesis, the response of neutrophils at each stage from production in the bone marrow to infiltration and death in the involuting mammary gland were examined through histology and gene expression analyses. Subsequently, the role of CXCL1 and CXCL2 were explored by investigating the effect of SB225002, a small molecule antagonist of CXCR2, on neutrophil infiltration.

Understanding the response of neutrophils to estrogen in the context of the involuting mammary gland could shed light on the development of PABC under influence of estrogen that may possibly uncover new targets for therapeutic intervention.

MATERIALS AND METHODS

Animal protocol

For each experiment, six to eight weeks old female BALB/c mice were bred. On lactation day 2-4 (LacD2-4), these monoparous mice underwent bilateral ovariectomy to eliminate effects of endogenous hormones. Litter sizes were standardized at 4-6 pups per lactating mouse. After recovering for at least 10 days, on LacD12-14, involution was initiated by forced weaning of the pups. After 24 hours of involution (InvD1) or 48 hours of involution (InvD2), the mice were randomly assigned into treatment or control groups. The mice were subsequently sacrificed under general anesthesia by cervical dislocation after 48 or 24 hours of treatment respectively for collection of tissues and blood for analysis. The 4^{th} and 9^{th} inguinal mammary glands were harvested for ribonucleic acid (RNA) and histological analyses. Mammary tissues collected for RNA analyses were snap frozen in liquid nitrogen and stored at -80°C until analysis while mammary glands collected for histological analyses were fixed in 4% paraformaldehyde for further processing.

Estrogen treatment

Starting from InvD1 or InvD2, mice in the treatment group underwent subcutaneous injection with 20μg/kg/day of 17β-estradiol-3-benzoate (E2B) (Sigma-Aldrich) diluted in sesame oil once daily for 48 hours or 24 hours respectively. The control group received the same dosage of sesame oil without E2B.

SB225002 treatment

CXCR2 antagonist, SB225002 (Sigma-Aldrich), was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 20mg/ml to make a stock solution. The working solution of SB225002 was prepared fresh each day by diluting the stock solution in 0.25% Tween 20 in phosphate-buffered saline (PBS). SB225002 was administered in mice intraperitoneally twice daily at a concentration of 0.3mg/kg. The control group was treated with the same dosage of vehicle control.

Cell isolation

Mammary tissue

Mammary gland tissues with draining lymph node removed were minced and digested for 1 hour at 37°C with gentle shaking in 2mL of Dulbecco's Modified Eagle Medium (DMEM) (with high glucose and no phenol red, HEPES or sodium pyruvate) (Nacalai Tesque) supplemented with HyClone™ L-glutamine (GE Healthcare Life Sciences), containing 1mg/mL collagenase (Sigma) and 120Kunitz of DNAse I (Sigma). Digested samples were filtered through a 100µm nylon sieve to obtain a single cell suspension. After centrifugation at 450g for 5 minutes, the supernatant was removed, and the pellet was resuspended in 1 mL of erythrocyte lysis buffer. Erythrocyte lysis buffer consists of tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) (20.59 g/L) and ammonium chloride (NH_4Cl) (4.15g/500mL) mixed in the ratio of 1:9. 9mL of Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque) was added to inactivate the lysis reaction. Cells were then pelleted and resuspended in 1mL of DMEM. The number of viable cells was counted in Trypan Blue using a hemocytometer.

Mammary neutrophils

Neutrophils were isolated from the mammary gland digests by positive selection with the Dynabeads® Biotin binder kit (Invitrogen) coupled with biotinylated antibodies raised against Ly6G (clone 1A8) based on established

techniques by our laboratory. Separation efficacy was determined by flow cytometry. Resulting cell mixtures deprived of neutrophils were kept as controls for subsequent analysis.

Blood neutrophils

Circulating leukocytes were obtained from heparinized blood extracted by cardiac puncture. Residual erythrocytes were removed by hypotonic lysis by incubating with shaking in erythrocyte lysis buffer at room temperature for 15 minutes. Erythrocyte lysis buffer consists of Tris-HCl (20.59 g/L) and NH_4Cl (4.15g/500mL) mixed in the ratio of 1:9. Erythrocyte lysis was inactivated by 20mL of 1X PBS. The suspension was then centrifuged at 450g for 5 minutes, followed by removal of supernatant and resuspension of cell pellet in 1X PBS for further analysis through flow cytometry.

Flow cytometry (performed by Or Yu Zuan or Lim Chew Leng)

Flow cytometry studies of single cell suspensions from blood and mammary tissue were performed on LSRII (BD Biosciences), and subsequently analyzed with FlowJo software (Tree Star). The following monoclonal antibodies purchased from either eBioscience or BioLegend were used: APC/Cy7 CD45 (clone 30-F11), biotin Gr-1 (clone RB6-8C5), Alexa Fluor 647 streptavidin, PE Ly6G (clone 1A8), BV605 CD11b (clone M1/70). Dead cells were identified with fixable viability dye eFluor® 450 (eBioscience).

Histological analysis of the mammary gland

The 4^{th} or 9^{th} inguinal mammary gland was harvested for histological analysis. Each mammary gland was fixed in 4% paraformaldehyde for 24 to 48 hours before transferring to 70% ethanol as part of the dehydration process. The mammary glands were then sent to the Institute of Molecular and Cell Biology Core

Histopathology Laboratory, the Advanced Molecular Pathology Laboratory for subsequent processing and paraffin embedding.

Paraffin-embedded mammary tissues were sectioned at 5µm. After rehydrating the sections, peroxidase activity was blocked with 3% hydrogen peroxide diluted in 100% methanol. The sections were then incubated with (i) biotinylated anti-Gr1 antibody (clone RB6-8C5) (e-Biosciences) diluted 1:100 overnight at 4°C, or (ii) biotinylated anti-Ly6G antibody (clone 1A8) (BioLegend) diluted 1:200 for two hours at room temperature. Visualization of binding was carried out using VECTASTAIN® Elite• ABC Kit (Vector Laboratories) and DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine (Vector Laboratories). The slides were then counterstained with Richard-Allan Scientific™ Signature Series Hematoxylin 2 (Thermo Fisher Scientific) and differentiated with 0.1% hydrochloric acid. After rinsing with distilled water, the slides were air-dried and mounted with distyrene plasticizer xylene (DPX) (Merck).

Images were viewed and captured using Carl Zeiss Axioscan.Z1 microscope and ZEN Blue Software with constant exposure and gain. Objective Plan-Apochromat 40x/0.95 Corr M27 lens was used for acquisition of images. Scanned images were analyzed using the ZEN Blue Software. Neutrophils were identified based on the positive brown immunohistochemical (IHC) stain. For Gr-1-stained cells, an additional criterion of a discernible banded or segmented nucleus stained blue by hematoxylin was included for greater specificity. For the mammary tissue, to maximize consistency across the different samples, an area of 20mm^2 around and excluding the lymph node was demarcated using the ZEN Blue software, and the number of neutrophils within the demarcated area was counted. Neutrophils in blood vessels were excluded from the counting. For the mammary lymph node, the number of neutrophils within the whole lymph node was counted and normalized against the area of the lymph node.

Histological analysis of the bone marrow

Femurs and tibias were harvested from mice and soft tissue attachments were removed. The bone marrow was flushed out from each bone by cutting the extreme tip of each end and forcing PBS through the bone using needle and syringe. The cell suspension was centrifuged at 500g for 10 minutes at room temperature, followed by removal of supernatant and resuspension of cell pellet.

Bone marrow smears

For each bone marrow smear, a drop (2μL) of the resultant cell suspension was placed on a glass slide. A smear was achieved by touching the drop of cell suspension with a cover slip, ensuring that the drop spreads evenly across the length of the cover slip, before pulling the cover slip across the surface of the glass slide. Each smear is approximately two-thirds the length of the slide. After air-drying, the bone marrow smears were fixed in 100% methanol for 10 minutes before staining.

Giemsa stain

The Giemsa working solution is prepared freshly by diluting stock Giemsa stain, modified solution (Sigma Aldrich) 1:20 in PBS pH 6.5. 1 mL of Giemsa working solution was used to stain each bone marrow smear. After staining for 45 minutes, each slide was rinsed with 2 mL of distilled water, before they were air-dried and mounted with DPX. Images were viewed and captured using Olympus Bx61 Microscope with Olympus UPlanFI 40x/0.75 lens, QImaging Retiga Exi Fast1394 Camera and Image Pro Plus 7.0 software at room temperature.

Tissue RNA isolation and reverse transcription

For isolation of total RNA, mammary gland samples were lysed in TRIzol reagent (Life Technologies). Reverse transcription was carried out using either random primers (Promega) and SuperScript II ™ reverse transcriptase (Life

Technologies) or qScript™ cDNA SuperMix (Quanta Biosciences) according to manufacturer's instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was carried out using SYBR Green master mix (KAPA Biosystems) on Quantstudio 6 Flex RT-PCR System (Applied Biosystems). RT-qPCR for each target gene was performed in duplicates. Acidic ribosomal phosphoprotein P0 (36B4) primers were included in each experiment to normalize the amount of complementary deoxyribonucleic acid (cDNA) used. For qualitative analysis, the comparative threshold cycle (C_t) method was used, by normalizing to C_t value of 36B4 in the same sample. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). The data is expressed as relative fold changes in arbitrary values. Primers used are as listed below:

Table 1. List of real-time PCR primers used and their nucleotide sequences.

