



# Developmental Stage-Specific Distribution of Macrophages in Mouse Mammary Gland

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Mammary gland development begins in the embryo and continues throughout the reproductive life of female mammals. Tissue macrophages (Mφs), dependent on signals from the Mφ colony stimulating factor 1 receptor (CSF1R), have been shown to regulate the generation, regression and regeneration of this organ, which is central for mammalian offspring survival. However, the distribution of Mφs in the pre- and post-natal mammary gland, as it undergoes distinct phases of development and regression, is unknown or has been inferred from immunostaining of thin tissue sections. Here, we used optical tissue clearing and 3-dimensional imaging of mammary tissue obtained from *Csf1r-EGFP* mice. Whilst tissue Mφs were observed at all developmental phases, their abundance, morphology, localization and association with luminal and basal epithelial cells exhibited stage-specific differences. Furthermore, sexual dimorphism was observed at E14.5, when the male mammary bud is severed from the overlying epidermis. These findings provide new insights into the localization and possible functions of heterogeneous tissue Mφ populations in mammogenesis.

**Keywords:** mammary gland, macrophages, development, embryonic mammary stem cells, adult mammary stem cells, stem cell niche

## INTRODUCTION

Mammary gland development is phasic, with distinct developmental periods occurring in the embryo, at puberty and during pregnancy/lactation (Watson and Khaled, 2008; Lloyd-Lewis et al., 2017). The formation of the milk lines occurs at approximately embryonic day (E) 10 in mice and within 36 h resolves into five pairs of disk-shaped thickenings known as mammary placodes (Cowin and Wysolmerski, 2010). At around E12.5, mammary placodes invaginate into the dermal mesenchyme forming the mammary buds, which later elongate and invade the fat pad precursor, creating a rudimentary epithelial tree (Cowin and Wysolmerski, 2010; Paine and Lewis, 2017; Lilja et al., 2018). During embryonic development, multipotent mammary stem cells are replaced by unipotent luminal and basal stem/progenitor cells (Lilja et al., 2018; Wuidart et al., 2018), with epithelial cell identities being resolved by E15.5 (Lilja et al., 2018).

Initial postnatal growth of the mammary epithelium is proportional to body size and it is not until puberty that ductal elongation occurs, fueled by proliferation of adult mammary stem/progenitor cells within terminal end bud (TEB) structures (Davis et al., 2016; Lloyd-Lewis et al., 2017, 2018; Paine and Lewis, 2017). Further epithelial expansion occurs during pregnancy to generate the functional (milk-producing) alveolar epithelium (Watson and Khaled, 2008; Davis et al., 2016). With the cessation of infant suckling, alveolar mammary epithelial cells undergo massive programmed cell death (a process known as post-lactational involution), returning the mammary gland to a near pre-pregnant state that is capable of supporting future pregnancies (Sargeant et al., 2014; Lloyd-Lewis et al., 2017).

M $\phi$ s are present in all adult tissues (Hume D. et al., 2019). These cells are first and foremost professional phagocytes, but also regulate tissue development, function and dysfunction (Hume, 2015; Naik et al., 2018; Yang et al., 2018). In the normal postnatal mammary gland, M $\phi$ s regulate ductal morphogenesis during puberty (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002; Ingman et al., 2006), alveolar budding during ovarian cycling (Chua et al., 2010), alveologensis in pregnancy (Pollard and Hennighausen, 1994) and tissue remodeling during post-lactational involution (O'Brien et al., 2010, 2012; Hughes et al., 2012), with many of these processes being impaired in mice deficient in tissue M $\phi$ s. Moreover, M $\phi$ s identified by fluorescence-activated cell sorting (FACS) of disaggregated tissue were detected within the embryonic mammary gland by E16.5 and fetal-derived M $\phi$ s were apparently retained and expanded by self-renewal in adult mammary tissue (Jäppinen et al., 2019).

With accumulating evidence demonstrating the dependence of the mammary epithelium on M $\phi$ s at all developmental stages, it is tempting to speculate that tissue-resident M $\phi$ s institute or influence a putative mammary stem cell niche, as has been shown for hematopoietic stem cells (Winkler et al., 2010), intestinal stem cells (Sehgal et al., 2018) and hair follicle stem cells (Castellana et al., 2014; Naik et al., 2018). Indeed, the activity of mammary "stem" or repopulating cells (defined as a subset of basal cells that are capable of recreating the bi-layered mammary epithelium upon limiting dilution transplantation) is reduced when cells are transplanted into the cleared fat pads of M $\phi$ -depleted recipient mice (Gyoriki et al., 2009). More recently, mammary repopulating cells were shown to express a Notch ligand Delta like 1 (DLL1) and *Dll1*-conditional knockout mice showed reduced mammary repopulating activity and lower levels of F4/80<sup>+</sup> M $\phi$ s (Chakrabarti et al., 2018). Thus, it has been suggested that DLL1-expressing basal cells activate Notch-expressing M $\phi$ s in a reciprocal stem cell-macrophage niche (Chakrabarti et al., 2018; Kannan and Eaves, 2018). Studies revealing developmental stage-dependent distribution of M $\phi$ s in the mammary gland, including their sites of confluence, would provide further evidence for the existence of a stem cell-macrophage niche in this organ and may help to reveal the specific and stage-dependent localization of mammary stem/progenitor cells within the dynamic, bilayered epithelium under physiological conditions. Here, we utilize a fluorescent reporter model and optical tissue clearing techniques to reveal the presence, prevalence and position of M $\phi$ s in the mammary gland at all phases of development.

## MATERIALS AND METHODS

### Reagents

Neutral buffered formalin (NBF), Quadrol<sup>®</sup>, triethanolamine and 4',6-diamidino-2-phenylindole (DAPI) dilactate were purchased from Sigma Aldrich. Normal goat serum was purchased from ThermoFisher. Urea and sucrose were purchased from Chem-Supply. Triton-X-100 was purchased from VWR International. The following primary antibodies were used for immunostaining: chicken anti-GFP (Abcam, ab13970, batch #s GR3190550-3 and -12), rat anti-F4/80 (Novus, NB600-404), rat anti-keratin 8 (DSHB, TROMA-I, batch #s 7/7/16 and 30/3/17), rabbit anti-keratin 5 (BioLegend, 905504, batch # B230397) and rabbit anti-SMA (Abcam, ab5694, batch # GR3183259-26). The following secondary antibodies were used: goat anti-chicken Alexa Fluor-488 (ThermoFisher, A21236), goat anti-rat Cy3 (ThermoFisher, A10522) and goat anti-rabbit Alexa Fluor-647 (ThermoFisher, A21245).

