Development 137, 4229-4238 (2010) doi:10.1242/dev.059261

Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium

Angela C. L. Chua, Leigh J. Hodson, Lachlan M. Moldenhauer, Sarah A. Robertson and Wendy V. Ingman*

SUMMARY

Each ovarian cycle, the mammary gland epithelium rotates through a sequence of hormonally regulated cell proliferation, differentiation and apoptosis. These studies investigate the role of macrophages in this cellular turnover. Macrophage populations and their spatial distribution were found to fluctuate across the cycle. The number of macrophages was highest at diestrus, and the greatest number of macrophages in direct contact with epithelial cells occurred at proestrus. The physiological necessity of macrophages in mammary gland morphogenesis during the estrous cycle was demonstrated in *Cd11b-Dtr* transgenic mice. Ovariectomised mice were treated with estradiol and progesterone to stimulate alveolar development, and with the progesterone receptor antagonist mifepristone to induce regression of the newly formed alveolar buds. Macrophage depletion during alveolar development resulted in a reduction in both ductal epithelial cell proliferation and the number of alveolar buds. Macrophage depletion during alveolar regression resulted in an increased number of branch points and an accumulation of TUNEL-positive cells. These studies show that macrophages have two roles in the cellular turnover of epithelial cells in the cycling mammary gland; following ovulation, they promote the development of alveolar buds in preparation for possible pregnancy, and they remodel the tissue back to its basic architecture in preparation for a new estrous cycle.

KEY WORDS: Mammary gland, Macrophage, Ovarian cycle, Morphogenesis, Mouse

INTRODUCTION

The mammary gland is a unique organ that undergoes the majority of its development postnatally (Hovey et al., 2002; Richert et al., 2000). During puberty, highly proliferative terminal end buds appear in response to increased circulating ovarian hormones, and elongation and branching of ductal epithelium occur. When the ductal tree has fully penetrated the mammary fat pad, these terminal end buds are replaced by terminal end ducts, with relatively low mitotic activity. With the onset of pregnancy, the mammary epithelium undergoes extensive proliferation and differentiation with the formation of alveolar buds that develop into milk-secreting elements by the end of pregnancy, to allow lactation to be initiated after parturition. Upon weaning, the mammary gland undergoes involution, which involves widespread apoptosis of the mammary epithelial cells and remodelling that returns the mammary gland back to the pre-pregnant state, ready to initiate new development with the onset of another pregnancy.

Compared with the extensive development that occurs during puberty and pregnancy, the non-pregnant adult mammary gland is relatively quiescent, leading this stage to be commonly described in the literature as a ‘resting state’. However, studies in humans and rodents show this to be a misnomer, with specific morphological and cellular changes documented in the mammary gland over the course of the ovarian cycle (Fata et al., 2001; Navarrete et al., 2005; Schedin et al., 2000; Strange et al., 2007). In mice, active secondary branching and alveolar budding occur throughout the estrous cycle (Fata et al., 2001), with the greatest number of alveolar buds observed in diestrus.

A number of studies have documented that macrophages are associated with mammary gland morphogenesis during puberty, pregnancy, lactation and involution (Gouon Evans et al., 2000; O’Brien et al., 2010; Pollard and Hennighausen, 1994; Van Nguyen and Pollard, 2002; Walker et al., 1989). In the mouse, macrophages localise mainly around the neck of the terminal end buds during puberty, and apoptotic bodies within the cytoplasm of some of these macrophages suggest that these cells might promote ductal extension by the clearance of apoptotic cells (Gouon Evans et al., 2000; Van Nguyen and Pollard, 2002). Macrophages are also observed in the interstitial space between alveolar buds throughout pregnancy and lactation (Pollard and Hennighausen, 1994). Although a precise role for macrophages in involution has yet to be established, there is a dramatic increase in macrophage density at this stage (O’Brien et al., 2010; Stein et al., 2004; Walker et al., 1989).

The colony-stimulating factor 1 (*Csf1*)-null mutant mouse model has been used extensively to study the effects of macrophage deficiency on the different stages of postnatal mammary gland development. CSF1 is a major regulator of macrophage survival, proliferation, differentiation and recruitment (Sapi and Kacinski, 1999; Stanley et al., 1997) and *Csf1*-null mice exhibit severe macrophage deficiency in many tissues (Pollard, 1997). The *Csf1*-null mutation also causes a range of reproductive defects, including delayed puberty and extended estrous cycles (Cecchini et al., 1994; Cohen et al., 1997; Pollard and Hennighausen, 1994; Sapi and Kacinski, 1999). During puberty, *Csf1*-null mice exhibit an atrophic mammary gland ductal system, with a reduced number of terminal end buds and reduced ductal length and branching (Gouon Evans et al., 2000). These phenotypes can be rescued with mammary

The Robinson Institute, Research Centre for Reproductive Health, and School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide 5005, Australia.

*Author for correspondence (wendy.ingman@adelaide.edu.au)

Accepted 15 October 2010

gland-specific expression of CSF1, emphasising the direct role of local macrophage populations in promoting pubertal mammary gland development (Van Nguyen and Pollard, 2002). Multi-photon imaging to observe three-dimensional collagen networks has implicated macrophages in the assembly of collagen into its fibrillous form (Ingman et al., 2006b). Macrophages are also involved in mammary gland development during pregnancy (Pollard and Hennighausen, 1994). Pregnant *Csf1*-null mice display reduced secondary and tertiary branching, with premature alveolar growth and milk protein expression; postpartum, the mammary gland does not switch to lactation and there is an accumulation of milk proteins within the alveolar cells that is neither secreted nor cleared from the ducts.

Clearly, macrophages are necessary for mammary gland development during puberty, pregnancy and lactation, and exert effects on a diverse array of epithelial cell processes including proliferation, differentiation, phagocytosis and tissue remodelling. As these same processes also occur in the non-pregnant adult mammary gland, we investigated the possibility that macrophages promote epithelial cell turnover during the ovarian cycle.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004). Mice were maintained in pathogen-free conditions with controlled light (12 hours light/12 hours dark cycle) and temperature at the University of Adelaide Medical School.

C57Bl/6 mice

Adult virgin female C57Bl/6 mice (9-10 weeks of age) were obtained from Laboratory Animal Services, University of Adelaide.

Cd11b-Dtr mice

Cd11b-Dtr mice on an inbred FVB background carry a transgene encoding the monkey diphtheria toxin receptor (Dtr) driven by the macrophage specific *Cd11b* (*Itgam* – Mouse Genome Informatics) promoter (Duffield et al., 2005a). This transgenic model exploits the difference in binding affinity of the monkey and mouse diphtheria toxin receptors. Monkey diphtheria toxin receptor binds diphtheria toxin with high affinity and causes cell death, whereas cells expressing the mouse form of the receptor are resistant. Administration of diphtheria toxin to *Cd11b-Dtr* mice causes acute and specific depletion of macrophages. Depletion lasts 48 hours, with longer depletion achieved by further administration of diphtheria toxin (Duffield et al., 2005b). Administration of the same dose of diphtheria toxin to non-transgenic FVB control mice has no effect on macrophage abundance and there are no toxic side effects of the treatment.

Cfms-GFP mice

Cfms-GFP mice carry a transgene encoding green fluorescent protein driven by the macrophage-specific *Cfms* (*Csf1r* – Mouse Genome Informatics) promoter (Sasmono et al., 2003). These mice are on a C57Bl/6 background.

Mammary gland dissection

Stages of the estrous cycle were determined by cytological evaluation of vaginal smears performed daily at 0930 h as previously described (Ingman et al., 2006a; Snell, 1956). The mice (aged 10-13 weeks) were tracked for at least 1 week through at least one normal 4- to 5-day estrous cycle. Mice were euthanised at each of the four stages of the estrous cycle (estrus, metestrus, diestrus and proestrus). At the time of euthanasia, 1 ml of blood was collected by cardiac puncture and the fourth abdominal pair of mammary glands was dissected. Mammary glands were enzymatically digested into single-cell suspensions for flow cytometry, fixed and processed in paraffin or OCT for immunohistochemistry, or whole-mounted for carmine alum staining. All samples were coded for subsequent blind analysis.