Gene	Forward (5' → 3')	Reverse (5' → 3')
36B4	GATCGGGTACCCAACTGTTGCC	CAGGGGCAGCAGCCGCAAATGC
AREG	CACAGCGAGGATGACAAGGA	GAGGATGATGGCAGAGACAAAGA
BAX	CATCTTTGTGGCTGGAGTCC	AAAATGCCTTTCCCCTTCCC
BCL2	CGTCAACAGGGAGATGTCAC	GGGCCATATAGTTCCACAAAGG
COX-2	CCAGCACTTCACCCATCAGTT	ACCCAGGTCCTCGCTTATGA
CTS B	TCCTTGATCCTTCTTTCTTGCC	ACAGTGCCACACAGCTTCTTC
CXCL1	ATCCAGAGCTTGAAGGTGTTG	GTCTGTCTTCTTTCTCCGTTACTT
CXCL2	CCAACCACCAGGCTACAG	GCGTCACACTCAAGCTCTG
CXCR2	ATGCCCTCTATTCTGCCAGAT	GTGCTCCGGTTGTATAAGATGAC
MMP3	TGGCCATCTCTTCCATCCAA	CCCAGAACTGATTTCCTTTAAAAATG
PR	CGCCATCTACCAGCCGCTC	TGAATCCGGCCTCAGGTAGTT
S100A8	CGAAAACTTGTTCAGAGAATTGGA	ACTTTTATCACCATCGCAAGGAA
S100A9	GTTGATCTTTGCCTGTCATGAG	AGCCATTCCCTTTAGACTTGG
TIMP1	GATATGCCCACAAGTCCCAGAACC	GCACACCCCACAGCCAGCACTAT

Statistics

Data for RT-qPCR and histological counts were expressed as Mean ± SEM. For studies on the effect of estrogen alone on the involuting mammary gland,

statistical significance of the data was determined by unpaired two-tailed Student's t-test using Microsoft Excel. Equal variance was assumed for all data sets for all statistical tests. For studies on the effect of estrogen and CXCR2 antagonist, SB225002, on the involuting mammary gland, statistical significance of the data was determined by one-way ANOVA least significant difference (LSD) post hoc test using SPSS. For all data sets, difference was considered significant if p value < 0.05. Outliers were determined by Grubbs test using GraphPad Software and removed from analysis.

RESULTS

Estrogen treatment promotes neutrophil infiltration into the mammary gland

Using flow cytometry, our laboratory had previously shown that 48 hours of E2B treatment starting from InvD1 resulted in a significant increase in the amount of neutrophils in the involuting mammary gland and an observable, albeit not statistically significant, increase in neutrophil percentage in the blood (Figure 6A). To ascertain that the increased mammary gland neutrophils observed through fluorescence-activated cell sorting (FACS) is due to actual neutrophil infiltration into the mammary tissue and not just neutrophils in the interspersing blood vessels, immunohistochemical (IHC) staining using biotinylated Gr-1 antibody was carried out for paraffin-embedded sections of the mammary glands harvested at InvD3. As the Gr-1 antibody has been reported to recognize both Ly6C and Ly6G markers, and may therefore stain for both monocytes and neutrophils (Lee, et al., 2013), to confirm that the positively-stained cells were neutrophils, an additional criterion of a discernible banded or segmented nuclear morphology that is characteristic of neutrophils was included (Figure 6B).

For a more detailed analysis of the neutrophil infiltration pattern, the number of Gr-1$^+$ neutrophils in 20mm^2 of mammary tissue near the lymph node was counted. In concordance with the FACS data, there was indeed a significant 4-fold increase (p = 0.00505) in neutrophils observed in mammary tissue from the E2B-treated group compared to controls. Furthermore, analysis of neutrophil infiltration by tissue compartment revealed a significant increase in the E2B-treated group by approximately 4.6 fold (p = 0.0309) within the alveolar and ductal lumen and 3.5 fold (p = 0.00028) in the interstitial space respectively (Figure 6C). The number of alveolar and ductal lumen with neutrophil infiltration was also

significantly increased in the E2B-treated group as compared to the control group by about 4 fold (p = 0.000851) (Figure 6D).

Interestingly, Gr-1$^+$ cells with discernible banded or segmented nuclei were also observed in the mammary lymph node. The lymph node can be divided into several tissue compartments, which can be differentiated through the structure and staining patterns. The darker staining regions at the rim of the lymph node are the cortical (follicular) regions, whilst the lighter staining regions just adjacent to the cortical regions are the paracortical regions. The most faintly stained region in the middle of the lymph interspersed with empty spaces is the medullary region. Amongst these three areas, neutrophils accumulated primarily in the medullary region, with less intensive infiltration in the cortical and paracortical regions (Figure 7A). The total number of neutrophils within the mammary lymph node was significantly increased by about 2.4 fold (p = 0.0186) in the E2B-treated group as compared to the control group (Figure 7B).

Estrogen-stimulated neutrophil influx is not associated with increased neutrophil production or lower rate of neutrophil death

Our previous data had shown increased gene expression of neutrophil chemoattractants (i.e. CXCL1, CXCL2, S100A8, S100A9) in involuting mammary glands from the E2B-treated group suggesting that the increased neutrophil sequestration in mammary tissue of the E2B-treated group is likely due to enhanced chemotaxis of neutrophils. However, it is also conceivable that the increased neutrophil accumulation in mammary tissues from the E2B-treated group could be attributed to an increased production of neutrophils in the bone marrow and/or reduced death of neutrophils within the mammary tissue.

The percentage of neutrophils in the bone marrow was determined by counting the number of neutrophils identified in Giemsa-stained bone marrow

smears. A set of criteria was used to identify and categorize neutrophils into three broad groups at various maturation stages based on their morphologies. All neutrophils identified had a clearing in the centre of each of their nucleus. Immature myelocytes and metamyelocytes (MM) were identified as cells with purple or pale pink cytoplasm, accompanied by a nuclear clearing with a diameter is less than 50% that of the nucleus. The more differentiated banded neutrophils (BN) were identified as cells with pale pink cytoplasm and a nuclear clearing with a diameter that is at least 50% that of the nucleus. Last but not least, mature neutrophils (MN) were identified as cells with pale pink cytoplasm, and whose nuclei are twisted, curled, or lobular with the presence of constrictions and/or thin linking chromatin threads (Zhou, et al., 2015) (Figure 8A). Estrogen treatment did not significantly affect the total percentage of cells of the neutrophilic lineage in the bone marrow, or the proportion of each neutrophil subtype (Figure 8B, C).

To determine if the rate of neutrophil death was affected by E2B treatment, a flow cytometry analysis of cells dispersed from whole involuting mammary gland was carried out (Figure 9A). Cell debris were first excluded based on their light scatter properties on a dot plot with forward scatter (area) (FSC-A) on the X-axis and side scatter (area) (SSC-A) on the Y-axis. Single cells were then gated for on a dot plot with forward scatter (width) (FSC-W) on the X-axis and forward scatter (height) (FSC-H) on the Y-axis by excluding doublets. These single cells were displayed on a dot plot with APC/Cy7 CD45 on the x-axis and SSC-A on the Y-axis. Leukocytes were gated for as cells with positive signals for CD45, the common leukocyte antigen. Neutrophils were selected for amongst the population of leukocytes by plotting PE Ly6G on the X-axis against BV605 CD11b on the Y-axis. CD11b is an antigen expressed on many leukocytes including neutrophils while Ly6G is a neutrophil-specific marker. Therefore, the population of cells with high signal for both PE Ly6G and BV605 CD11b were identified as neutrophils. To discriminate between viable and dead neutrophils, the population of neutrophils

were visualized on a dot plot with signals from fixable viability dye eFluor® 450 (dead stain) on the X-axis and PE Ly6G on the Y-axis. Higher dead stain signals indicate greater membrane permeability and are therefore likely to represent dead cells. As expected, the percentage of $CD11b^+$ $Ly6G^+$ neutrophils among $CD45^+$ cells increased (p = 0.0561) in the E2B-treated group (Figure 9B). However, the percentages of $Ly6G^+$ dead $stain^-$ and $Ly6G^+$ dead $stain^+$ cells were not significantly different between the control and E2B-treated groups (Figure 9C).

To confirm that there was no significant change in the rate of neutrophil death, expression of known apoptosis-related genes (i.e. Bax and Bcl2) in neutrophils isolated from the involuting mammary glands using biotinylated anti-Ly6G antibody coupled with Dynabeads® Streptavidin was examined. Instead of looking at the individual expression of each apoptosis-related gene, comparing the Bax-to-Bcl2 (Bax/Bcl2) ratio between control and treatment group provides a greater indication of neutrophil death (van der Vliet, et al., 1997). Interestingly, while there was no significant difference in the gene expression of Bcl2, expression of Bax and thereby the ratio of Bax/Bcl2 showed a decreasing, although not significant, trend with E2B treatment (Figure 9D, E).

Estrogen-induced neutrophil infiltration is mediated by CXCR2

As mentioned above, various types of neutrophil chemoattractants (CXCL1, CXCL2, S100A8, S100A9) were shown to be upregulated in the involuting mammary gland with E2B treatment and each subtype of neutrophil chemoattractant could have a different role to play in facilitating the increased neutrophil influx. Since a CXCR2 antagonist was identified as an ideal candidate for unraveling the role of the CXC chemokines, gene expression of the CXCR2 was first validated by RT-qPCR. Analyses of total mammary glands from mice subjected to 24 hours and 48 hours of E2B treatment both revealed an increasing, albeit insignificant, trend in CXCR2 expression (Figure 10A). CXCR2 expression has also been seen in other leukocytes,

such as monocytes, macrophages, basophils and T-lymphocytes, as well as on endothelial cells (Campbell, et al., 2013). Thus, expression of CXCR2 in neutrophils was also examined. Neutrophils were isolated from involuting mammary glands of mice treated with 48 hours of E2B or vehicle control using biotinylated anti-Ly6G antibody coupled with Dynabeads® Streptavidin. Indeed, isolated neutrophils from estrogen-stimulated glands showed a significant increase (p = 0.0472) in CXCR2 expression by about 6 fold compared to controls (Figure 10B).