### Animal Models

Animal experimentation was carried out in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes* and the *Queensland Animal Care and Protection Act (2001)*, with local animal ethics committee approval. Animals were housed in individually ventilated cages with a 12 h light/dark cycle. Food and water were available *ad libitum*. *Csf1r-EGFP* (MacGreen) (Sasmono et al., 2003) mice were a kind gift from A/Prof Allison Pettit (Mater Research Institute-UQ). Mice were maintained as hemizygotes on a C57BL6/J background. C57BL6/J mice were obtained from the Animal Resources Center (Western Australia).

To obtain mammary tissue during gestation, female mice were mated and tissue harvested 14.5 days-post-coitus (mean no. embryos: 7; range: 6–9). GFP<sup>+</sup> embryos (E14.5) were also harvested and analyzed after PCR-sexing. To obtain tissue during lactation, female mice were mated, allowed to litter naturally and lactating mammary tissue harvested on day 10 of lactation. For studies during involution, females were allowed to nurse for 10 days and mammary glands harvested 96 h post forced involution. Litter sizes were not standardized (mean litter size: 7; range: 5–10). Mammary glands from pre-pubertal female GFP<sup>+</sup> mice (postnatal day 10), pubertal (6.5 weeks) and post-pubertal (12 weeks) were also harvested and analyzed. No estrus staging was performed in these studies. In all mice the 2nd, 3rd, 4th, and 5th mammary glands were excised and fixed as described above; 2nd/3rd and 5th mammary glands were preferentially selected for 3D imaging, owing to their smaller size.

### CUBIC-Based Tissue Clearing and IHC

Tissue clearing was performed as previously optimized and described (Davis et al., 2016; Lloyd-Lewis et al., 2016). Briefly, mammary tissue was spread on foam biopsy pads and fixed for 6–9 h in NBF (10%). Embryos were fixed whole. For CUBIC-based clearing, tissue was immersed in Reagent 1A (Susaki et al., 2014; Lloyd-Lewis et al., 2016) at 37°C for 3 days before

washing and blocking in goat serum (10%) in PBS with Triton-X-100 (0.5%) overnight at 4°C. Tissue was incubated in primary antibody in blocking buffer for 4 days and secondary antibody in blocking buffer for 2 days at 4°C. DAPI (5 µg/mL) treatment was performed for 2–3 h at room temperature [omitted for second harmonic generation (SHG)] and tissue was immersed in modified Reagent 2 (Lloyd-Lewis et al., 2016) at 37°C for at least 24 h prior to imaging.

## Immunohistochemistry (FFPE Slides)

IHC on FFPE slides was performed as previously described in detail (Stewart et al., 2019). Wholemout immunostaining using anti-GFP antibody was performed prior to processing for paraffin embedding.

## Microscopy

Immunostained tissue sections were imaged using an Olympus BX63 upright epifluorescence microscope using UPlanSAPO 10 × /0.4, 20 × /0.75, 40 × /0.95, 60 × /1.35, and 100 × /1.35 objective lenses. Immunostained optically cleared tissue was imaged using an Olympus FV3000 laser scanning confocal microscope with UPLSAPO 10 × /0.40, UPLSAPO 20 × /0.75, UPLSAPO 30 × /1.05, and UPLFLN 40 × /0.75 objective lenses. 3D de-noising was performed as previously described (Boulanger et al., 2010). For SHG, images were acquired using a Mai Tai DeepSee multiphoton laser on a Zeiss 710 laser scanning inverted microscope. Visualization and image processing was performed in ImageJ (v1.52e, National Institutes of Health) (Linkert et al., 2010; Schindelin et al., 2012).

## RESULTS

### Mφs Are Present in the Embryonic Bud and Early Postnatal Gland With Sexually Dimorphic Distribution

Mφs have never been visualized in the embryonic mammary gland. A recent study by Jäppinen et al. revealed the presence of F4/80<sup>+</sup> cells in digested mammary tissue by E16.5 by flow cytometry (Jäppinen et al., 2019). However, in the absence of *in situ* imaging, it is currently unclear whether these embryonic Mφs physically associate with the developing mammary epithelium, as has been observed in the postnatal gland.

To assess Mφ distribution in 3-dimensions in intact mammary tissue, we used a *Csf1r-EGFP* mouse model (Sasmono et al., 2003), combined with methods for optical tissue clearing and deep tissue imaging (**Supplementary Figure S1**) (Davis et al., 2016; Lloyd-Lewis et al., 2016, 2018). In this model, green fluorescent protein (GFP) expression in tissues is restricted to monocytes and Mφs in the developing embryo, starting with yolk sac-derived phagocytes, and in all adult tissues (Sasmono et al., 2003; Hume D. A. et al., 2019). Much lower expression in granulocytes and some B lymphocytes is detectable by FACS, but not in tissues. Multi-color fluorescence immunostaining of tissue sections from mouse spleen confirmed that the majority