Acute macrophage depletion

To investigate the direct effect of macrophages on early alveolar development in the mammary gland, the *Cd11b-Dtr* macrophage depletion transgenic mouse was utilised. However, macrophages are important regulators of ovarian function (Wu et al., 2004) and our recent studies have shown that macrophage depletion leads to reduced ovarian secretion of progesterone (A. S. Care, W.V.I., M. J. Jasper and S.A.R., unpublished). To eliminate the confounding influence of indirect effects of macrophage depletion on the ovary, a hormone replacement model that mimics the cellular turnover in the mammary gland that occurs across the ovarian cycle was utilised (L.J.H., A.C.L.C., S.A.R. and W.V.I., unpublished). Female adult virgin mice (aged 12-14 weeks) were ovariectomised and allowed to recover for 1 week. To mimic the early alveolar development that occurs during the metestrus/diestrus phase of the cycle, ovariectomised mice were injected daily for 3 days with estradiol (1 μg in sesame oil) and progesterone (1 mg in sesame oil), administered subcutaneously. To mimic the epithelial apoptosis and remodelling that occur at the proestrus stage of the cycle, ovariectomised mice were stimulated by a 3-day treatment with exogenous estradiol and progesterone as above, and then on the fourth day mice were injected subcutaneously with the progesterone receptor antagonist mifepristone (200 μg in 100 μl sesame oil).

To investigate the physiological role of macrophages in the development of early alveolar buds, *Cd11b-Dtr* ovariectomised mice were stimulated with estradiol and progesterone for 3 days. On days 1 and 3, one group of mice was also given an intraperitoneal injection of diphtheria toxin (100 ng/10 g body weight), whereas the control group was given PBS. Mice were sacrificed on day 4, 1 hour after intraperitoneal administration of 100 μl of 10 mg/ml BrdU (Sigma, St Louis, MO, USA). The fourth abdominal mammary glands were dissected and either fixed and processed as whole-mounts or paraffin-embedded for immunohistochemistry.

To investigate the role of macrophages in the regression and remodelling of the alveolar epithelium, *Cd11b-Dtr* ovariectomised mice were stimulated with estradiol and progesterone for 3 days. On the fourth day, all mice were treated with mifepristone and a single injection of diphtheria toxin (100 ng/10 g body weight) or PBS as control. Forty-eight hours following mifepristone treatment, mice were sacrificed and the fourth abdominal mammary glands were dissected and either fixed and processed as whole-mounts or paraffin-embedded for immunohistochemistry.

Serum progesterone and estradiol analysis

Approximately 1 ml of blood was collected by cardiac puncture under deep Avertin anaesthesia. The blood was incubated at 4°C overnight, and then centrifuged at 4000 rpm (1500 ***g***) for 4 minutes to recover serum. Serum samples were stored at –80°C until serum progesterone and estradiol concentrations were measured using commercial radioimmunoassay kits DSL-4800 and DSL-3400, respectively, according to the instructions of the manufacturer (Diagnostic Systems Laboratories, Webster, TX, USA). All samples were measured in a single assay. The within-assay coefficients of variation were 10.0% in the estradiol assay and 7.9% in the progesterone assay. The lower limits of detection were 5 pg/ml estradiol and 0.3 ng/ml progesterone.

Flow cytometry analysis

To determine the abundance and phenotype of macrophages, single-cell suspensions of mammary gland tissue were analysed by flow cytometry. The lymph nodes were removed from the fourth abdominal mammary glands and the gland was minced finely and digested in 20 ml PBS containing 10% heat-inactivated fetal calf serum (Sigma), 20 U/ml collagenase (Roche Diagnostics, Mannheim, Germany), 0.04 g/ml dispase II (Roche Diagnostics) and 25 μg/ml DNase I (Sigma) for 60 minutes at 37°C. The digested tissue was filtered through a 70-μm nylon cell strainer to produce a single-cell suspension.

Cell suspensions of digested mammary gland tissue were incubated for 30 minutes at 4°C with phycoerythrin (PE)-conjugated macrophage-specific F4/80 antibody (1:50 dilution; BD Biosciences, San Diego, CA, USA). CountBright Absolute Count Beads (Molecular Probes, Carlsbad,

CA, USA) were added to the cell suspension (51,000 beads per 50 µl sample). The number of F4/80-positive cells was calculated by reference to the count beads according to the manufacturer's instructions.

To investigate the effect of macrophage depletion on the abundance of immune cells in the mammary gland, mammary gland cells were incubated with PE-conjugated anti-F4/80 (EMR1 – Mouse Genome Informatics), anti-CD3 (BD Biosciences) and RB6 (which detects LY6G/C) (BD Biosciences) antibodies for flow cytometry using FACS Canto and FACS Diva software (BD Biosciences).

Immunohistochemistry

Mammary glands were prepared as whole-mounts on a glass slide and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Glands were removed from the slide and embedded in paraffin. For macrophage localisation studies, sections (7 µm) were mounted on glass slides and blocked with 15% normal mouse serum and 15% normal rabbit serum for 30 minutes before immunostaining. The sections were immunostained with 1:50 anti-F4/80 rat monoclonal antibody (Caltag Laboratories, Burlingame, CA, USA) overnight at 4°C or with isotype-matched irrelevant antibody as a negative control, followed by 1:100 biotinylated rabbit anti-rat IgG (Vector Laboratories) for 40 minutes at room temperature, and detection by the ABC Elite Kit (Vector Laboratories) with DAB peroxidase (Sigma).

Images of the stained sections were captured using a Hamamatsu Protonics Nanozoomer and blinded analysis was performed. Epithelium was categorised as ductal or alveolar. The classification system was similar to that of Fata et al. (Fata et al., 2001), whereby ductal epithelium was defined as a single epithelial cell layer (analogous to Grade 0), and alveolar epithelium was defined as clusters of epithelial structures containing alveolar lumens (analogous to Grade 3). Five ducts and five clusters of alveolar buds in each section were randomly chosen from within the area between the lymph node and the distal end of the gland (n=6-9 mice per cycle stage). For both ductal and alveolar epithelia, the number of F4/80-positive cells found in direct contact with the epithelium was manually counted. Only F4/80-positive cells with visible Hematoxylin-stained nuclei were included, and the assessment was made by an assessor blinded to the estrous cycle stage. Secondly, the number of macrophages located within a 150-µm zone around the epithelia was counted using the same inclusion criteria. Five random fields of connective tissue from each section were also selected and the number of F4/80-positive cells counted. Results were expressed as F4/80-positive cells per mm^2. The mean density of F4/80-positive cells around the five ductal, five alveolar and five connective tissue fields for each mouse was calculated and grouped according to the estrous cycle stage.

To localise smooth muscle actin, 5-µm paraffin sections were blocked with 2% bovine serum albumin (Sigma) for 30 minutes at room temperature before immunostaining with 1:30 alkaline phosphatase-conjugated monoclonal antibody to alpha smooth muscle actin (Sigma) for 1 hour at room temperate. Slides were washed in PBS before incubating with Fast Red TR/Naphthol AS-MX phosphate (Sigma) for 10 minutes and counterstained with Hematoxylin solution (Sigma).

Proliferating cells were identified by BrdU immunostaining of paraffin-embedded mammary gland tissue. Sections were stained for BrdU incorporation into DNA using a BrdU In-Situ Detection Kit (BD Pharmingen) according to the manufacturer's instructions. Apoptotic cells were identified by TUNEL staining of paraffin-embedded mammary glands. Sections were stained using the In-Situ Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions. BrdU-positive and TUNEL-positive epithelial cells were manually counted in five randomly selected ducts and alveolar clusters for each mouse by a blinded assessor. The number of BrdU-positive or TUNEL-positive cells was expressed as a percentage of the total number of epithelial cell nuclei. Data were averaged to give a single value for ductal and alveolar epithelium for each mouse.

Whole-mount preparations

Mammary glands from *Cd11b-Dtr* mice were prepared as whole-mounts on a glass slide and fixed with Carnoy's fixative (2-4 hours) followed by carmine alum staining overnight according to http://mammary.nih.gov/tools/histological/histology. Images of the glands were captured using a light microscope (Leica MZ16FA stereo microscope, Adelaide Microscopy). To quantify secondary branching and alveolar budding, blinded analysis of photographed whole-mounts was performed using ImageJ. Three primary ducts between the lymph node and the distal end of the gland were selected for each mouse and the numbers of secondary branches along a known length of primary duct were counted. Three secondary ducts were also selected and the numbers of alveolar buds along a known length of secondary duct were counted. The average number of secondary branches or alveolar buds per mm was calculated for each mouse, giving one value per mouse for statistical analysis.