To examine the involvement of CXCL1/CXCL2/CXCR2 in estrogen-induced neutrophil infiltration, involuting mice were treated with E2B, SB225002 (a CXCR2 antagonist) and/or their corresponding controls (hereafter known as Ctrl and DMSO respectively) for 48 hours starting from InvD1. SB225002 has been shown to be a specific and effective CXCR2 antagonist that impairs neutrophil chemotaxis in mice (Bento, et al., 2008; White, et al., 1998). To determine the efficacy of SB225002, the percentage of neutrophils in mammary glands harvested at InvD3 was determined using FACS analysis (Figure 11A). In this set of experiment, FACS analysis revealed that E2B+DMSO treatment resulted in an increasing, albeit insignificant, trend of neutrophil influx in the mammary gland as compared to Ctrl+DMSO. This effect of estrogen on neutrophil infiltration was abolished in the presence of SB225002, as neutrophil recruitment in involuting glands in E2B+SB225002 group was severely hampered as compared to the Ctrl+SB225002 group (p = 0.0373). The reduction of neutrophil percentage in the E2B+SB225002 group was also significant when compared to the E2B+DMSO group (p = 0.0159). In contrast, Ctrl+SB225002 did not result in significant changes in neutrophil percentages as compared to Ctrl+DMSO (Figure 11B).

The results from FACS analysis were corroborated by histological analysis of neutrophil infiltration in the mammary tissue. E2B+DMSO treatment significantly increased neutrophil influx in both alveolar and ductal lumen as well as interstitial

space as compared to Ctrl+DMSO and Ctrl+SB225002 treatment. However, E2B+SB225002 treatment significantly abolished the E2B-stimulated increase in neutrophil numbers, particularly in the alveolar and ductal lumen (Figure 11C).

CXCR2 antagonist, SB225002, effectively abolishes estrogen-stimulated changes in inflammatory and tissue remodeling genes

In our previous study, we looked at the effects of estrogen stimulation and Ly6G depletion on a panel of genes, including those involved in cell proliferation, inflammation and tissue remodeling in the mammary gland. To understand the role of neutrophils in mediating these changes, we examined a similar panel of genes in involuting mammary glands from mice treated with estrogen in the presence and absence of SB225002. RT-qPCR analysis revealed that the well-established estrogen target genes, AREG and PR, as well as one of the driving factors for lysosomal-mediated mammary epithelial cell death during involution, CTS B, were significantly upregulated under treatment of E2B. Estrogen-induced expression of these genes were not significantly affected by SB225002 treatment (Figure 12). Moreover, neutrophil chemoattractants CXCL1, CXCL2, S100A8 and S100A9, as well as the cognate receptor for CXCL1 and CXCL2, CXCR2, showed significant or close to significant upregulation in the E2B+DMSO group as compared to the Ctrl+DMSO or Ctrl+SB225002 groups. In the E2B+SB225002 group, expression of these genes decreased to levels similar to that of the Ctrl+DMSO and Ctrl+SB225002 groups, although these changes were not significant (Figure 12). For COX2, which is involved in both inflammation and adipogenesis, treatment of SB225002 significantly prevented the E2B-induced upregulation of COX2 (Figure 12). Interestingly, the expression of two other tissue remodeling genes, MMP3 and TIMP1, showed no significant changes across all four groups, but the decrease in MMP3/TIMP1 ratio in the E2B+DMSO treatment group was close to significant in comparison with the Ctrl+DMSO and Ctrl+SB225002 treatment groups (Figure 12).

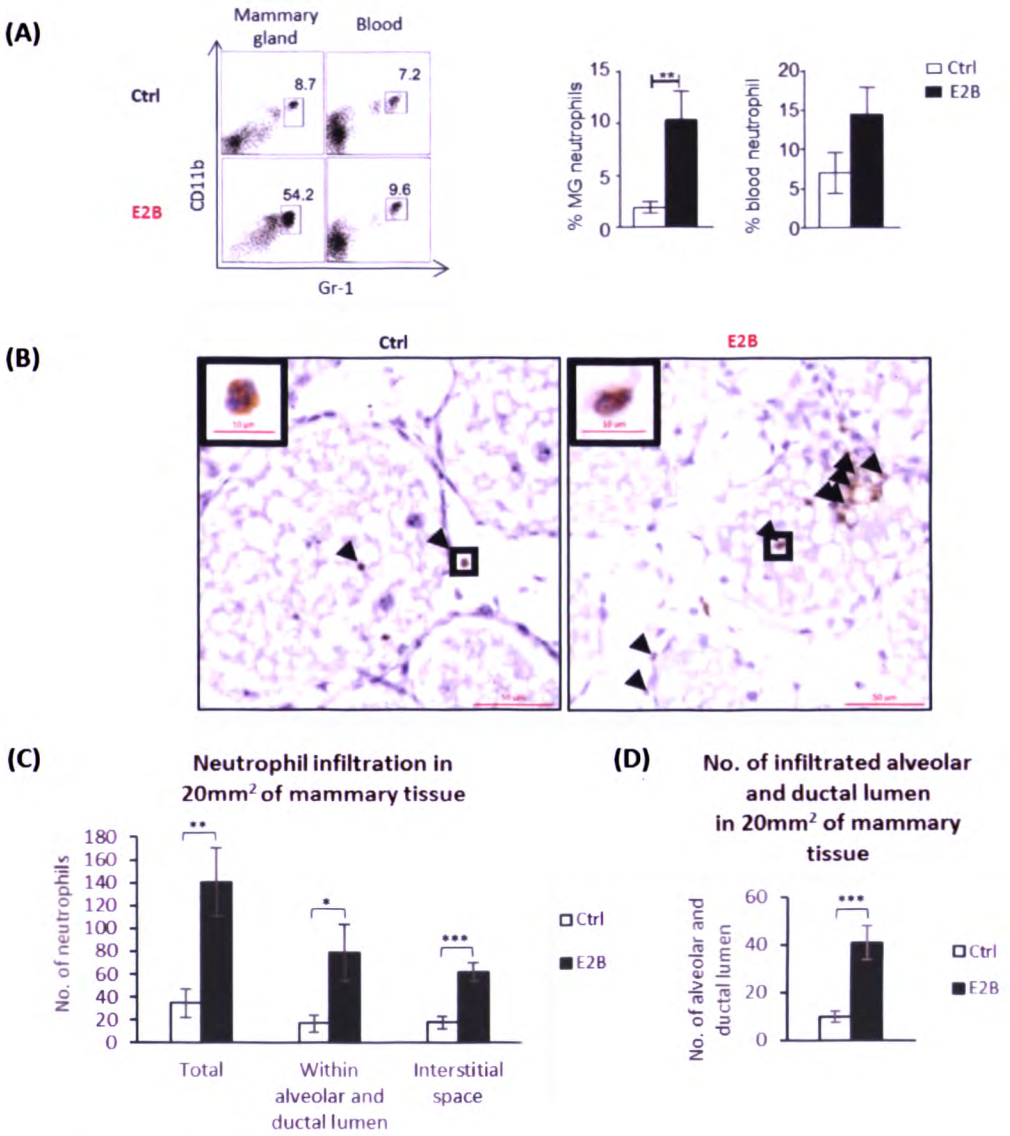

Figure 6. Estrogen significantly increases neutrophil infiltration into the mammary tissue. Involuting mammary glands and peripheral blood were collected after 48 hours of vehicle control (Ctrl) or estrogen benzoate (E2B) treatment at InvD1. Single cell suspension of mammary cells and blood samples were stained with various cell surface markers. Immunohistochemical staining with biotinylated Gr-1 antibody was also performed on mammary tissue sections. **(A)** Estrogen induced significant neutrophil infiltration into the involuting mammary gland (Credits to Or Yu Zuan). Representative dot plots and bar graphs of neutrophils ($CD11b^+$ $Gr\text{-}1^{hi}$) amongst leukocytes in mammary glands and peripheral blood. Values in the dot plot represent percentages of neutrophils. For neutrophils in the involuting mammary gland, Ctrl n = 13, E2B n = 12. For neutrophils in the peripheral blood, Ctrl n= 10, E2B n = 9. **(B)** Representative images of neutrophil infiltration into mammary tissue in Ctrl (left) and E2B (right) groups. Arrow heads represent neutrophils and scale bar represents 50µm and 10µm (inset). **(C, D)** Bar graphs showing a significant increase in average number of neutrophils in both alveolar and ductal lumen and interstitial space **(C)** and average number of infiltrated alveolar and ductal lumen **(D)** in $20mm^2$ of mammary tissue in E2B (n = 9) as compared to Ctrl (n = 9) groups. Results are represented as Mean ± SEM. Statistical significance was evaluated by unpaired 2-tailed Student's t-test; $*p<0.05$, $**p<0.01$, $***p<0.001$.