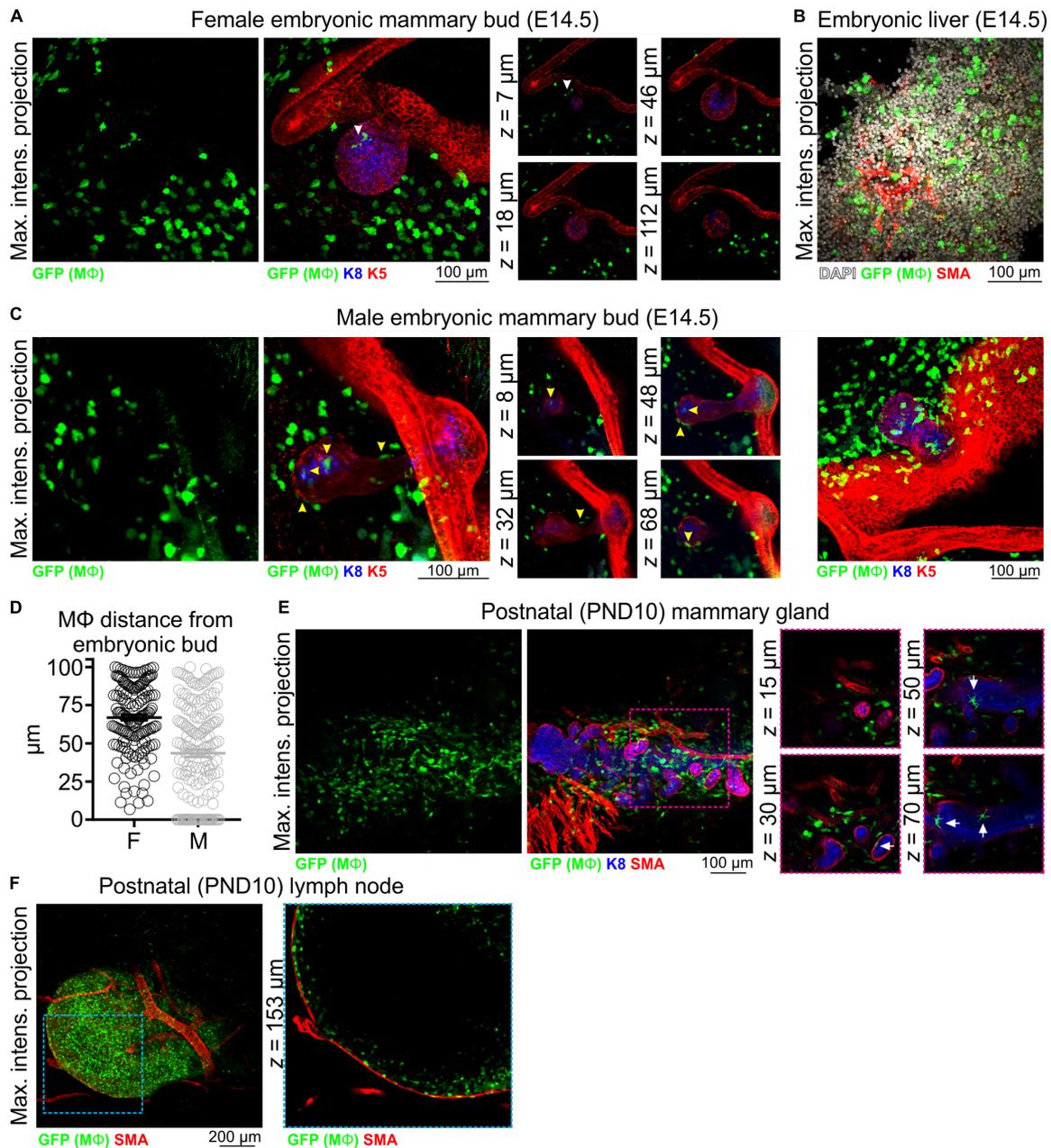
of GFP<sup>+</sup> cells were also positive for the Mφ cell surface marker, F4/80 (**Supplementary Figure S2**). Previous studies using digested mammary tissue from *Csf1r-EGFP* mice analyzed by flow cytometry have shown that >90% of GFP<sup>+</sup> cells in the mammary gland react with F4/80 (Chua et al., 2010; Hodson et al., 2013).

In 3D image stacks of female *Csf1r-EGFP* embryos, Mφs were detected in the mammary and dermal mesenchyme surrounding the mammary epithelial bud as early as E14.5 (**Figure 1A** and **Supplementary Figure S3A**). As expected (Sasmono et al., 2003), Mφs were also present in the embryonic liver at this stage (**Figure 1B**), and it has been suggested that these fetal liver-derived Mφs contribute extensively to the pool of tissue Mφs present in the adult gland (Jäppinen et al., 2019). Our data show that Mφs were positioned adjacent to the embryonic mammary epithelium around the time of lineage segregation (Lilja et al., 2018; Wuidart et al., 2018). Interestingly, although Mφs were positioned around the embryonic bud, they were rarely observed to directly interact with the developing epithelium of female embryos (**Figure 1A** and **Supplementary Figure S3A**). In contrast, Mφs directly contacted and invaded the mammary bud of male mice at E14.5, the developmental period when the male bud is severed from the overlying epidermis in mice and begins to regress (**Figures 1C,D** and **Supplementary Figure S3B**) (Dunbar et al., 1999; Heuberger et al., 2006; Cowin and Wysolmerski, 2010). Mammary Mφs were also observed in the early postnatal period in female mice (**Figures 1E,F**). By this stage, however, Mφs were positioned around and inside of this rudimentary structure, apparently interacting with the epithelium (**Figure 1E**).

### Mφs Envelope and Infiltrate the Elongating Terminal End Bud During Ductal Morphogenesis

Mφs are essential for normal ductal morphogenesis during puberty (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002; Ingman et al., 2006). Pre-pubertal leukocyte depletion using sub-lethal  $\gamma$ -irradiation is associated with impaired ductal development and in Mφ-deficient *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice, misshapen TEBs fail to properly invade the mammary fat pad at the rate observed in age-matched controls (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002; Ingman et al., 2006). Previous studies analyzing Mφ density and distribution in mouse mammary tissue sections have shown recruitment of F4/80<sup>+</sup> Mφs to the pubertal epithelium and their convergence around the neck of TEBs (Gouon-Evans et al., 2000; Schwertfeger et al., 2006), where adult mammary stem/progenitor cells are thought to reside (Sreekumar et al., 2015; Lloyd-Lewis et al., 2017).