Statistical analysis

Data were analysed by Student's t-test; P<0.05. Outliers present in the ovarian hormone analysis were defined as those with a serum hormone concentration greater than two standard deviations from the mean. As the present study aimed to classify macrophage abundance and location over the course of normal estrous cycles, all tissue from these outlier mice was excluded from subsequent analysis.

RESULTS

Estrous cycling affects mammary epithelium structure

To confirm normal estrous cyclicity in the C57Bl/6 mice, serum levels of the ovarian hormones progesterone and estradiol were assessed. In four mice, progesterone and/or estradiol concentration was two standard deviations from the mean, and the mammary gland tissue from these mice was excluded from subsequent analysis (see Materials and methods). In the remaining mice, as expected, there was a 3-fold increase in the mean serum concentration of progesterone from estrus (1.2±0.3 ng/ml, mean ± s.e.m.) to metestrus (3.3±0.7 ng/ml; P<0.01), and then a decrease at proestrus (1.1±0.1 ng/ml; P=0.01). By contrast, the mean serum concentration of estradiol increased 2-fold from diestrus (18.8±3.2 pg/ml) to estrus (35.3±3.3 pg/ml; P<0.01).

As described previously (Fata et al., 2001), the mammary gland contained both ductal and alveolar epithelium. Ductal epithelium was evident as a single epithelial cell layer, and alveolar epithelium was evident as clusters of small lobuloalveolar structures. These were designated ductal epithelium (Fig. 1A,C) and alveolar epithelium (Fig. 1B,D), respectively. The ductal and alveolar epithelia were both enveloped in a layer of smooth muscle actin (SMA) (Fig. 1E,F). The cyclical effect of the estrous cycle stage on the differentiation of mammary gland epithelium was determined by analysis of Hematoxylin and Eosin (H&E)-stained paraffin sections. Between 60 and 80 ducts per mouse were counted and categorised as either ductal or alveolar (n=6-10 mice per estrous cycle stage). The percentage of alveolar epithelium was found to fluctuate during the estrous cycle, with a 2-fold increase at diestrus (27.8±2.5%) compared with estrus (16±2.8%) (Fig. 1G). However, metestrus (22±4.5%) and proestrus (25.9±5.4%) did not show a significant increase in the percentage of alveolar epithelium. The percentage of alveolar epithelial ducts in the mammary gland correlated positively with serum progesterone (r^2=0.228; P=0.01) but not with serum estradiol. The highest percentage of alveolar epithelium being at diestrus, and the positive correlation between the percentage of alveolar epithelium and serum progesterone but not estradiol, confirmed previous findings (Fata et al., 2001) and demonstrate that this is a robust cohort for analysis of macrophages in the mammary gland.

Macrophage abundance fluctuates over the course of the ovarian cycle

To determine the effect of the estrous cycle phase on macrophage abundance, mammary glands from C57Bl/6 females were digested into single-cell suspensions and the cells were stained with F4/80

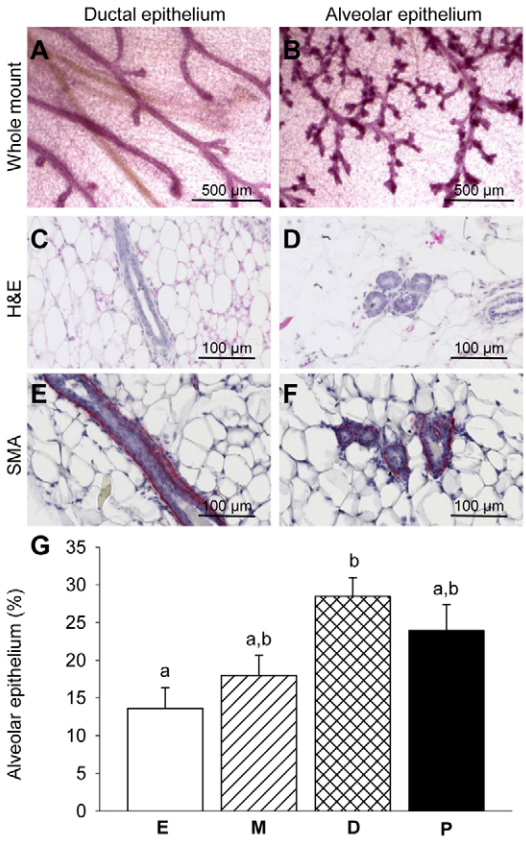

Fig. 1. The effect of estrous cycle stage on the development of mouse mammary gland alveolar epithelium. (**A-F**) Whole-mount carmine alum staining of ductal epithelium in the estrus phase of the cycle (A) and alveolar buds in the diestrus phase (B). The extent of alveolar budding is variable within different parts of the mammary gland. The images presented are not representative of the entire mammary gland, but rather have been selected to highlight ductal and alveolar epithelium. The ductal epithelium had a single layer of epithelial cells (C) and alveolar epithelium was observed in clusters (D) in H&E-stained thin sections. Both ductal (E) and alveolar (F) epithelium was surrounded by smooth muscle actin (SMA). (**G**) The percentage of epithelium classified as alveolar in H&E-stained sections. E, estrus; M, metestrus; D, diestrus; P, proestrus. *n*=6-10 mice per group. a and b are significantly different, whereas a,b is not different to a or b ($P<0.05$). Data are presented as mean + s.e.m.

antibody for flow cytometry. The number of F4/80-positive cells was quantified by adding a known concentration of count beads into the cell suspension. Three distinct populations of cells were observed: F4/80^{neg}, F4/80^{low} and F4/80^{high} (Fig. 2A). Macrophages with relatively low expression of F4/80 tended to be small in size, whereas macrophages with higher F4/80 expression were larger, as measured by flow cytometry. An isotype control showed no non-specific binding (Fig. 2B). To confirm the identity of the F4/80-positive cells, mammary gland cells from *Cfms-GFP* mice were stained for F4/80. In these mice, GFP is expressed only by cells of the mononuclear phagocyte lineage (Sasmono et al., 2003). The majority of cells with F4/80^{low} and F4/80^{high} expression also had high GFP expression (84.8% and 86.9% positive, respectively) (Fig. 2C), confirming that both these F4/80 populations are indeed macrophages.

The abundance of macrophages in the mammary gland fluctuated over the course of the estrous cycle, with both F4/80^{low} and F4/80^{high} macrophage populations showing similar trends, peaking at diestrus (Fig. 2D,E). Overall, the total number of F4/80-positive macrophages (combined F4/80^{low} and F4/80^{high} populations) increased by 2-fold at diestrus (17800±3050) compared with estrus (8300±2200; $P=0.03$) and proestrus (8050±1370; $P<0.01$) (Fig. 2F).

Macrophage location fluctuates over the course of the estrous cycle

The spatial distribution of macrophages in the mammary glands of C57Bl/6 virgin adults was determined by F4/80 antibody immunostaining (Fig. 3A). Macrophages were found to be principally localised in three areas: in close or direct contact with ductal epithelium (Fig. 3B) or alveolar epithelium (Fig. 3C) and in the connective tissue (Fig. 3D). Macrophages were also observed within the adipose tissue, as noted by others (Schwertfeger et al., 2006a). The isotype control antibody showed no non-specific staining (Fig. 3E).

When data from all four stages of the estrous cycle were combined, the alveolar epithelium was found to be in direct contact with a significantly higher number of macrophages than the ductal epithelium (833±62 versus 479±46 macrophages/mm^2) (Fig. 4A). However, there was no difference in the number of macrophages within the 150-μm radium around ductal epithelium compared with alveolar epithelium (96±8 versus 117±9 macrophages/mm^2). Across the four stages of the estrous cycle, there was a significant increase in macrophages in direct contact with the epithelium at proestrus (824±100 macrophages/mm^2) compared with estrus (468±75 macrophages/mm^2), metestrus (487±36 macrophages/mm^2) and diestrus (482±94 macrophages/mm^2) (Fig. 4B). No effect of estrous cycle stage on the number of macrophages within 150 μm around the epithelium (Fig. 4C) or in the connective tissue (Fig. 4D) was observed.