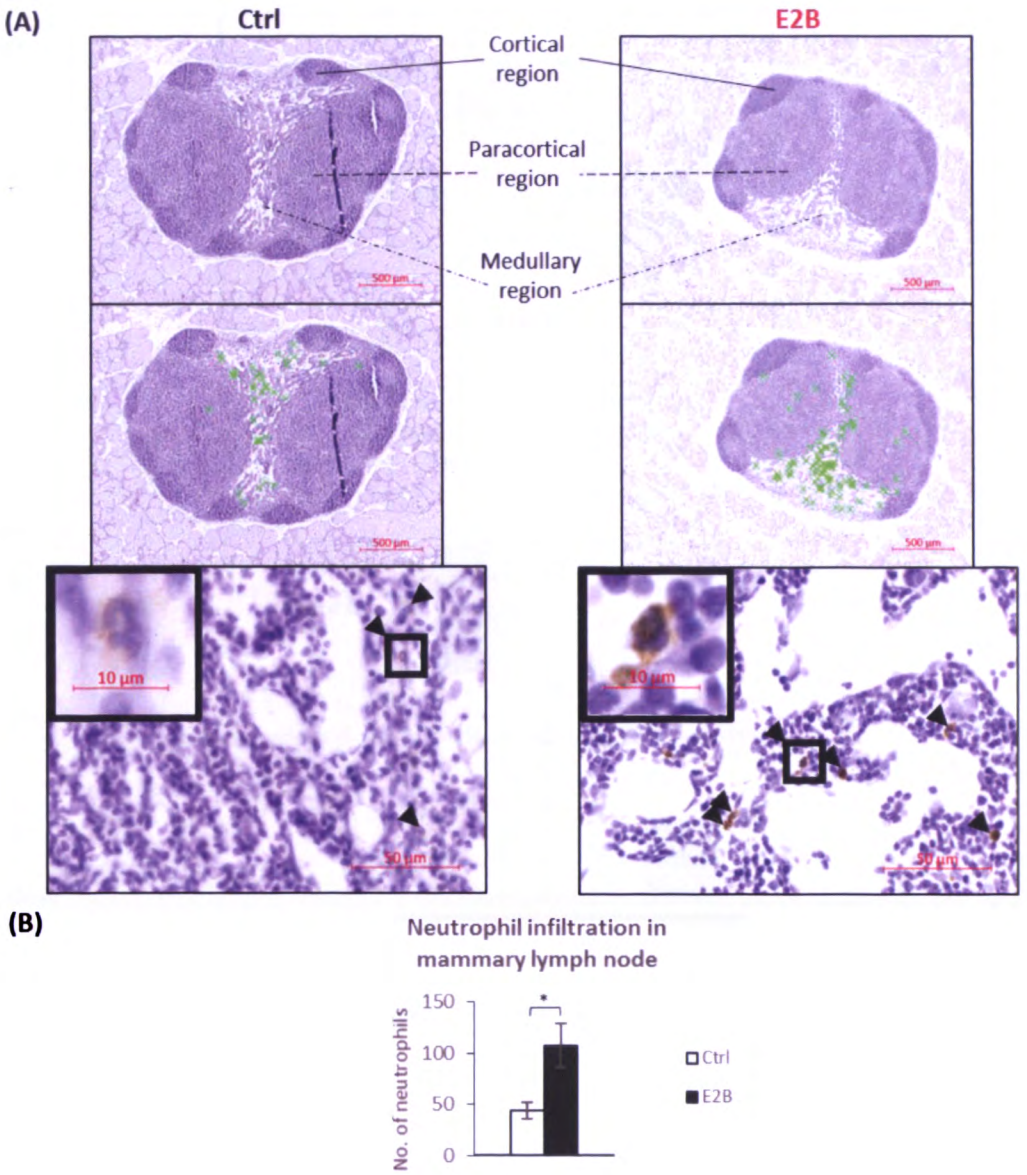

Figure 7. Estrogen significantly increases neutrophil infiltration into the mammary lymph node. Involuting mammary glands were collected after 48 hours of vehicle control (Ctrl) or estrogen benzoate (E2B) treatment at InvD1. Immunohistochemical staining with biotinylated Gr-1 antibody was performed on mammary tissue sections. **(A)** Representative images of neutrophil infiltration into mammary lymph node in Ctrl (left) and E2B (right) groups. Wide view of mammary lymph node (top) with green crosses marking neutrophils to show localization of neutrophils within the lymph node (middle); scale bar represents 500µm. Magnified image of neutrophils within the mammary lymph node (bottom) where arrowheads represent neutrophils and scale bar represents 50µm and 10µm (inset). **(B)** Bar graph showing a significant increase in average number of neutrophils within the mammary lymph node from E2B (n = 9) as compared to Ctrl (n = 9) groups. Number of neutrophils for each sample were normalized against the area of the mammary lymph node. Results are represented as Mean ± SEM. Statistical significance was evaluated by unpaired 2-tailed Student's t-test; *p<0.05.

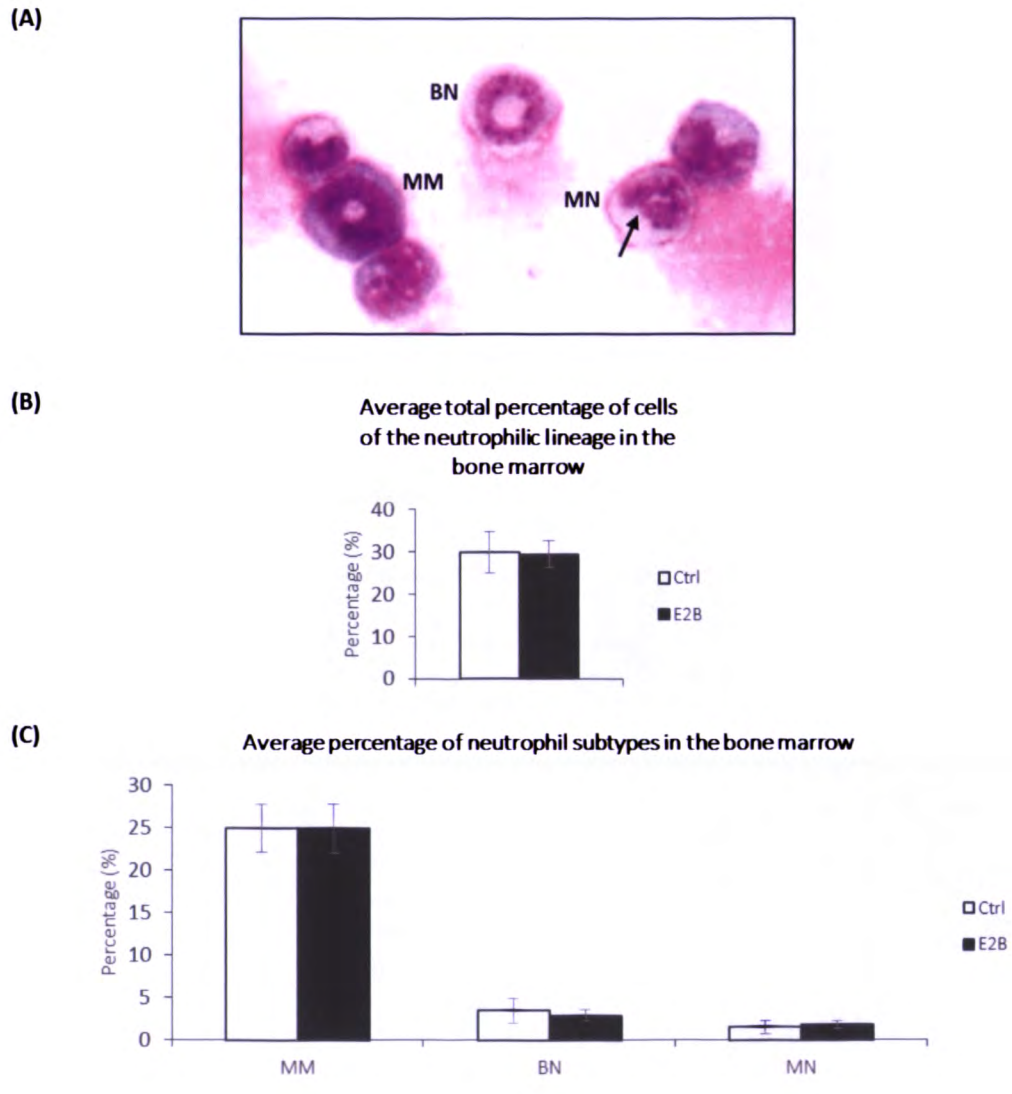

Figure 8. Estrogen does not significantly increase neutrophil percentage in the bone marrow. Bone marrows from femurs and tibias of involuting mice were collected at InvD3 after 48 hours of vehicle control (Ctrl) or estradiol benzoate (E2B) treatment. The cell suspension was centrifuged and the supernatant was removed before the pellet was resuspended. 2µl of each final cell suspension was used to make a smear, which was then stained with Giemsa. **(A)** Representative image of each neutrophil subtype in a bone marrow smear. MM = myelocytes and metamyelocytes; BN = banded neutrophils; MN = mature neutrophils. Arrow represents constriction in mature neutrophil. **(B)** Bar graph showing average total percentage of cells of the neutrophilic lineage in the bone marrow. Estrogen treatment did not significantly affect percentage of total neutrophils. Ctrl (white bar) n = 4, E2B (black bar) n = 4. **(C)** Bar graph showing average percentages of neutrophil subtypes in the bone marrow. MM = myelocytes and metamyelocytes; BN = banded neutrophils; MN = mature neutrophils. Ctrl (white bar) n = 4, E2B (black bar) n = 4. Results are represented as Mean ± SEM. Statistical significance was evaluated by unpaired 2-tailed Student's t-test.