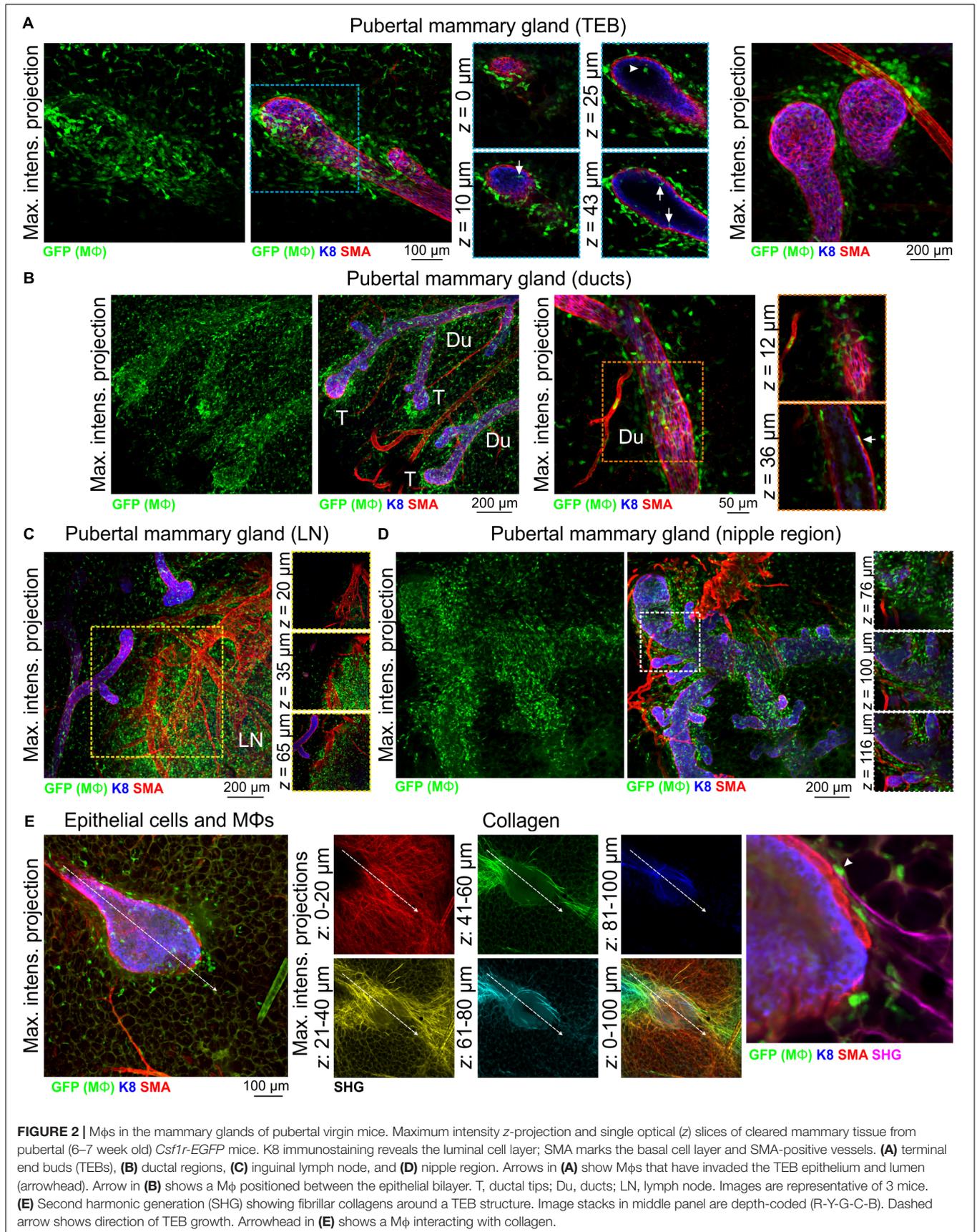
3D imaging of mammary tissue from pubertal *Csf1r-EGFP* mice revealed that mammary TEBs were enveloped by Mφs, with spatial clustering observed (**Figure 2A** and **Supplementary Figure S4A**). Previous studies using the F4/80 marker indicated that Mφs were mainly distributed at the neck of TEBs, whereas eosinophils (distinguished by their eosinophilic cytoplasm and bi-lobed nuclei) were concentrated at the TEB head (Gouon-Evans et al., 2000, 2002). By contrast, in this study GFP<sup>+</sup> Mφs in both locations shared stellate morphology

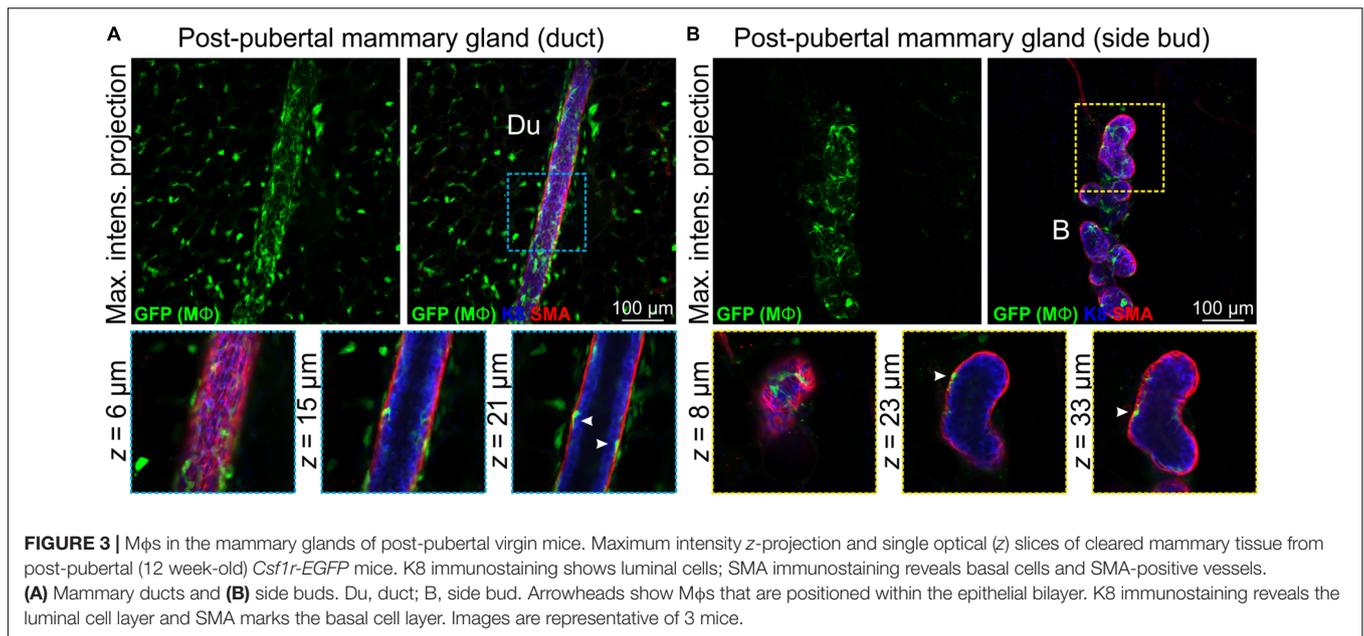


**FIGURE 1** | Mφs in the embryonic and early postnatal mouse mammary gland. Maximum intensity z-projection and single optical (z) slices of cleared tissue from **(A,B)** embryonic (E14.5) female mice and **(C)** embryonic (E14.5) male mice. **(D)** The distance of Mφs (within a 100 μm radius) of the female and male embryonic buds. Mφs contacting the bud or inside of the bud were assigned a value of 0; this was only observed in male embryos. **(E)** Mammary tissue from postnatal day (PND) 10 *Csf1r-EGFP* female mice. **(F)** Inguinal lymph node from PND10 mice showing subcapsular sinus Mφs. Keratin (K) 8 immunostaining shows K8-positive luminal cells; K5 immunostaining reveals K5-expressing basal cells; smooth muscle actin (SMA) immunostaining reveals basal cells and SMA-positive vessels. White arrowhead in **(A)** points to a Mφ that appears to be in contact with the embryonic bud in the maximum intensity projection, but is revealed to be positioned in the mammary mesenchyme above the bud in optical slices. Yellow arrowheads in **(C)** point to Mφs that are in direct contact with the embryonic bud. Arrows in **(E)** point to Mφs that are in contact with the PND10 mammary epithelium. Images are representative of 3 mice/embryos at each developmental stage.