The functional role of macrophages in the mammary gland

To investigate the physiological significance of macrophages in the cyclical turnover of epithelial cells in the mammary gland, macrophages were depleted during alveolar development and alveolar regression. Ovariectomised mice were stimulated with exogenous hormones estradiol and progesterone to mimic development of early alveolar buds, followed by the progesterone receptor antagonist mifepristone to stimulate alveolar regression. The mammary glands of unstimulated ovariectomised mice had a basic ductal structure and very little alveolar budding (Fig. 5A). After 3 days of estradiol and progesterone treatment, early alveolar buds appeared similar to those observed in the diestrus phase of the cycle (Fig. 5B). Analysis of H&E-stained sections (not shown) revealed that 27.7%±3.5 of the epithelium was alveolar, which is similar to the 27.8±2.5% observed at diestrus (Fig. 1G). Mifepristone administration resulted in alveolar regression and the gland was remodelled back to its basic architecture (Fig. 5C). Macrophages were observed in low abundance in unstimulated mammary glands (Fig. 5D); however, they were recruited to the epithelium upon stimulation with estradiol and progesterone (Fig. 5E) and were also observed in close contact with the epithelium during mifepristone-induced alveolar regression (Fig. 5F). Therefore, macrophages are observed in close contact with the epithelium during alveolar development and regression, similar to what is observed in naturally cycling mice.

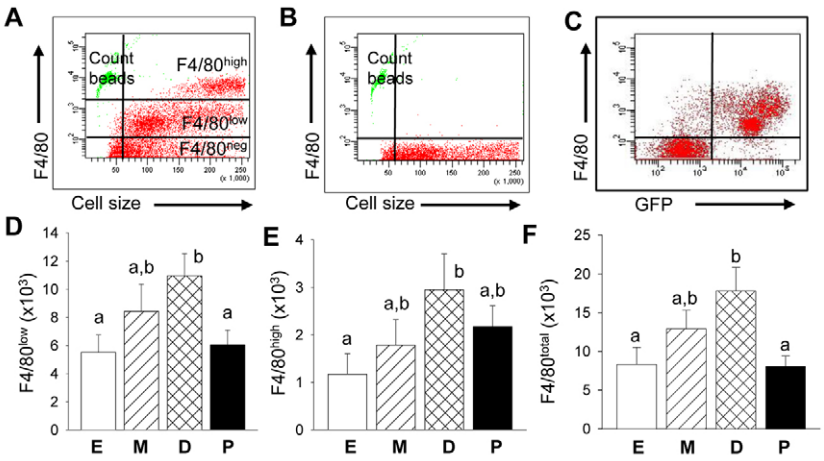

Fig. 2. The effect of estrous cycle stage on the number of macrophages in the mammary gland. (**A-C**) Two distinct F4/80-positive populations were designated F4/80^{low} and F4/80^{high} (A). These populations were not present in the isotype-matched negative control (B). The F4/80-positive populations were confirmed to be of the mononuclear phagocyte lineage by green fluorescence of cells from mammary glands from *Cfms-GFP* transgenic mice (C). (**D-F**) The number of F4/80^{low} (D), F4/80^{high} (E) and total F4/80-positive (F) macrophages were quantified. E, estrus; M, metestrus; D, diestrus; P, proestrus. a and b are significantly different, whereas a,b is not different to a or b (P<0.05). Data are presented as mean + s.e.m.

Acute macrophage depletion was achieved using the *Cd11b-Dtr* transgenic mouse, in which macrophages can be acutely and specifically depleted with nanogram quantities of diphtheria toxin (DT). Ovariectomised mice were treated with estradiol and progesterone for 3 days together with either PBS or DT on days 1 and 3 (Fig. 6A,B), or treated with estradiol and progesterone followed by mifepristone together with PBS or DT (Fig. 6C,D). Macrophages were visible in the mammary gland of *Cd11b-Dtr* mice treated with PBS control (Fig. 6A,C), but were absent from the mammary glands of DT-treated *Cd11b-Dtr* mice (Fig. 6B,D). The depletion of macrophages in the mammary gland was also demonstrated by flow cytometry. F4/80-positive macrophages constituted 7.7±1.4% of total cells in the mammary gland in PBS-treated naturally cycling *Cd11b-Dtr* mice (Fig. 6E,G), which was reduced to 1.8±0.5% following 24 hours of DT treatment (P<0.01) (Fig. 6F,G). No differences in the abundance of RB6-positive neutrophils or CD3-positive T cells were observed (Fig. 6G). Macrophages were not depleted in non-transgenic control mice given DT (data not shown).

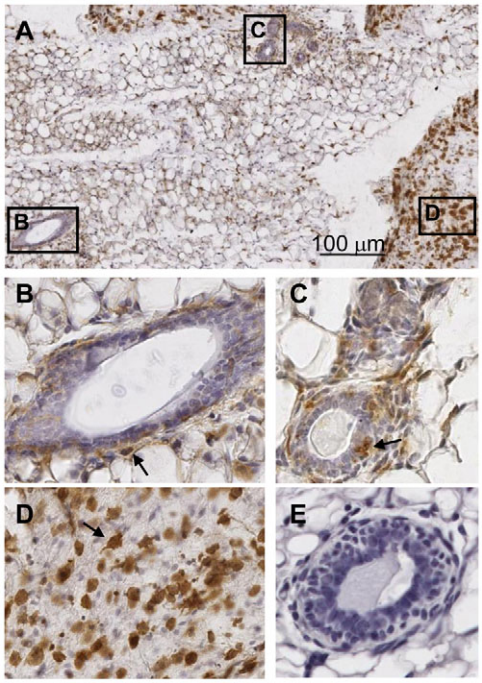

Fig. 3. Localisation of macrophages in the adult virgin mammary gland. (**A-E**) Paraffin sections of C57Bl/6 virgin adult mouse mammary glands were stained with macrophage-specific F4/80 antibody (A). Macrophages were localised in three areas: in direct contact with ductal epithelium (B) or alveolar epithelium (C) and in the connective tissue (D). Negative control stained with isotype-matched irrelevant antibody shows no F4/80 staining (E). Macrophages are indicated by arrows.

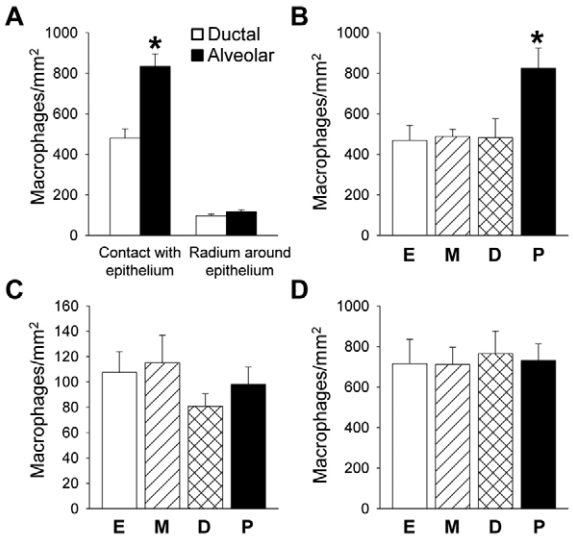

Fig. 4. The effect of estrous cycle stage on the localisation of macrophages in the mammary gland. (**A**) The numbers of F4/80-stained macrophages in direct contact with the epithelium and within a 150 μm radium around the ductal and alveolar epithelium were quantified, for which data from all cycle stages were combined. (**B-D**) The density of macrophages in direct contact with epithelium (B), within a 150 μm radium around the epithelium (C) and in the connective tissue (D) were quantified in F4/80-stained sections. E, estrus; M, metestrus; D, diestrus; P, proestrus. n=6-10 mice per group. *, P<0.05, for (A) ductal versus alveolar epithelium and (B) proestrus versus the three other cycle stages. Data are presented as mean + s.e.m.