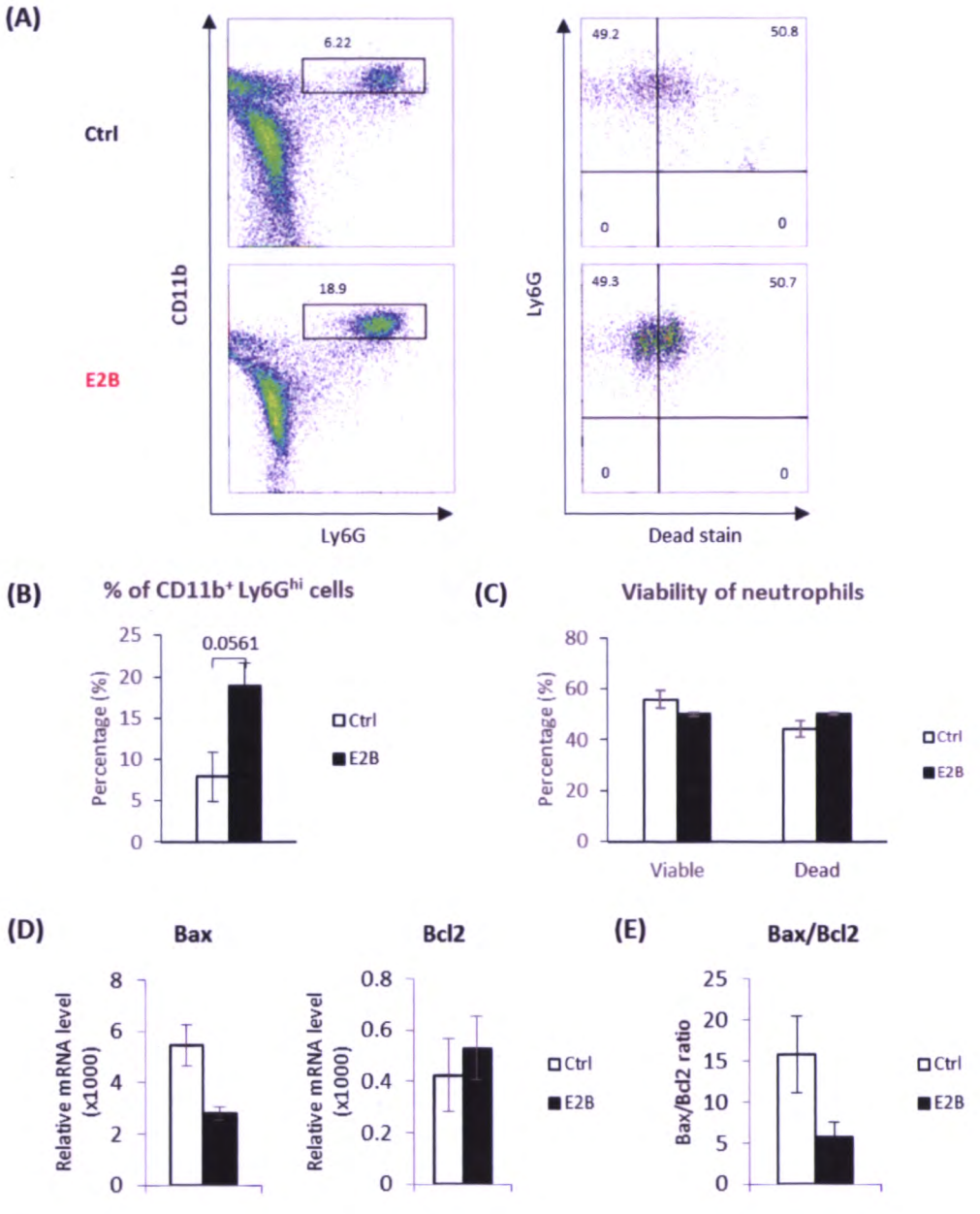

Figure 9. Estrogen does not significantly affect rate of neutrophil death. Involuting mammary glands were collected on InvD3 after 48 hours of treatment with vehicle control (Ctrl) or estradiol benzoate (E2B) starting from InvD1. **(A)** Representative dot plots of neutrophil ($CD11b^+$ $Ly6G^+$) percentage amongst leukocytes and neutrophil viability. **(B, C)** Bar graphs showing increase in average percentage of neutrophils ($CD45^+$ $CD11b^+$ $Ly6G^+$) **(B)** and no significant difference in percentage of viable or dead neutrophils **(C)** in the Ctrl (n = 3) and E2B (n = 3) groups. **(D)** Bar graphs showing mRNA expression of apoptosis-related genes, Bax and Bcl2, in neutrophils isolated from involuting mammary glands in Ctrl (n = 3) and E2B (n = 2) groups. **(E)** Bar graph showing Bax/Bcl2 ratio in neutrophils isolated from involuting mammary glands in Ctrl (n = 3) and E2B (n = 2) groups. Estrogen treatment did not result in significant decrease of Bax/Bcl2 ratio. Results are represented as Mean ± SEM. Statistical significance was evaluated by unpaired 2-tailed Student's t-test.

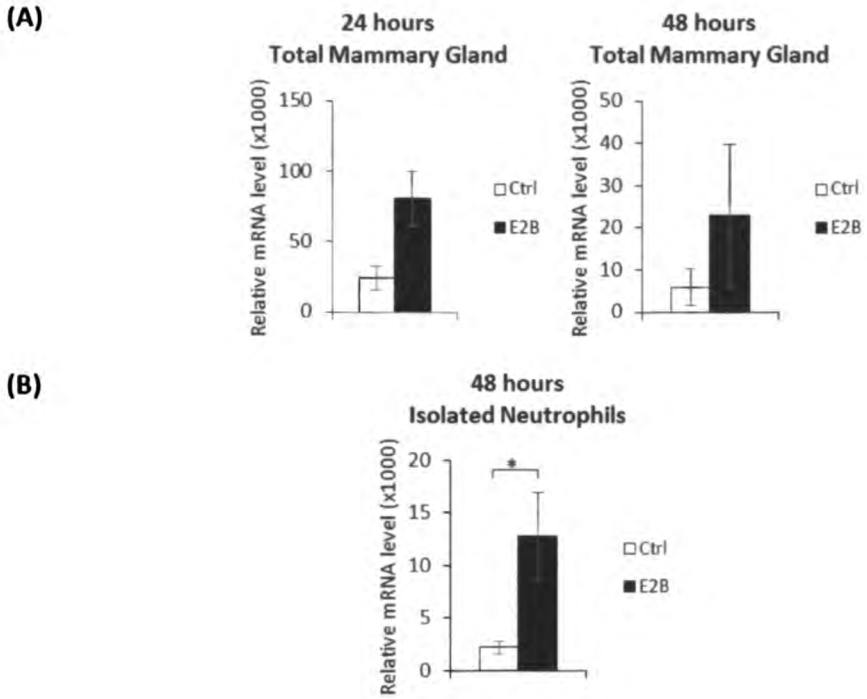

Figure 10. Estrogen enhances CXCR2 expression in involuting mammary glands. Involuting mammary glands were collected on InvD3 after 24 or 48 hours of E2B treatment. Real-time PCR analysis of CXCR2 expression was conducted and normalized against housekeeping gene, 36B4. **(A)** Bar graphs showing increasing, but not significant, trend of CXCR2 mRNA expression in total mammary glands from mice after 24 hours (left) and 48 hours (right) of E2B treatment. For the 24 hours treatment set, Ctrl n = 2, E2B n = 3. For the 48 hours treatment set, Ctrl n = 7, E2B n = 7. **(B)** Bar graph showing significant increase in CXCR2 mRNA expression in neutrophils isolated from involuting mammary glands of mice treated with 48 hours of E2B (n = 2) as compared to Ctrl (n = 3). Neutrophils were isolated using biotinylated anti-Ly6G antibody coupled with Dynabeads® Streptavidin. Results are represented as Mean ± SEM. Statistical significance was evaluated by unpaired two-tailed Student's t-test; *p<0.05.