(Figure 2A and Supplementary Figure S4A) and neither showed any evidence of segmented nuclei (Supplementary Figure S4A). A small number of mammary Mφs were observed inside the body of TEBs (Figure 2A), where they may contribute to clearance of apoptotic cells from the TEB lumen

(Humphreys et al., 1996; Gouon-Evans et al., 2000; Paine and Lewis, 2017). GFP<sup>+</sup> Mφs were found along the length of the ductal epithelium in the pubertal gland (Figure 2B and Supplementary Figure S4B) and in some cases appeared to be positioned between the luminal and basal cell layers





(Figure 2B, arrow). Intraepithelial Mφs, detected with F4/80, are a feature of ductal epithelia throughout the body (Hume D. A. et al., 1984). It is currently unclear how these interposed Mφs affect luminal-basal cell connections [e.g., desmosomes and gap junctions (Shamir and Ewald, 2015)] and their precise function within the epithelial bilayer. GFP<sup>+</sup> cells were also dispersed throughout the mammary fat pad (Figure 2 and Supplementary Figure S4; Schwertfeger et al., 2006; Chua et al., 2010) and were densely packed in the inguinal lymph node (Figure 2C and Supplementary Figure S4B) and nipple region (Figure 2D).

Mammary Mφs have been shown to organize collagen into fibrillar bundles to steer TEB growth through the stromal fat pad (Ingman et al., 2006). We therefore examined fibrillar collagens with SHG (Williams et al., 2005) in tissue from *Csf1r-EGFP* mice at depth using an immersion-based optical clearing approach, which preserves endogenous fluorescence and tissue architecture (Lloyd-Lewis et al., 2016; Vigouroux et al., 2017). Although surface collagen fibers in the mammary gland were dense and multi-directional [Figure 2E (red)], deeper collagen fibers proximal to the growing TEB were aligned along its perimeter, extended in the direction of TEB growth and were associated with Mφs (Figure 2E). These data provide further evidence that mechanical forces from the stroma guide epithelial development in the normal mammary gland (Ingman et al., 2006; Stewart et al., 2019).

## Mφs Are Intimately Associated With the Mature Ductal Epithelium

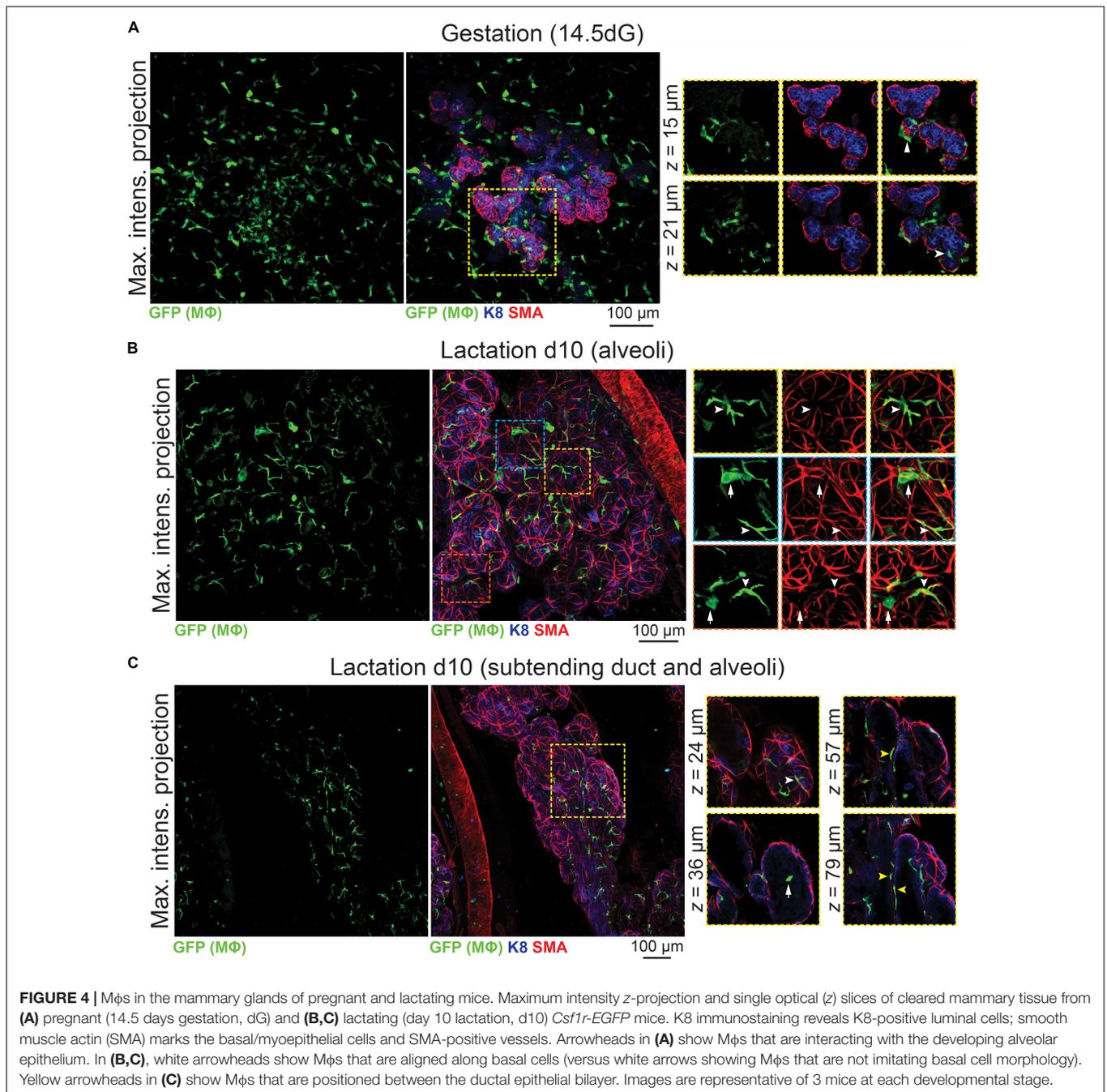
Mφs are present in the post-pubertal mouse mammary gland at all phases of the estrus cycle, with the numbers being highest in diestrus (Chua et al., 2010). In tissue sections at all estrus stages, F4/80<sup>+</sup> cells are detectable around alveolar