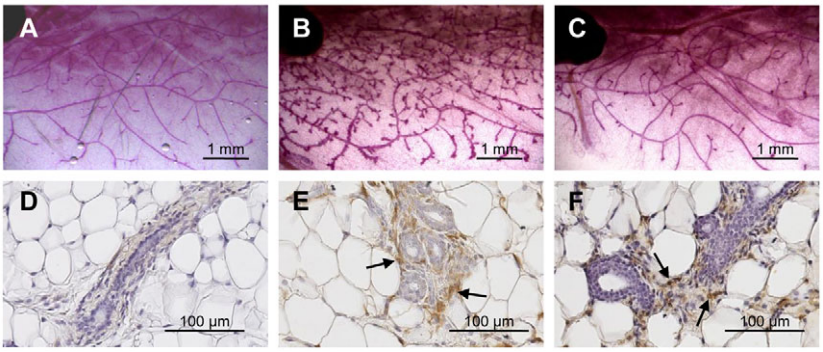

Fig. 5. The effect of exogenous hormone treatment on mammary gland morphogenesis. (**A-F**) Carmine alum-stained whole-mounts (A-C) and F4/80 staining (D-F) from ovariectomised unstimulated mice (A,D), mice administered exogenous estradiol and progesterone (B,E) and estradiol and progesterone followed by mifepristone (C,F). Arrows indicate F4/80-stained macrophages. Representative images from *n*=4 mice per group.

Macrophages promote the development of alveolar buds

To investigate the role of macrophages in the development of alveolar buds, hormone-stimulated *Cd11b-Dtr* mice were administered DT or PBS as a control. Alveolar buds were observed in whole-mount preparations (Fig. 7A,B) and H&E-stained sections (Fig. 7C,D). No difference was observed in the frequency of secondary branch points along primary branches in the control and macrophage-depleted groups (2.7±0.18 versus 2.8±0.42 branch points/mm) in mammary gland whole-mounts (Fig. 7E). However, there was a 53% decrease in the frequency of alveolar buds along the ducts in the macrophage-depleted group compared with the control group (1.0±0.32 versus 1.9±0.35 buds/mm; P=0.005) (Fig. 7E). Similarly, there was a 52% decrease in alveolar epithelium quantified in H&E-stained sections of macrophage-depleted mice (P=0.01) (Fig. 7F).

Proliferation in ductal and alveolar epithelium was quantified by BrdU incorporation in mammary glands from control (Fig. 7G,I) and macrophage-depleted (Fig. 7H,J) mice. There was a 47% decrease in the percentage of ductal epithelial cells undergoing proliferation (P=0.05) and no significant difference in proliferation of the alveolar epithelium (Fig. 7K).

Macrophages promote progesterone withdrawal-induced alveolar bud regression

To investigate the role of macrophages in alveolar bud regression in the mammary gland during progesterone withdrawal, ovariectomised *Cd11b-Dtr* mice were hormone stimulated as before, followed by a single injection of mifepristone. At the same time as mifepristone, DT or control PBS was administered. Mifepristone administration caused regression of alveolar buds and a ductal branching pattern similar to that observed in the estrus stage of the cycle (Fig. 1A; Fig. 8A,C). Mammary glands from mice depleted of macrophages at the same time as mifepristone administration (Fig. 8B,D) displayed a 46% increase in the frequency of branch points along primary ducts when compared with the control group (4.1±0.54 versus 2.8±0.23 branch points/mm; P=0.026) (Fig. 8E). Many of the branch points in macrophage-depleted mammary glands appeared to be incompletely regressed alveolar buds (Fig. 8D, arrows). When observed histologically, some alveolar buds were still visible in PBS-treated mice (Fig. 8F). Alveolar buds in macrophage-depleted mice were also observed, although many had a number of pyknotic nuclei, which are indicative of cells undergoing apoptosis (Fig. 8G,H). It is expected that the SMA-secreting myoepithelial cell layer that encapsulates the alveolar epithelium would be remodelled as alveolar buds regress. In control mice, SMA was observed incompletely surrounding alveolar buds; however, the lumen was always observed to be completely surrounded by SMA-negative alveolar epithelium (Fig. 8I). In macrophage-depleted mice, SMA-positive myoepithelium was often observed directly adjacent to the lumen (Fig. 8J) or in very low abundance surrounding the alveolar epithelium (Fig. 8K).

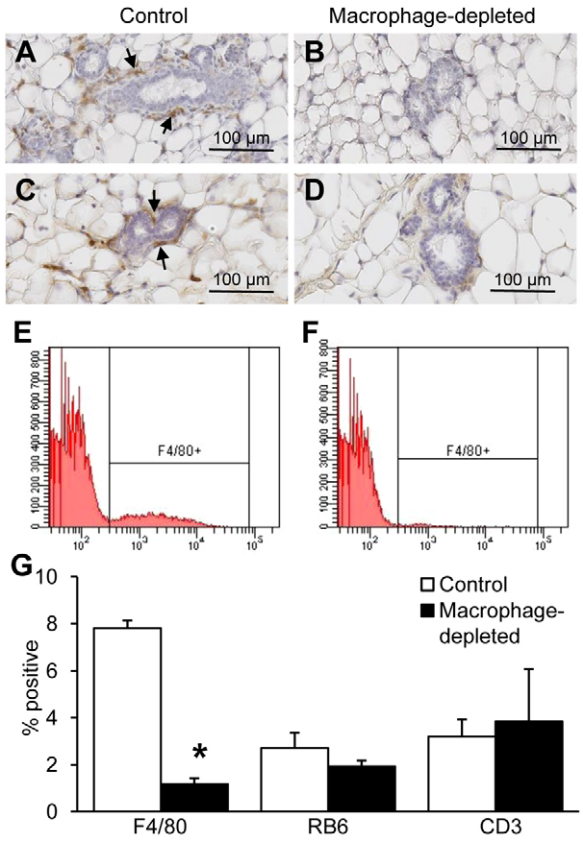

Fig. 6. Effect of DT administration on *Cd11b-Dtr* mice. (**A-D**) F4/80-stained macrophages (arrows) were observed in direct contact with mammary gland epithelium in PBS-treated control mice (A,C), but were not observed in diphtheria toxin (DT)-treated mice (B,D) during alveolar development (A,B) and alveolar regression (C,D). (**E**,**F**) Flow cytometry analysis of F4/80-positive cells in mammary glands from PBS-treated (E) and DT-treated (F) naturally cycling *Cd11b-Dtr* mice. (**G**) Quantification of F4/80-, RB6- and CD3-positive cells in the mammary gland 24 hours following DT or PBS administration (*n*=5 mice/group). *, P<0.05 versus control. Data are presented as mean + s.e.m.

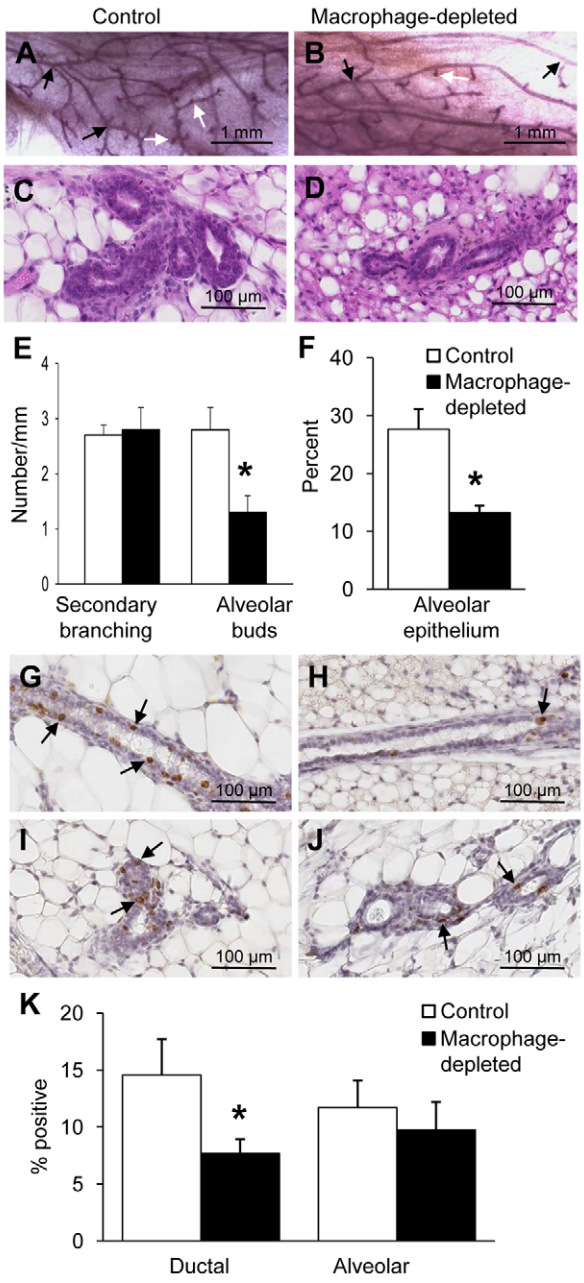

Fig. 7. Effect of macrophage depletion on alveolar bud development. (**A-D**) Whole-mount preparations showed secondary branching (black arrows) and alveoli buds (white arrows) in control (A,C) and macrophage-depleted (B,D) alveolar buds observed by H&E staining at low (A,B) and high (C,D) magnification. (**E**) Secondary branching and alveolar bud formation were quantified in control and macrophage-depleted mice (n=7 and 8 mice, respectively). (**F**) Alveolar epithelium as a percentage of total epithelium quantified in H&E-stained sections. (**G-J**) BrdU staining of ductal (G,H) and alveolar (I,J) epithelium indicative of proliferating cells in control (G,I) and macrophage-depleted (H,J) mice. Arrows indicate BrdU-positive cells. (**K**) Manually counted BrdU-positive cells as a percentage of total nuclei. *, P<0.05 versus control. Data are presented as mean + s.e.m.