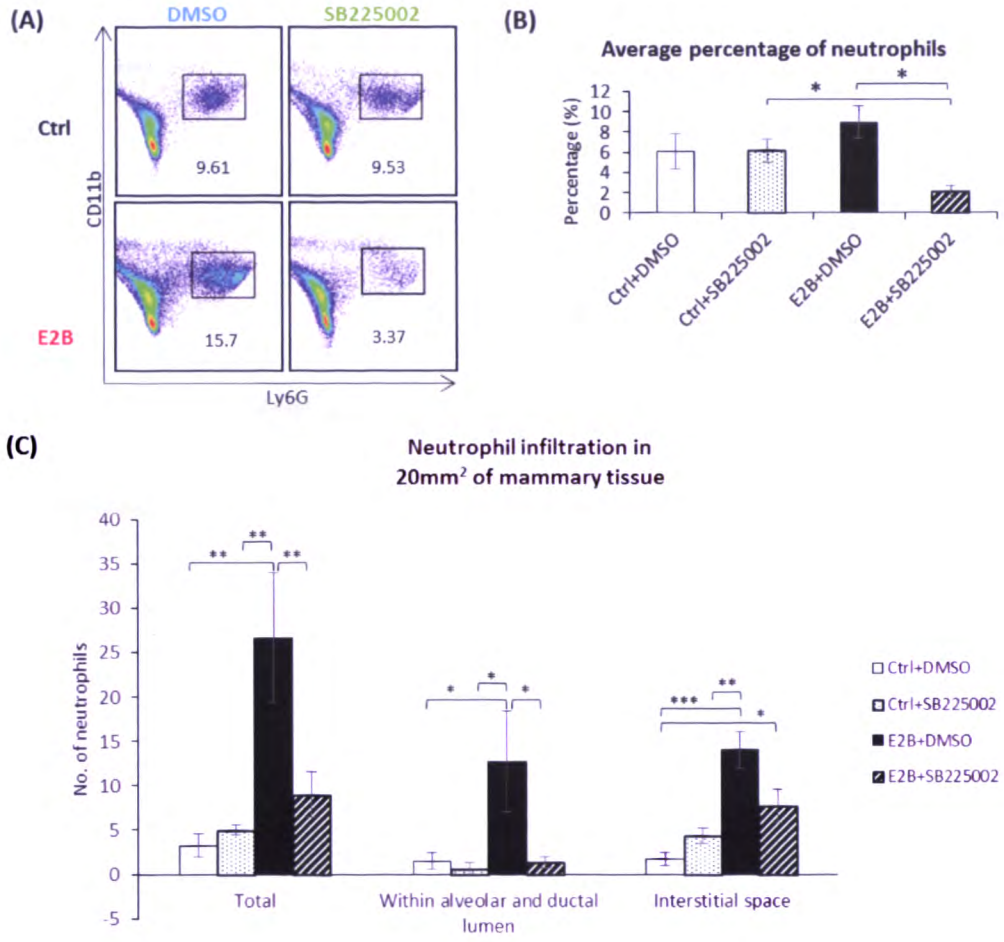

Figure 11. CXCR2 is required for E2B-mediated neutrophil infiltration into involuting mammary glands. Involuting mammary glands were collected on InvD3 after 48 hours of treatment with E2B, CXCR2 antagonist SB225002, and/or their corresponding controls (Ctrl and DMSO respectively). Single cell suspensions of mammary gland digests were stained with various cell surface markers. Mammary gland sections were stained with biotinylated Ly6G antibody. **(A)** Representative dot plots of neutrophil ($CD11b^+$ $Ly6G^+$) percentage amongst leukocytes. **(B)** Bar graph showing average percentage of neutrophils in involuting mammary glands from mice treated with E2B and/or SB225002 and their respective controls. E2B+DMSO (black bar, n = 3) increased, albeit not significantly, neutrophil percentage as compared to Ctrl+DMSO (white bar, n = 4). E2B+SB225002 treatment (striped bar, n = 4) significantly reduced neutrophil infiltration as compared to E2B+DMSO and Ctrl+SB225002.Ctrl+SB225002 treatment (stippled bar, n = 3) did not significantly affect neutrophil percentage as compared to Ctrl+DMSO. **(C)** Bar graph showing number of infiltrated neutrophils in $20mm^2$ of mammary tissue. E2B+DMSO treatment (black bar, n = 3) significantly increased neutrophil infiltration as compared to Ctrl+DMSO (white bar, n = 4) and Ctrl+SB225002 (stippled bar, n = 3) treatments. E2B+SB225002 treatment (striped bar, n = 3) significantly reduced total neutrophil infiltration, particularly in alveolar and ductal lumen. Results are represented as Mean ± SEM. Statistical significance was evaluated by one-way ANOVA LSD post hoc test; *p<0.05, **p<0.01, ***p<0.001

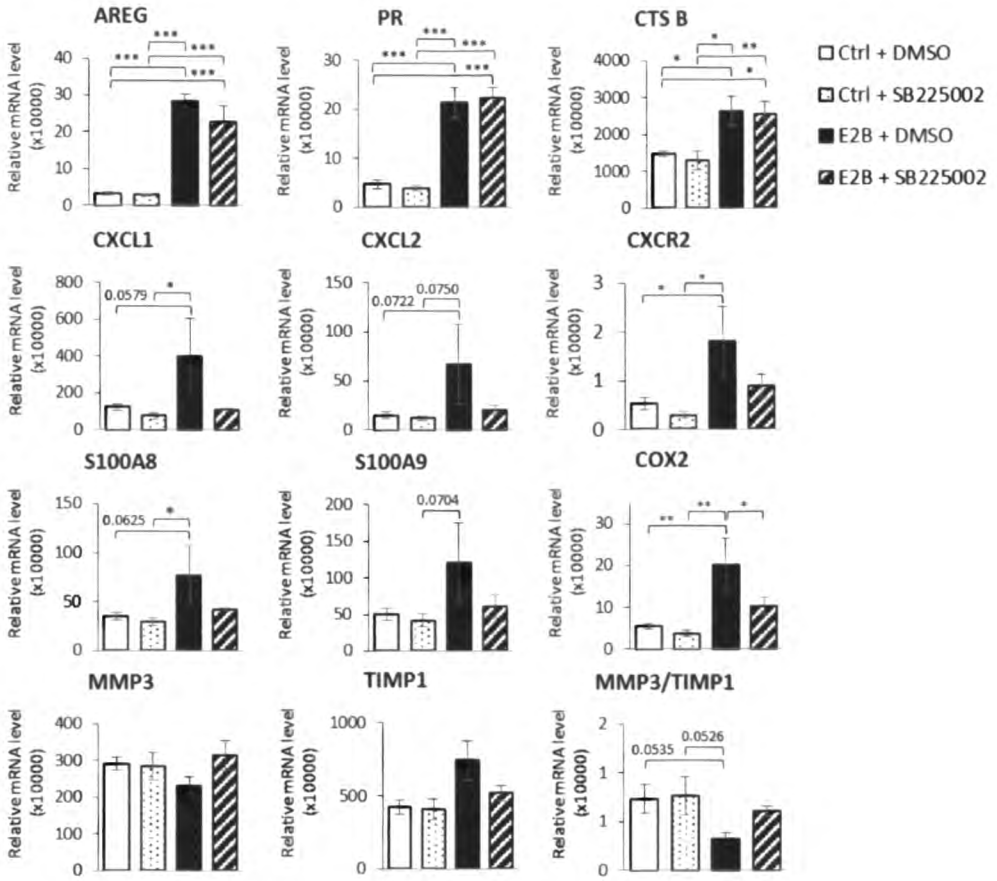

Figure 12. SB225002 treatment abolished estrogen-stimulated upregulation of inflammatory genes and downregulation of MMP3/TIMP1 ratio. Involuting mammary glands were collected on InvD3 after 48 hours of treatment with E2B, CXCR2 antagonist SB225002, and/or their corresponding controls (Ctrl and DMSO respectively). Real-time PCR analysis was conducted for a panel of genes and normalized against housekeeping gene, 36B4. Estrogen target genes, AREG and PR, as well as one of the driving factors for lysosomal-mediated mammary epithelial cell death, CTS B, showed significant upregulation with E2B treatment regardless of SB225002 treatment. In comparison to the Ctrl+DMSO and Ctrl+SB225002 groups, genes involved in neutrophil chemotaxis, CXCL1, CXCL2, CXCR2, S100A8 and S100A9 showed significant or close to significant upregulation in the E2B+DMSO group, and a decreasing, albeit not significant, trend in the E2B+SB225002 group. COX2 was significantly upregulated in the E2B+DMSO group as compared to all three other treatment groups. Individual expression of MMP3 and TIMP1 was did not show significant changes, while the decrease in MMP3/TIMP1 ratio in the E2B+DMSO treatment group was close to significant compared to the Ctrl+DMSO and Ctrl+SB225002 groups. Ctrl+DMSO (white bar) n = 4, Ctrl+SB225002 (stippled bar) n = 3, E2B+DMSO (black bar) n = 3, E2B+SB225002 (striped bar) n = 3. Results are represented as Mean ± SEM. Statistical significance was evaluated by one-way ANOVA LSD post hoc test; *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Estrogen significantly increases neutrophil infiltration and thus neutrophil function within the mammary gland

The effect of estrogen on various physiological states have been controversial. The results presented in this study unveils a new dimension of estrogenic action and its downstream inflammatory response mediated by neutrophils during mammary gland involution. The effect of estrogen on neutrophils appears to be pro-inflammatory, as can be seen through the significant increase in neutrophil influx within the mammary gland under stimulus of estrogen. However, this amplification of inflammation appears to occur only locally within the mammary gland, as the bone marrow did not reflect a significant depletion of neutrophil reserves, which is a hallmark of systemic inflammation (Christensen, et al., 1982).

Within the mammary gland, the pattern of neutrophil infiltration is illuminating regarding the function of neutrophils and a possible mechanism through which estrogen may promote involution. The localization of neutrophils within the alveolar and ductal lumens which contain milk and shed cells supports the idea that neutrophils are involved in the clearance of milk proteins and shed apoptotic cells (Atabai, et al., 2007; Sadik, et al., 2011). At the same time, the effect of estrogen on neutrophil infiltration appears to be twofold, as both the frequency (number of infiltrated alveoli and ducts) and intensity (number of neutrophils per alveolus or duct) of neutrophil infiltration were increased in the E2B-treated group. It is therefore plausible to suggest that estrogen promotes involution by stimulating more extensive neutrophil infiltration to augment their function in the clearance of

alveolar and ductal lumens and thereby accelerate the return of the mammary gland to the pre-pregnant state.