side buds versus ducts, where they are thought to promote the development and regression of these transient structures (Chua et al., 2010). Using 3D imaging of mammary tissue from *Csf1r-EGFP* mice, we observed similar numbers of Mφs closely associated with mammary ducts (Figure 3A and Supplementary Figure S5) and side buds (Figure 3B and Supplementary Figure S5A). As in the pubertal epithelium, Mφs were also positioned between the luminal and basal cell layers in mature ducts and buds (Figures 3A,B and Supplementary Figure S5B, arrowheads) with some evidence of periodicity in intraepithelial Mφ placement (Supplementary Figure S5B). This is consistent with regular distributions of Mφs in many locations throughout the body (Hume D. et al., 2019). SHG of mature ducts revealed some fibrillar collagens that were located around the ducts and vessels (Supplementary Figure S5C).

## Mφs Surround Alveolar Units in Gestation and Lactation

Mφ deficient *Csf1<sup>op</sup>/Csf1<sup>op</sup>* female mice have compromised fertility (Pollard et al., 1991). Amongst those that do generate offspring, none are able to nurture a full litter, despite normal maternal behaviors (Pollard and Hennighausen, 1994). In-depth analyses of mammary tissue from pregnant and lactating *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice showed incomplete branching and precocious alveolar development (Pollard and Hennighausen, 1994) and F4/80<sup>+</sup> cells have been detected around the developing and functional alveolar units during pregnancy and late gestation (Gouon-Evans et al., 2002).

3D analysis of mammary tissue from pregnant *Csf1r-EGFP* mice (day 14.5 gestation, dG) confirmed Mφ localization around the expanding alveolar structures (Figure 4A and Supplementary Figure S6). By lactation, Mφs were observed immediately adjacent to alveolar basal cells, where they frequently imitated basal cell morphology (Figures 4B,C, white



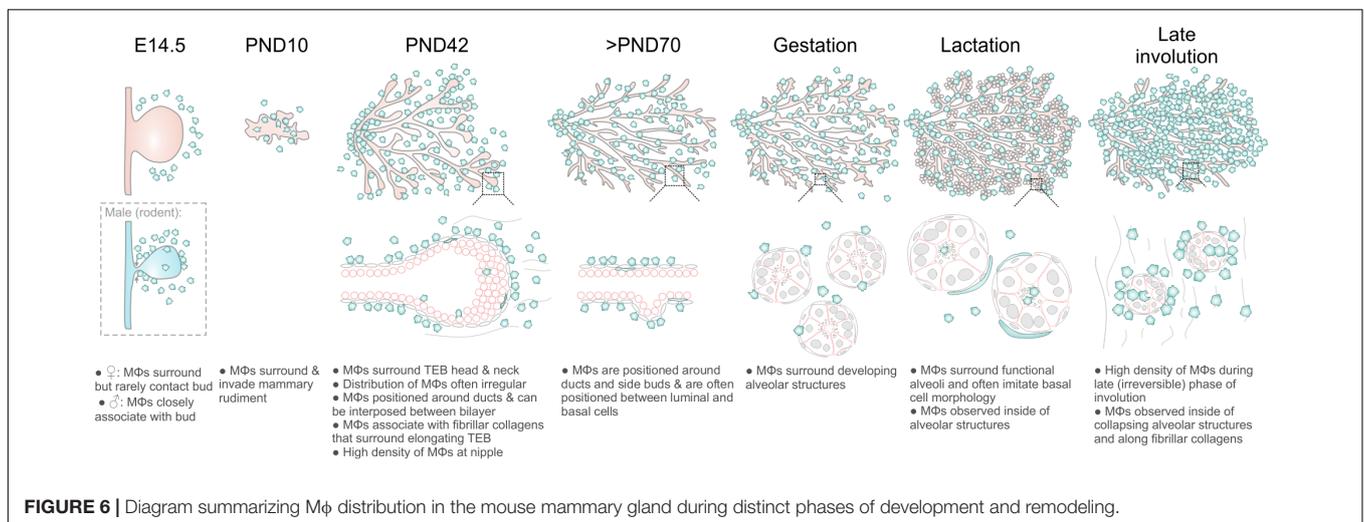
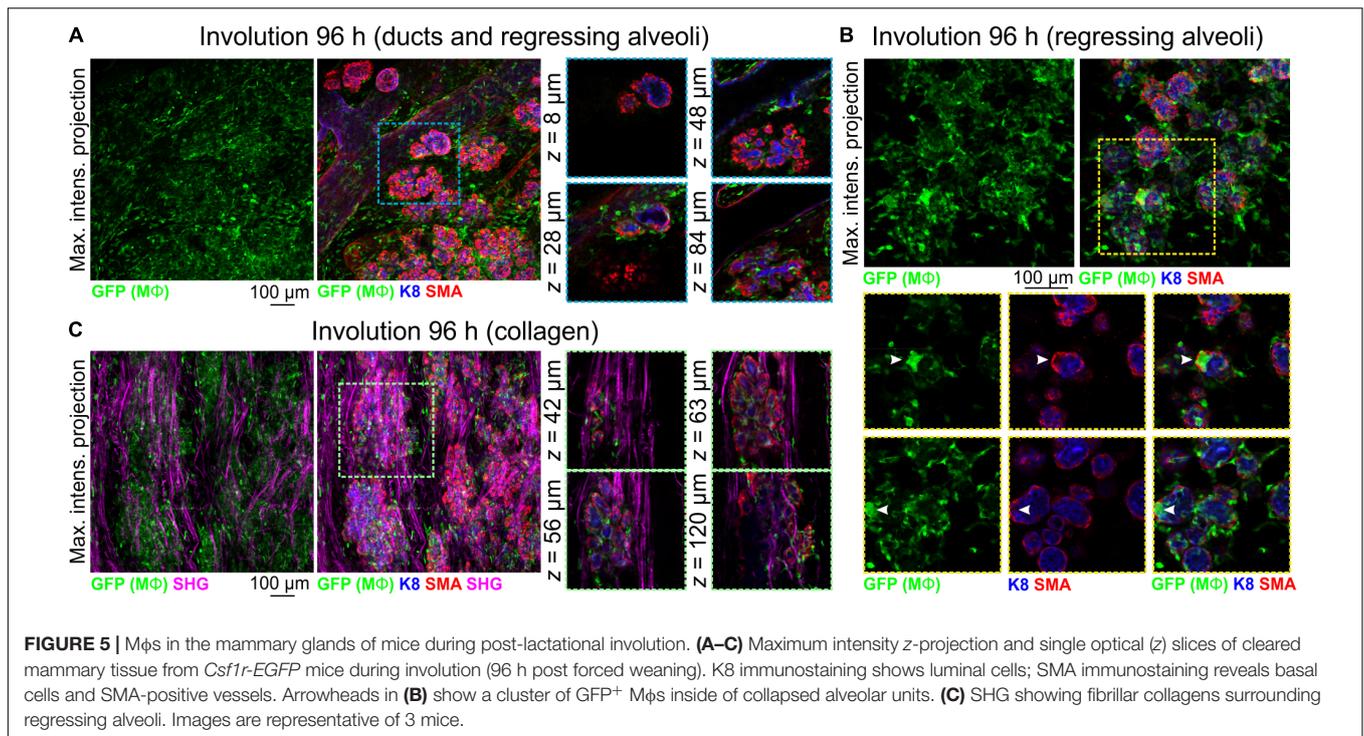
arrowheads). M $\Phi$ s were also present within lactational alveoli (Figure 4C, arrow), consistent with their enrichment in breast milk (Field, 2005).