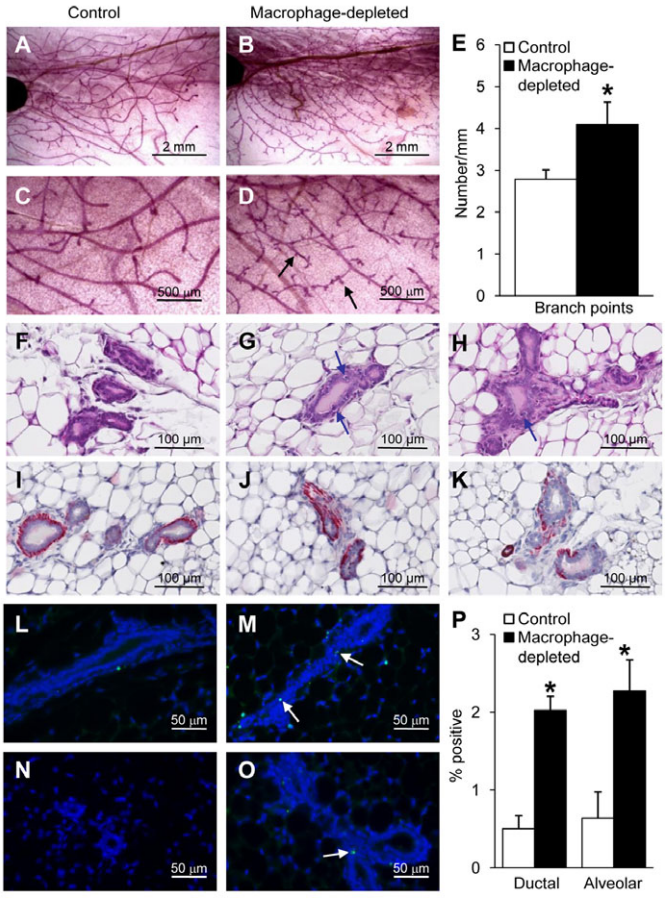

Fig. 8. Effect of macrophage depletion on alveolar bud regression. (**A-D**) Whole-mount mammary glands from control (A,C) and macrophage-depleted (B,D) mice at low (A,B) and high (C,D) magnification. Branch points persisting in macrophage-depleted mice appeared to be incompletely regressed alveolar buds (D, black arrows) that were not present in control mice (C). (**E**) Quantification of branch points from macrophage-depleted (n=11) and control (n=14) mice. (**F-H**) H&E staining of mammary gland sections showed some alveolar buds in control (F) and macrophage-depleted (G,H) mice. Pyknotic nuclei (blue arrows) were evident in macrophage-depleted mice. (**I-K**) SMA staining in control (I) and macrophage-depleted (J,K) mice. (**L-O**) TUNEL staining of ductal (L,M) and alveolar (N,O) epithelium indicative of dying cells in control (L,N) and macrophage-depleted (M,O) mice. Arrows indicate TUNEL-positive cells. (**P**) Manually counted TUNEL-positive cells as a percentage of total nuclei. *, P<0.05 versus control. Data are presented as mean + s.e.m.

Apoptotic nuclei were observed by TUNEL staining in ductal and alveolar epithelium in control (Fig. 8L,N) and macrophage-depleted (Fig. 8M,O) mammary gland sections. There was a 4-fold increase in the number of TUNEL-positive nuclei in both ductal and alveolar epithelium from macrophage-depleted mammary glands compared with the control (P<0.001) (Fig. 8P).

DISCUSSION

Investigation of the role of macrophages in mammary gland development during puberty, pregnancy and lactation using *Csf1*-null mice has implicated macrophages in the proliferation and differentiation of mammary epithelial cells, the phagocytosis of apoptotic cell bodies, and in the tissue remodelling events that occur during mammary gland development (Cecchini et al., 1994; Gouon Evans et al., 2000; Pollard and Hennighausen, 1994; Van Nguyen and Pollard, 2002). Given that the cycling adult mammary

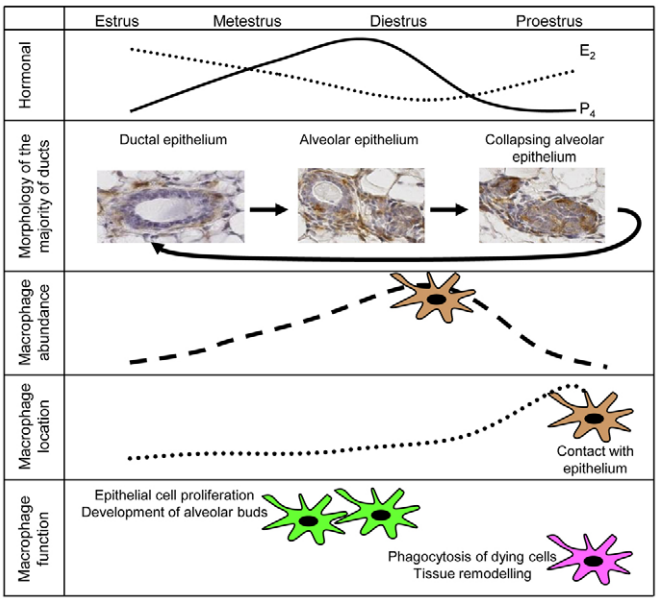

Fig. 9. Fluctuations in mammary gland macrophages over the course of the estrous cycle. As progesterone (P_4) rises, alveolar buds develop, with the number of buds peaking at diestrus. Macrophages in the mammary gland are most abundant at this stage. Following the fall in serum progesterone at proestrus, alveolar epithelium regresses, and the density of macrophages in direct contact with the epithelium is highest at this stage. Macrophages assist in hormone-stimulated alveolar development and in the tissue remodelling associated with alveolar bud regression to return the gland back to its basic architecture. E_2, estradiol.

gland undergoes active epithelial proliferation, differentiation and apoptosis (Fata et al., 2001; Schedin et al., 2000; Strange et al., 2007), it has been hypothesised that macrophages might have roles in epithelial cell turnover in the adult non-pregnant mammary gland. Here, we describe the spatial distribution and abundance of macrophages in the cycling adult mammary gland, and provide evidence of two distinct functional roles for macrophages in this dynamic tissue (Fig. 9). Macrophages promote the development of alveolar buds during the metestrus/diestrus phase of the cycle (analogous to the early to mid-luteal phase of the menstrual cycle in humans), when serum progesterone is rising. Macrophages also facilitate alveolar regression and tissue remodelling during the proestrus stage of the cycle (analogous to the menstrual phase in humans), when progesterone levels fall.

Macrophages are required for early alveolar development

Macrophages accumulate in high density around alveolar epithelium. As alveolar buds are most prevalent at diestrus, it was not surprising that total macrophage abundance, as measured by flow cytometry, was also highest during this stage of the cycle. The increase we observe in macrophage abundance at diestrus, together with the increased density of macrophages around the alveolar epithelium compared with the ductal epithelium, suggests that macrophages have a role in the proliferation and/or differentiation required for early alveolar development.