While neutrophils are conventionally thought to undergo apoptosis within peripheral tissues after completing their function, more recent studies have shown that neutrophils can also perform reverse transmigration back to the bone marrow, or travel to secondary lymphoid organs to interact with the adaptive immune system (Maletto, et al., 2006; Powell & Huttenlocher, 2016). In fact, Brackett and colleagues (2013) have also identified a IL-17, and subsequently, CXCR2, -dependent mechanism for the delivery of neutrophils into tumor-draining lymph nodes. In this study, the observation of neutrophils within the mammary lymph node **implies that neutrophils in the mammary gland do drain towards the secondary lymphoid organ. It also suggests that effects of estrogen on neutrophils in the involuting mammary gland could have longer-lasting effects due to their ability to further influence cells of the adaptive immune system, thereby highlighting the importance of studying the role of neutrophils in the context of estrogen-stimulated involution. Specifically, the localization of neutrophils within the cortical, paracortical and medullary regions suggests that neutrophils could have a role in activating and/or suppressing both B and T cells in the mammary lymph node, as these regions are rich in B and T cells (Abbas, et al., 2014).**

Estrogen does not significantly affect cell death of neutrophils

The function of neutrophil extends beyond phagocytosis. Apoptotic neutrophils are known to interact with the immune system by acting as anti-inflammatory signals to promote the resolution of inflammation (Fox, et al., 2010). Delays in apoptosis in neutrophils and hence resolution of inflammation can be a cause for concern as inflammation provides a conducive environment for neoplastic progression (de Visser, et al., 2006; Hanahan & Weinburg, 2011). Lifespan of neutrophils can be altered by changes in hormonal exposure. For

example, elevation in levels of female sex hormones during pregnancy has been found to delay cell death of neutrophils (Molloy, et al., 2003). In this study however, both gene expression analyses of Bax/Bcl2 ratio in isolated neutrophils and FACS analyses of neutrophil viability between control and E2B-treated groups did not reveals statistically significant differences. This suggests that estrogen does not significantly affect cell death of neutrophils and that the increased accumulation of neutrophils within the estrogen-treated involuting mammary gland is unlikely to be due to a longer lifespan of neutrophils.

CXCR2 is involved in estrogen-induced neutrophil infiltration

CXCR2 inhibition through small molecule inhibitors has been investigated in numerous *in vivo* cancer models in preclinical studies, including breast cancer and colorectal cancer xenografts (Campbell, et al., 2013). Specifically, the small molecule CXCR2 antagonist SB225002 has been successfully used *in vivo* to significantly reduce neutrophil influx and tissue damage in acute experimental colitis in mice (Bento, et al., 2008). This highlights the potential for SB225002 in attenuating the estrogen-stimulated inflammation in the involuting mammary gland. Indeed, gene expression of CXCR2 showed an increasing, albeit not significant, trend in total mammary tissue with both 24 hours and 48 hours of E2B treatment, and this can be attributed, at least in part, to the mammary neutrophils which demonstrated a significant upregulation of CXCR2 expression. This result supports the idea that SB225002 may be able to inhibit estrogen-induced neutrophil infiltration and inflammation that has been observed thus far in our studies.

This idea was confirmed in the next part of our study, in which a significant decrease in neutrophil infiltration was observed through both FACS and histological analyses in the E2B+SB225002 group as compared to the E2B+DMSO group. The reduction was especially apparent in the number of neutrophils within alveolar and

ductal lumen. This not only indicates that SB225002 is effective at this dosage, but also validates the role of CXCR2 ligands in the recruitment of neutrophils into the involuting mammary gland, especially in mediating neutrophils' crossing into the alveolar and ductal lumen, as inhibition of CXCR2 resulted in increased accumulation of neutrophils in the interstitial space. Remarkably, this difference was not significant between the Ctrl+SB225002 group and the Ctrl+DMSO group. This observation, coupled with a significant decrease in neutrophil percentage in the E2B+SB225002 group as compared to the Ctrl+SB225002 group, seems to suggest that the estrogen-induced neutrophil infiltration into the involuting mammary gland is predominantly and uniquely dependent on CXCR2. Thus far, studies have shown that IL-8 (human homologue of mouse CXCL1/KC, CXCL2/MIP and CXCL5-6/LIX) signaling can enhance tumor progression by activating signaling pathways to promote proliferation, angiogenesis, migration, invasion and cell survival, and even regulate breast cancer stem cell activity (Campbell, et al., 2013; Singh, et al., 2013; Sparmann & Bar-Sagi, 2004). CXCR2 expression has also been found on the surface of tumor cells and tumor-associated stromal cells of several cancers (Campbell, et al., 2013). It is therefore plausible to suggest that the upregulation of CXCR2 expression under stimulus of estrogen during involution can help to foster a tumorigenic environment which may contribute to the development of PABC.

The significant upregulation of AREG, PR and CTS B with E2B treatment regardless of SB225002 treatment indicates that estrogen regulation of cell growth (AREG and PR) and death (CTS B) in the mammary gland are not under the influence of mammary neutrophils. In contrast, the similar pattern between the average percentage of neutrophils in the mammary gland and expression of inflammatory genes (CXCL1, CXCL2, CXCR2, S100A8, S100A9 and COX2 – positive correlation) and tissue remodeling genes (COX2 – positive correlation; MMP3/TIMP1 ratio – negative correlation) suggests that the expression of these genes are contributed

predominantly by the recruited neutrophils. Interestingly, although the average percentage of neutrophils was significantly lower in the E2B+SB225002 treatment group than the E2B+DMSO treatment group, this significant difference was not reflected in the expression of these inflammatory genes. It is thus possible that estrogen not only induces the influx of more neutrophils, but also regulates the activity of neutrophils by modulating the expression of these inflammatory genes.

The neutrophil-dependent, estrogen-induced changes in gene expression supports our previous hypothesis that E2B promotes involution through increasing neutrophil function. The upregulation of inflammatory genes contributes to a highly inflammatory profile. In addition, upregulation of COX2 and downregulation of MMP3/TIMP1 result in increased collagen fibrillogenesis and adipogenesis. All of these are contributing factors of carcinogenesis and tumor progression (Alexander, et al., 2001; Lyons, et al., 2011; van Kruijsdijk, et al., 2009). The ability of SB225002 to impair these estrogen-induced effects thus not only proves that the effect of estrogen on neutrophils and their function in the involuting mammary gland is mediated by CXCR2, but also that CXCR2 inhibition may be a potent way to prevent PABC.

Proposed mechanism of estrogen-induced neutrophil infiltration into involuting mammary gland

Neutrophils are able to express both ERα and ERβ and these ER subtypes have been implicated in mediating estrogen-induced changes of neutrophil functions including chemotaxis as well as expression of nitric oxide synthases and proinflammatory mediators (Molero, et al., 2002; Nuedling, et al., 2001; Xing, et al., 2007). As such, it is highly plausible to suggest that estrogen acts directly on neutrophils to stimulate increased neutrophil infiltration in the involuting mammary gland.

We have previously shown that estrogen increases expression of CXCL1 and CXCL2 in neutrophils isolated from the involuting mammary gland. In this study, we followed up by showing that estrogen increased expression of CXCR2 in neutrophils isolated from the involuting mammary gland, and that inhibition of CXCR2 impaired expression of proinflammatory molecules in the involuting mammary gland. Thus, we propose the following mechanism for estrogen-induced neutrophil infiltration into the involuting mammary gland (**Figure 13**):

Estrogen increases expression of CXCR2, CXCL1 and CXCL2 by both resident neutrophils that are normally within the mammary gland and circulating neutrophils in the blood. The production of CXCL1 and CXCL2 may be supplemented by other cell types such as adipocytes and mammary epithelial cells to set up a high concentration of these neutrophil chemoattractants in the involuting mammary gland (Kerr, et al., 2006; Tourniaire, et al., 2013). The resultant steep gradient of CXCL1 and CXCL2 between blood (low) and involuting mammary gland (high) facilitates chemotaxis and hence recruitment of the circulating neutrophils into the involuting mammary gland. Within the involuting mammary gland, resident and recruited neutrophils with CXCL1 or CXCL2 bound to CXCR2 are then stimulated to produce more proinflammatory molecules, including CXCL1, CXCL2, S100A8, S100A9 and COX2. Neutrophil chemoattractants CXCL1, CXCL2, S100A8 and S100A9 may also be direct targets of estrogen, as they contain estrogen response elements and have been verified as estrogen target genes (Bourdeau, et al., 2004; Tang, et al., 2004). The production of these neutrophil chemoattractants can in turn aid in the recruitment of even more neutrophils into the involuting mammary gland.