### The Irreversible Phase of Involution Is Associated With an Increase in M $\Phi$ Number in and Around Regressing Alveolar Structures

The number of M $\Phi$ s surrounding the mammary epithelium increases drastically from days 3–4 of involution

(Lund et al., 1996; Stein et al., 2004; Hughes et al., 2012), and involution-associated M $\Phi$ s appear polarized toward tissue repair (O'Brien et al., 2010). The recruitment and polarization of M $\Phi$ s in the involuting mammary gland is regulated by epithelial *Stat3* expression (Hughes et al., 2012). Moreover, pre-weaning depletion of CSF1R-expressing cells reduces mammary epithelial cell death during post-lactational involution, an effect that can be reversed by orthotopic transplantation of bone marrow-derived M $\Phi$ s (O'Brien et al., 2012).

To further examine M $\Phi$  number, morphology and distribution in the regressing mammary gland in 3-dimensions, we analyzed



optically clear tissue from *Csf1r-EGFP* mice during the irreversible phase of involution. Relative to other developmental stages, Mφ density was high at 96 h involution and Mφs were observed around and inside ducts and regressing alveoli (Figures 5A,B). Large aggregates of GFP<sup>+</sup> cells, reminiscent of homotypic fusion (MacLauchlan et al., 2009), were also observed inside degenerating alveolar structures (Figure 5B arrowheads). Similar aggregates of GFP<sup>+</sup> Mφs have been observed in a model of epithelial regeneration in the kidney following transient ischemia (Joo et al., 2016).

Collagen density increases during mammary gland involution and partially degraded non-fibrillar collagens have been suggested to be chemotactic for Mφs (O'Brien et al., 2010). Intra-

and interlobular fibrillar collagens were observed with SHG in *Csf1r-EGFP* mice and GFP<sup>+</sup> Mφs were observed to be associated with collagen fibrils (Figure 5C).

## DISCUSSION

Mφs contribute to mammary gland development and remodeling at all developmental stages (Pollard and Hennighausen, 1994; Gouon-Evans et al., 2000; Dai et al., 2002; Van Nguyen and Pollard, 2002; Ingman et al., 2006; Chua et al., 2010; O'Brien et al., 2010, 2012; Hughes et al., 2012). The exact mechanisms by which tissue Mφs regulate these processes are still being elucidated

(Schwertfeger et al., 2006) and may be linked to their phagocytic, trophic and/or matrix remodeling functions (Sternlicht, 2006; Pollard, 2009). A comprehensive characterization of the stage-specific physiological roles of M $\phi$ s in the mammary gland depends upon knowledge of their precise anatomical location within this organ. In this study, we provide new insights into the allocation, morphology and distribution of M $\phi$ s in the embryonic, pre-pubertal, pubertal, post-pubertal, pregnant, lactating and involuting mammary glands of fluorescent reporter-positive mice *in situ* in 3-dimensions (**Figure 6**). Our study yields a number of important observations that could only be revealed by multi-dimensional imaging using a tamoxifen-independent, cell type-specific fluorescent reporter model (Hume D. et al., 2019; Hume D. A. et al., 2019). Firstly, in contrast to previous reports (Gouon-Evans et al., 2000, 2002), we demonstrate that M $\phi$ s are not concentrated at the TEB neck, although some polarity in their distribution around TEBs was observed. These findings suggest that M $\phi$ s may regulate mammary epithelial cells both within the head and neck of the TEB structure (Paine et al., 2016). Studies performing intravital imaging of TEB dynamics in *Csf1r-EGFP* mice are an aim for the future and may help to reveal possible correlations between M $\phi$  density and TEB behavior (e.g., turning and bifurcation events).

Mammary M $\phi$ s were also frequently embedded between luminal and basal cells of the ductal epithelium. This has previously been observed in mammalian ductal epithelia, including the bile duct, salivary gland, tracheobronchial gland and mammary gland using thin sections prepared from formalin-fixed paraffin-embedded or frozen tissue (Hume D. A. et al., 1984; Sun et al., 2013). Regularity in the spacing of these intraepithelial M $\phi$ s was also noted, which may arise through mutual repulsion (Hume D. et al., 2019) and could potentially contribute to regular distribution of adjacent populations of heterogeneous luminal and basal cells (Ismail et al., 2002; Davis et al., 2016). In sum, the work presented here suggests a close functional relationship between M $\phi$ s and ductal epithelial cells, and possible communication between morphologically related M $\phi$  populations. Further studies are needed to determine whether these intraepithelial M $\phi$ s share similar gene and protein expression patterns and whether this information can be used to probe their function, retention and passage within the epithelium. Tissue M $\phi$ s have been shown to be influenced by properties of their specific niche within each tissue (e.g., anchoring scaffolds and local cues) (Chakarov et al., 2019; Mondor et al., 2019). Single cell sequencing of isolated mammary M $\phi$ s from *Csf1r-EGFP* mice at distinct developmental stages, as exemplified by recent studies of other tissues (Chakarov et al., 2019; Mondor et al., 2019), might help to reveal the extent of functional diversity within M $\phi$  populations in this organ.