Through acute macrophage depletion, we have demonstrated a physiological role for macrophages in alveolar bud formation, which is associated with a reduction in ductal, but not alveolar, epithelial cell proliferation. Macrophages might promote alveolar development through the support of alveolar progenitor cell activity. The ductal epithelium consists of progesterone receptor-positive and -negative populations, with the former suggested to be alveolar progenitor cells (Smith, 1996). In the present study, we did not differentiate between these two populations within the ductal epithelial cell population, although it is likely that the ductal epithelial cells that proliferated in response to hormone stimulation were alveolar progenitors. Macrophages are known to have an important role in the stem cell niche (Gyorki et al., 2009) and might support alveolar progenitor cell activity through the production of various cytokines and growth factors (Cecchini et al., 1994; Nathan, 1987), such as fibroblast growth factor (Schwertfeger et al., 2006a; Schwertfeger et al., 2006b) and transforming growth factor beta 1 (TGFβ1) (Ingman and Robertson, 2008).

Macrophages are required for alveolar breakdown and remodelling

If pregnancy does not occur following ovulation, the corpus luteum undergoes luteolysis, causing a dramatic fall in serum progesterone. In the human uterus this is associated with menstruation, as the endometrial epithelium breaks down and is remodelled in preparation for the next menstrual cycle. The mammary gland also turns over epithelium (Fata et al., 2001), with progesterone withdrawal initiating alveolar bud regression and tissue remodelling that returns the gland back to its basic architecture (L.J.H., A.C.L.C., S.A.R. and W.V.I., unpublished).

The increase in macrophages in direct contact with the epithelium in the proestrus phase of the cycle, when alveolar buds regress, suggests that macrophages might participate in the alveolar breakdown process. When macrophages were depleted at the same time as progesterone withdrawal, there was an increase in the number of branch points compared with macrophage-replete controls. As many of the branch points were connected to very short secondary ducts, and the SMA-expressing myoepithelial layer appeared disorganised, we reasonably infer that the alveolar buds were not fully remodelled. The accumulation of apoptotic nuclei in the epithelium of macrophage-depleted mammary glands suggests that macrophages are involved in the phagocytosis of dying cells. A similar role of macrophages might exist in human breast tissue, where regression of the epithelium during the menstrual phase of the cycle is also accompanied by an infiltration of inflammatory cells, including macrophages (Ramakrishnan et al., 2002), which might be the cause of the breast tenderness that many women report just prior to the onset of menstruation (Yonkers et al., 2008).

Hormonal regulation of macrophage function

The distinct functions of macrophage populations across the ovarian cycle are likely to be regulated, directly or indirectly, by ovarian hormones. Mouse peritoneal macrophages express receptors for both estradiol (both the alpha and beta isoforms) and progesterone (Huang et al., 2008), and are responsive to these hormones in vitro. Progesterone promotes the expression of proinflammatory cytokines, including tumour necrosis factor alpha (TNFα), CCL2 (also known as monocyte chemoattractant protein 1) and interleukin 1 beta (IL1β), by mouse and human macrophages, whereas estradiol suppresses expression (Frazier-Jessen and Kovacs, 1995; Huang et al., 2008; Yuan et al., 2008). Alternatively, indirect hormonal regulation might be mediated by the epithelium. KIM2 mammary epithelial cells secrete IL12, interferon gamma (INFγ) and TNFα unless stimulated by prolactin,

whereupon they switch to IL4, IL5 and IL13 secretion (Khaled et al., 2007). CCL2, IL4 and IL13 are produced by the rat mammary gland and fluctuate during involution (O'Brien et al., 2010). Little is known about cytokine expression by the mammary gland epithelium during the estrous cycle, although latent and active TGFβ1 is most highly expressed by the epithelium during the diestrus phase (Ewan et al., 2002). These cytokines and chemokines are locally produced and would be expected to influence macrophage function. This mechanism, in which hormonally regulated epithelial cell cytokine expression controls macrophage activity, is similar to that utilised in the uterine endometrium during both the ovarian cycle and the embryo implantation phase of early pregnancy (Robertson et al., 1997).

Significance of macrophages in breast cancer susceptibility

Epidemiological studies indicate that the number and regularity of menstrual cycles affect breast cancer susceptibility (Butler et al., 2000; Chavez-MacGregor et al., 2005; Titus-Ernstoff et al., 1998): the greater the cumulative number of menstrual cycles a woman experiences, the greater the breast cancer risk. However, the underlying cellular mechanisms that lead to this increased risk are poorly understood.

Macrophages have a diverse array of potential roles in both protecting the mammary gland from cancer and in promoting it. Macrophage infiltration has been correlated with poor prognosis in breast cancer, and previous studies have demonstrated the involvement of macrophages in tumour growth, angiogenesis, metastasis and immunosuppression (Lewis and Pollard, 2006; Lin et al., 2006). However, not all macrophage functions should be considered deleterious. Lipopolysaccharide-stimulated macrophages express NKG2D (also known as KLRK1) (Diefenbach et al., 2000), a receptor that recognises the ligands H60 and RAE1 beta (Diefenbach et al., 2000), which are expressed on the surface of DNA-damaged cells (Gasser et al., 2005). Interaction between NKG2D on macrophages and these DNA damage-response ligands induces release of nitric oxide and production of TNFα by macrophages, and is a mechanism by which the innate immune system eliminates potentially oncogenic cells (Diefenbach et al., 2000).

The function of macrophages is controlled by an array of cytokines and hormones, and in the mammary gland macrophages promote both the development and regression of alveolar buds over the course of the ovarian cycle. The dependence of this tissue on macrophage-mediated morphogenesis is likely to affect the cytokine microenvironment of the mammary gland, potentially diverting both macrophages and the wider immune system from appropriately responding to DNA damage. Over the course of many years and menstrual cycles, this would lead to an accumulation of DNA mutations in the mammary gland epithelium, increasing the risk of breast cancer.

Conclusions

These studies show that the location, abundance and function of macrophages fluctuate during the ovarian cycle in the mammary gland. We propose that macrophages have two distinct roles in the cyclical turnover of epithelial cells: (1) the promotion of hormone-induced alveolar development; and (2) tissue remodelling and phagocytosis following progesterone withdrawal. Future studies will examine the cytokine microenvironment that regulates macrophage function within the mammary gland and the role of macrophages in the protection of this tissue from DNA damage. Understanding how the diverse functions of macrophages are regulated will shed light on the cellular mechanisms that underpin the susceptibility of the breast to cancer.

Acknowledgements

We thank Prof. Richard Lang for the generous gift of *Cd11b-Dtr* transgenic mice; Ms Alison Care for help with *Cd11b-Dtr* mice; and Mr Fred Amato from the University of Adelaide for conducting radioimmunoassays of serum hormones. These studies were supported by an Australian NHMRC project grant awarded to W.V.I.

Competing interests statement

The authors declare no competing financial interests.

References

Butler, L. M., Potischman, N. A., Newman, B., Millikan, R. C., Brogan, D., Gammon, M. D., Swanson, C. A. and Brinton, L. A. (2000). Menstrual risk factors and early-onset breast cancer. *Cancer Causes Control* **11**, 451-458.

Cecchini, M. G., Dominguez, M. G., Mocci, S., Wetterwald, A., Felix, R., Fleisch, H., Chisholm, O., Hofstetter, W., Pollard, J. W. and Stanley, E. R. (1994). Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* **120**, 1357-1372.

Chavez-MacGregor, M., Elias, S. G., Onland-Moret, N. C., van der Schouw, Y. T., Van Gils, C. H., Monninkhof, E., Grobbee, D. E. and Peeters, P. H. (2005). Postmenopausal breast cancer risk and cumulative number of menstrual cycles. *Cancer Epidemiol. Biomarkers Prev.* **14**, 799-804.

Cohen, P. E., Zhu, L. and Pollard, J. W. (1997). Absence of colony stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice disrupts estrous cycles and ovulation. *Biol. Reprod.* **56**, 110-118.

Diefenbach, A., Jamieson, A. M., Liu, S. D., Shastri, N. and Raulet, D. H. (2000). Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* **1**, 119-126.

Duffield, J. S., Forbes, S. J., Constandinou, C. M., Clay, S., Partolina, M., Vuthoori, S., Wu, S., Lang, R. and Iredale, J. P. (2005a). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J. Clin. Invest.* **115**, 56-65.