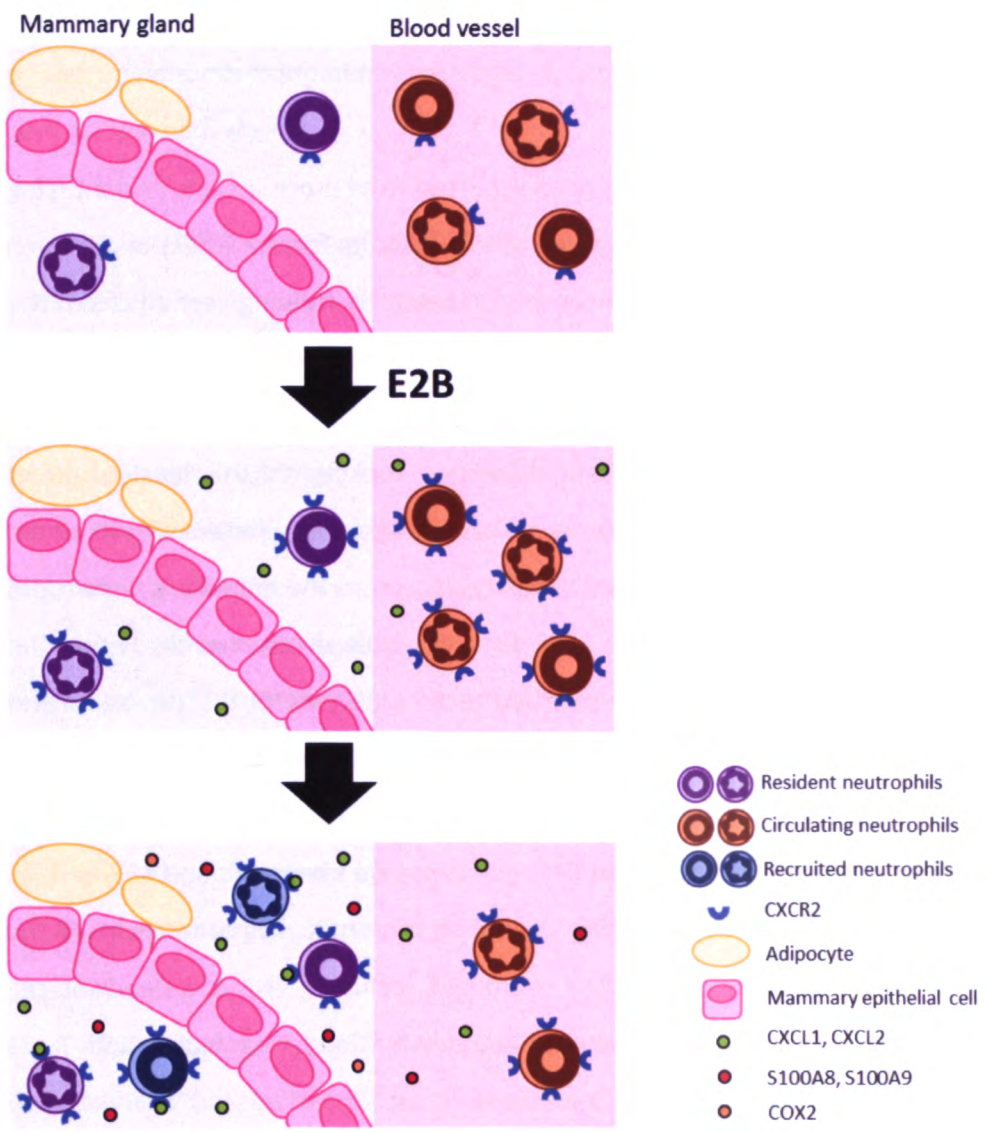

Figure 13. Proposed mechanism of estrogen-induced neutrophil infiltration into involuting mammary gland. Upon treatment with E2B, neutrophils increase expression of CXCR2 on their cell surface and produce more neutrophil chemoattractants CXCL1 and CXCL2. Within the involuting mammary gland, other cell types such as mammary epithelial cells and adipocytes may also be stimulated to produce more CXCL1 and CXCL2 alongside resident neutrophils. This sets up a high concentration of CXCL1 and CXCL2 within the mammary gland that drives the infiltration of circulating neutrophils into the mammary gland. Recruited neutrophils and resident neutrophils in turn secrete more proinflammatory molecules, including CXCL1, CXCL2, S100A8, S100A9 and COX2 within the involuting mammary gland.

FUTURE WORK

Since the Ly6G antibody has been reported to be more specific in identifying neutrophils than the Gr-1 antibody, current IHC results for the effect of estrogen on the involuting mammary gland can be validated by staining sections from the same samples with a biotinylated Ly6G antibody instead.

While IHC has proven to be useful in looking at the localization of neutrophils, there could be sample variation arising from different depth of the sections, especially in the mammary lymph node, where the medullary regions are only revealed at a greater depth. Moreover, the role of neutrophils within the mammary lymph node are only speculative based on Gr-1 IHC. This can be further explored by looking at the gene expression profile of the mammary lymph node. Genes of interest include B-cell stimulating factors such as BAFF, APRIL, CD40L, IL-21 and NETs, as well as T-cell stimulating factors like chemerin, IL-17, and IL-23 (Cerutti, et al., 2013; Nathan, 2006). Signaling molecules for other cells of the adaptive immune system can also be examined, including TNF-α, a dendritic cell stimulating factor, as well as IFN-γ, a monocyte stimulating factor and T-cell differentiation stimulating factor (Brackett, et al., 2013).

Analyses on SB225002 require more experimental replicates to fortify statistical power. The observed changes in gene expression in the presence of SB225002 can be validated through histological means, for example, by studying the differences in adipocyte repopulation and the number of shed, apoptotic cells within the alveolar and ductal lumens. Last but not least, to affirm that CXCR2-mediated neutrophil infiltration is unique to the context of estrogen-treated involuting mice and that CXCR2 inhibition is capable of abolishing the tumorigenic

microenvironment induced by estrogen, it would be necessary to follow up with parallel studies of CXCR2 and the effect of SB225002 on adipogenesis and cell death, as well as tumor development in estrogen-treated nulliparous mice and the tumor context respectively.

Since PABC tumors tend to be ER-negative, it is likely that surrounding ER-positive cells in the mammary glands are responsible for promoting tumorigenesis with estrogen exposure during mammary involution. It is known that the effect of estrogen can be tissue- and receptor-specific (Yu, et al., 2006), but it is yet unknown which specific ER subtype(s) are responsible for the development of PABC during mammary involution. Even for neutrophils, which have been found to express both ERα and ERβ, the effect of estrogen on each specific ER subtype and their respective influence on neutrophil function have been controversial thus far (Du, 2002). The expression of ER subtypes on various tissues during mammary involution can be revealed through histological analyses, whilst their respective roles in contributing to PABC can be elucidated by studying the effect of various ER subtype agonists such as ERα agonist propyl pyrazole triol (PPT) and ERβ agonist diarylpropionitrile (DPN) on mammary involution.

Progesterone is another ovarian hormone involved in normal mammary development that has been increasingly recognized to have a role in breast cancer (Brisken, 2013). Exposure to exogenous progesterone can occur through combination type (estrogen plus progesterone) hormone replacement therapy and oral contraceptives, and the addition of progesterone has been associated with an increased risk of breast cancer as compared to estrogen-only alternatives (Pike, et al., 1983; Ross, et al., 2000). However, other studies on estrogen/progesterone crosstalk have yielded controversial results under different contexts. For example, while progesterone has been found to synergize estrogen-induced neuroprotection, estrogen signaling in ER-positive breast tumors and estrogen-induced impairment

of neutrophil chemotaxis was antagonized by progesterone (Miyagi, et al., 1992; Nilsen & Brinton, 2002; Singhal, et al., 2016). Since the effect of progesterone on estrogen-induced effects can vary across different contexts, it would be interesting to study the effects of progesterone or combination treatment of estrogen and progesterone on the involuting mammary gland to understand if they work synergistically or antagonistically in the context of mammary involution and PABC.

CONCLUSION

The modern trend of delayed childbirth is associated with increased incidence of PABC, and this has in part been attributed to the increased estrogen exposure through delaying childbirth to a later age and increasing use of contraceptives containing estrogen. The poor prognosis and survival rates of women with PABC highlights the importance and urgency of uncovering the factors and mechanisms that contribute to the development of PABC. In particular, since estrogen has long been known to be a breast cancer risk factor, it would be interesting and illuminating to elucidate the specific effect of estrogen exposure on increasing breast cancer risk during mammary gland involution.

In this study, it was shown that E2B treatment during mammary involution resulted in a significant increase in neutrophil infiltration within the mammary tissue. This appears to be a localized effect within the involuting mammary gland, as the percentage of neutrophils in blood (previous observation) and bone marrow (current study) were not significantly affected by estrogen exposure. Conceivably, the increased accumulation of neutrophils in the involuting mammary gland upon estrogen exposure could be due to decreased apoptosis of neutrophils, and/or increased chemotaxis of neutrophils. However, estrogen did not result in statistically significant changes in Bax/Bcl2 ratio in neutrophils within the mammary gland, indicating that a decrease in apoptosis of neutrophils was unlikely to be a possible mechanism. In contrast, it was confirmed through the significant upregulation of CXCR2 expression with E2B treatment and the CXCR2 antagonist study that increased neutrophil infiltration into the involuting mammary gland upon E2B treatment is in part dependent on CXCR2.

Within the involuting mammary gland, the significant or close to significant upregulation of genes responsible for cell growth and death (AREG, PR, CTS B) and inflammatory genes (CXCL1, CXCL2, CXCR2, S100A8, S100A9, COX2), as well as downregulation of MMP3/TIMP1 ratio, suggests that estrogen accelerates involution by promoting inflammation and tissue remodeling. The concurrent reduction in neutrophil influx and abolishment of estrogen-induced upregulation in inflammatory genes and downregulation of MMP3/TIMP1 ratio with SB225002 treatment in turn indicates that the estrogen-induced recruitment of more neutrophils and increased adipocyte repopulation is mediated by neutrophils. The involvement of neutrophils in roles related to breast cancer risk factors such as inflammation and adipogenesis further highlights the importance of having a better understanding about the mechanisms and impacts of the estrogen-induced increased neutrophil infiltration during mammary involution.

While the current study still requires more experimental replicates and more in-depth studies about the effect of SB225002 in a tumor model, the results presented in this work highlight a potential CXCR2-dependent mechanism for increased neutrophil sequestration in the involuting mammary gland that is specific to the context of estrogen exposure. The unveiling of a mechanism that is truly unique to the context of estrogen-stimulated involution could pave the way for novel therapeutics for PABC, by targeting estrogen-stimulated pro-tumorigenic neutrophil responses in the involuting mammary gland without affecting the normal function of neutrophils in fighting off infections in other tissues.

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