We reveal that M $\phi$ s alter their morphology at distinct developmental stages, including the transition from gestation to lactation. The localization of M $\phi$ s around growing alveolar units during gestation and the observation that M $\phi$ -deficient *Csf1<sup>OP</sup>/Csf1<sup>OP</sup>* mice exhibit precocious alveolar development, suggests that during this phase, alveolar-associated M $\phi$ s may restrain alveologenesis. By analogy, M $\phi$ s in the diaphragm appear to constrain the growth of lymphatic vessels and *Csf1r* mutation

promoted branch formation of lymphatic sprouts (Ochsenbein et al., 2016). During lactation, M $\phi$ s altered their anatomical position and were observed to closely imitate the morphology of adjacent, differentiated alveolar basal cells. Whether these cells specifically align themselves with oxytocin-responsive basal cells during lactation to modify basal cell function (Davis et al., 2015; Stevenson et al., 2019) or more simply to occupy the physical space that these force-exerting cells create within the alveolar epithelium (Davis, 2016; Stewart et al., 2019), remains to be seen. Such a function might be analogous to the role of a distinct population of CSF1-dependent M $\phi$ s in the regulation of peristalsis in the muscularis externa of the intestine (Muller et al., 2014). Interestingly, in this study muscularis M $\phi$ s and intestinal motility could be reversibly modified by lumen factors (Muller et al., 2014). Whether mammary M $\phi$ s, positioned alongside alveolar basal cells, are capable of sampling the alveolar lumen environment to constrain basal cell-mediated alveolar contractility (e.g., in mastitis) has not yet been determined. Another possibility is that basal cell contractility may instead alter the function of alveolar M $\phi$ s. Such an effect has been observed in the lung, another organ that is subject to cyclical mechanical stimulation, although this phenomenon was restricted to newly recruited monocytes and not the population of resident alveolar M $\phi$ s (Solis et al., 2019). Finally, we were able to visualize for the first time tissue-resident M $\phi$ s in the mesenchyme surrounding the mammary epithelial bud in 14.5 day-old female embryos. Intriguingly, these embryonic M $\phi$ s rarely contacted the epithelial cells of the developing mammary bud at this stage of embryogenesis. This is in striking contrast to epithelial-M $\phi$  interactions in the early postnatal period, where M $\phi$ s surround and invade the rudimentary ductal epithelium. This also contrasts with the male embryo, where M $\phi$ s were often observed to both contact and infiltrate the epithelial bud at the time when its connection to the overlying epidermis is severed and the structure begins to regress (Dunbar et al., 1999; Heuberger et al., 2006; Cowin and Wysolmerski, 2010). At this stage, M $\phi$ s may have an important role in clearing apoptotic epithelial and mesenchymal cells (Dunbar et al., 1999; Henson and Hume, 2006).

Mammary stem/progenitor cells are located within the mammary bud (in the embryo) and TEBs (in puberty). After ductal elongation is complete and TEBs regress, however, the location of long-lived mammary stem/progenitor cells and their putative niche remains unknown, although it has been suggested that these cells are deposited along the ductal epithelium by elongating TEBs (Davis et al., 2016; Lloyd-Lewis et al., 2017). In the 14.5 day embryo, M $\phi$ s were positioned uniformly around, but not in contact with, the mammary bud. These data suggest that if a mammary stem cell-macrophage niche exists in the embryo around the time of lineage segregation, it operates over the scale of tens of micrometers and is fairly homogeneous. M $\phi$ s were also positioned around pubertal TEBs, however, in contrast to the embryo, these cells contacted and infiltrated TEBs, were more densely arranged around these structures and often showed spatial clustering. Future studies combining tamoxifen-independent *Dll1-mCherry* (Chakrabarti et al., 2018) and *Csf1r-EGFP* mouse models with optical tissue clearing and 3D imaging may help to reveal the precise location of mammary

stem/progenitor cells within TEBs and the post-pubertal ductal epithelium. It should be noted, however, that whilst ductal elongation is delayed in *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice, these structures are still capable of invading the fat pad and by 12 weeks of age have reached the fat pad limits (Gouon-Evans et al., 2000). These findings imply that mammary epithelial cells have mechanisms to overcome insufficiencies in niche signaling. One candidate is the alternative CSF1R ligand, IL34, which may also be expressed by mammary epithelial cells (DeNardo et al., 2011). Studies investigating the activation and roles of the CSF1R in mammary development have been thwarted by the severe postnatal phenotype of *Csf1r<sup>-</sup>/Csf1r<sup>-</sup>* mice (Chitu and Stanley, 2017), but may be more amenable to study in recently described *Csf1r<sup>-</sup>/Csf1r<sup>-</sup>* rats (Pridans et al., 2019). Alternatively, these findings may reflect a long-term plasticity in mammary epithelial cells (Lilja et al., 2018) and a shifting definition of “stemness” in some tissues away from a unidirectional, top-down model to a model where stemness is considered as a cell state that may be acquired or extinguished under specific microenvironmental conditions (Laplane and Solary, 2019). A closer examination of mammary cell behaviors—including lineage segregation—under conditions of M $\phi$  depletion may provide important insights into epithelial plasticity in this vital mammalian organ.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/Supplementary Files.

## ETHICS STATEMENT

The animal study was reviewed and approved by the University of Queensland Health Sciences Animal Ethics Committee. In accordance with the Australian Code for the Care and Use of

Animals for Scientific Purposes and the Queensland Animal Care and Protection Act (2001).

## AUTHOR CONTRIBUTIONS

FD and TS performed all the experiments. FD, DH, and TS, conceived and designed the experiments. TS, KH, DH, and FD analyzed the results. FD wrote the manuscript. DH, KH, and TS edited the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2019.00250/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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