Duffield, J. S., Tipping, P. G., Kipari, T., Cailhier, J. F., Clay, S., Lang, R., Bonventre, J. V. and Hughes, J. (2005b). Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *Am. J. Pathol.* **167**, 1207-1219.

Ewan, K. B., Shyamala, G., Ravani, S. A., Tang, Y., Akhurst, R., Wakefield, L. and Barcellos-Hoff, M. H. (2002). Latent transforming growth factor-beta activation in mammary gland: regulation by ovarian hormones affects ductal and alveolar proliferation. *Am. J. Pathol.* **160**, 2081-2093.

Fata, J. E., Chaudhary, V. and Khokha, R. (2001). Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle. *Biol. Reprod.* **65**, 680-688.

Frazier-Jessen, M. R. and Kovacs, E. J. (1995). Estrogen modulation of JE/monocyte chemoattractant protein-1 mRNA expression in murine macrophages. *J. Immunol.* **154**, 1838-1845.

Gasser, S., Orsulic, S., Brown, E. J. and Raulet, D. H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* **436**, 1186-1190.

Gouon Evans, V., Rothenberg, M. E. and Pollard, J. W. (2000). Postnatal mammary gland development requires macrophages and eosinophils. *Development* **127**, 2269-2282.

Gyorki, D. E., Asselin-Labat, M. L., van Rooijen, N., Lindeman, G. J. and Visvader, J. E. (2009). Resident macrophages influence stem cell activity in the mammary gland. *Breast Cancer Res.* **11**, R62.

Hovey, R. C., Trott, J. F. and Vonderhaar, B. K. (2002). Establishing a framework for the functional mammary gland: from endocrinology to morphology. *J. Mammary Gland Biol. Neoplasia* **7**, 17-38.

Huang, H., He, J., Yuan, Y., Aoyagi, E., Takenaka, H., Itagaki, T., Sannomiya, K., Tamaki, K., Harada, N., Shono, M. et al. (2008). Opposing effects of estradiol and progesterone on the oxidative stress-induced production of chemokine and proinflammatory cytokines in murine peritoneal macrophages. *J. Med. Invest.* **55**, 133-141.

Ingman, W. V. and Robertson, S. A. (2008). Mammary gland development in transforming growth factor beta1 null mutant mice: systemic and epithelial effects. *Biol. Reprod.* **79**, 711-717.

Ingman, W. V., Robker, R. L., Woittiez, K. and Robertson, S. A. (2006a). Null mutation in transforming growth factor beta1 disrupts ovarian function and causes oocyte incompetence and early embryo arrest. *Endocrinology* **147**, 835-845.

Ingman, W. V., Wyckoff, J., Gouon-Evans, V., Condeelis, J. and Pollard, J. W. (2006b). Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev. Dyn.* **235**, 3222-3229.

Khaled, W. T., Read, E. K., Nicholson, S. E., Baxter, F. O., Brennan, A. J., Came, P. J., Sprigg, N., McKenzie, A. N. and Watson, C. J. (2007). The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development. *Development* **134**, 2739-2750.
Lewis, C. E. and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* **66**, 605-612.
Lin, E. Y., Li, J. F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., Qian, H., Xue, X. N. and Pollard, J. W. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res.* **66**, 11238-11246.
Nathan, C. F. (1987). Secretory products of macrophages. *J. Clin. Invest.* **79**, 319-326.
Navarrete, M. A., Maier, C. M., Falzoni, R., Quadros, L. G., Lima, G. R., Baracat, E. C. and Nazario, A. C. (2005). Assessment of the proliferative, apoptotic and cellular renovation indices of the human mammary epithelium during the follicular and luteal phases of the menstrual cycle. *Breast Cancer Res.* **7**, R306-R313.
O'Brien, J., Lyons, T., Monks, J., Lucia, M. S., Wilson, R. S., Hines, L., Man, Y. G., Borges, V. and Schedin, P. (2010). Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am. J. Pathol.* **176**, 1241-1255.
Pollard, J. W. (1997). Role of colony-stimulating factor-1 in reproduction and development. *Mol. Reprod. Dev.* **46**, 54-60.
Pollard, J. W. and Hennighausen, L. (1994). Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc. Natl. Acad. Sci. USA* **91**, 9312-9316.
Ramakrishnan, R., Khan, S. A. and Badve, S. (2002). Morphological changes in breast tissue with menstrual cycle. *Mod. Pathol.* **15**, 1348-1356.
Richert, M. M., Schwertfeger, K. L., Ryder, J. W. and Anderson, S. M. (2000). An atlas of mouse mammary gland development. *J. Mammary Gland Biol. Neoplasia* **5**, 227-241.
Robertson, S. A., Mau, V. J., Hudson, S. N. and Tremellen, K. P. (1997). Cytokine-leukocyte networks and the establishment of pregnancy. *Am. J. Reprod. Immunol.* **37**, 438-442.
Sapi, E. and Kacinski, B. M. (1999). The role of CSF-1 in normal and neoplastic breast physiology. *Proc. Soc. Exp. Biol. Med.* **220**, 1-8.
Sasmono, R. T., Oceandy, D., Pollard, J. W., Tong, W., Pavli, P., Wainwright, B. J., Ostrowski, M. C., Himes, S. R. and Hume, D. A. (2003). A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* **101**, 1155-1163.
Schedin, P., Mitrenga, T. and Kaeck, M. (2000). Estrous cycle regulation of mammary epithelial cell proliferation, differentiation, and death in the Sprague-Dawley rat: a model for investigating the role of estrous cycling in mammary carcinogenesis. *J. Mammary Gland Biol. Neoplasia* **5**, 211-225.
Schwertfeger, K. L., Rosen, J. M. and Cohen, D. A. (2006a). Mammary gland macrophages: pleiotropic functions in mammary development. *J. Mammary Gland Biol. Neoplasia* **11**, 229-238.
Schwertfeger, K. L., Xian, W., Kaplan, A. M., Burnett, S. H., Cohen, D. A. and Rosen, J. M. (2006b). A critical role for the inflammatory response in a mouse model of preneoplastic progression. *Cancer Res.* **66**, 5676-5685.
Smith, G. H. (1996). Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. *Breast Cancer Res. Treat.* **39**, 21-31.
Snell, G. D. (1956). *Biology of the Laboratory Mouse.* Philadelphia: Blakiston.
Stanley, E. R., Berg, K. L., Einstein, D. B., Lee, P. S., Pixley, F. J., Wang, Y. and Yeung, Y. G. (1997). Biology and action of colony-stimulating factor-1. *Mol. Reprod. Dev.* **46**, 4-10.
Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M. A., Heath, V. J., Bell, A. K., Ferrier, R. K., Sandilands, G. P. and Gusterson, B. A. (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res.* **6**, R75-R91.
Strange, R., Westerlind, K. C., Ziemiecki, A. and Andres, A. C. (2007). Proliferation and apoptosis in mammary epithelium during the rat oestrous cycle. *Acta Physiol.* **190**, 137-149.
Titus-Ernstoff, L., Longnecker, M. P., Newcomb, P. A., Dain, B., Greenberg, E. R., Mittendorf, R., Stampfer, M. and Willett, W. (1998). Menstrual factors in relation to breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **7**, 783-789.
Van Nguyen, A. and Pollard, J. W. (2002). Colony stimulating factor-1 is required to recruit macrophages into the mammary gland to facilitate mammary ductal outgrowth. *Dev. Biol.* **247**, 11-25.
Walker, N. I., Bennett, R. E. and Kerr, J. F. (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am. J. Anat.* **185**, 19-32.
Wu, R., Van der Hoek, K. H., Ryan, N. K., Norman, R. J. and Robker, R. L. (2004). Macrophage contributions to ovarian function. *Hum. Reprod. Update* **10**, 119-133.
Yonkers, K. A., O'Brien, P. M. and Eriksson, E. (2008). Premenstrual syndrome. *Lancet* **371**, 1200-1210.
Yuan, Y., Shimizu, I., Shen, M., Aoyagi, E., Takenaka, H., Itagaki, T., Urata, M., Sannomiya, K., Kohno, N., Tamaki, K. et al. (2008). Effects of estradiol and progesterone on the proinflammatory cytokine production by mononuclear cells from patients with chronic hepatitis C. *World J. Gastroenterol.* **14**, 2200-2207.