Title
Adaptive Immune Regulation of Mammary Postnatal Organogenesis

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Authors
Plaks, V
Boldajipour, B
Linnemann, JR
et al.

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Adaptive Immune Regulation of Mammary Postnatal Organogenesis

Highlights

- Adaptive immune responses regulate postnatal organogenesis of an epithelial tissue
- Epithelial-associated antigen-presenting cells negatively regulate MG organogenesis
- Antigen-mediated interactions with CD4+ T helper 1 cells regulate MG organogenesis
- IFNγ, produced by T helper 1 cells, affects luminal epithelial differentiation

Authors

Vicki Plaks, Bijan Boldajipour, Jelena R. Linnemann, ..., Andrew C. Melton, Matthew F. Krummel, Zena Werb

Correspondence

vicki.plaks@ucsf.edu (V.P.), zena.werb@ucsf.edu (Z.W.)

In Brief

Plaks et al. provide evidence that antigen-mediated interactions between mammary CD11c+ antigen-presenting cells and interferon-γ (IFNγ)-producing CD4+ T helper 1 cells provide signals that negatively regulate mammary postnatal organogenesis. This response participates in orchestrating epithelial rearrangement by locally affecting luminal epithelial differentiation.

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Adaptive Immune Regulation of Mammary Postnatal Organogenesis

Vicki Plaks, Bijan Boldajipour, Jelena R. Linnemann, Nguyen H. Nguyen, Kelly Kersten, Yochai Wolf, Amy-Jo Casbon, Niwen Kong, Renske J.E. van den Bijgaart, Dean Sheppard, Andrew C. Melton, Matthew F. Krummel, and Zena Werb

1Department of Anatomy
2Department of Pathology
3Lung Biology Center, Department of Medicine
University of California, San Francisco, San Francisco, CA 94143, USA
4Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel
*Correspondence: vicki.plaks@ucsf.edu (V.P.), zena.werb@ucsf.edu (Z.W.)
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SUMMARY

Postnatal organogenesis occurs in an immune competent environment and is tightly controlled by interplay between positive and negative regulators. Innate immune cells have beneficial roles in postnatal tissue remodeling, but roles for the adaptive immune system are currently unexplored. Here we show that adaptive immune responses participate in the normal postnatal development of a non-lymphoid epithelial tissue. Since the mammary gland (MG) is the only organ developing predominantly after birth, we utilized it as a powerful system to study adaptive immune regulation of organogenesis. We found that antigen-mediated interactions between mammary antigen-presenting cells and interferon-γ (IFNγ)-producing CD4+ T helper 1 cells participate in MG postnatal organogenesis as negative regulators, locally orchestrating epithelial rearrangement. IFNγ then affects luminal lineage differentiation. This function of adaptive immune responses, regulating normal development, changes the paradigm for studying players of postnatal organogenesis and provides insights into immune surveillance and cancer transformation.

INTRODUCTION

Prenatal organ development takes place in the presence of an immature immune system and continues after birth as the immune system reaches its full potential. In epithelial organs such as lung, breast, and intestine, postnatal organogenesis is regulated by communication between epithelial cells and innate immune cells (Reed and Schwertfeger, 2010; Renz et al., 2012). During this period, the previously immature adaptive immune system is exposed to antigens and rapidly develops. Therefore, postnatal development of various epithelial tissues occurs in an immune-competent environment. However, whether the adaptive immune system regulates normal organ development in non-pathologic conditions has yet to be explored. Whereas other epithelial organs have relatively minor postnatal developmental phases, the mammary gland (MG) is the only organ that develops predominantly after birth, during puberty (Wiseman and Werb, 2002). At birth, the MG consists of an epithelial rudimentary tree. In mice, mammary postnatal organogenesis starts with onset of puberty, at 3–4 weeks of age, and is characterized by ductal invasion until the growing and branching epithelial ducts reach the fat pad limits by 8–9 weeks of age (Figure S1A) (Sternlicht et al., 2006). A secondary expansion occurs with each estrus and menstrual cycle in rodents and humans, respectively, as well as during pregnancy and lactation. This secondary expansion regresses during the process of involution (Watson et al., 2011). Although steroid and peptide hormones are major initiators and regulators of mammary postnatal organogenesis, it is also tightly regulated by intracellular and extracellular signals between epithelial cells and the surrounding microenvironment or stroma, which includes extracellular matrix, fibroblasts, adipocytes, and immune cells (Lu and Werb, 2008).

Innate immune cells such as colony-stimulating factor-1 (CSF-1)-dependent macrophages, eotaxin-dependent eosinophils (Gouon-Evans et al., 2002), and mast cells (Lilla and Werb, 2010) are involved in the positive regulation of pubertal mammary ductal expansion. Interestingly, based on the immune cells involved, postnatal MG development has been compared to a wound healing process (Reed and Schwertfeger, 2010). During wound healing, antigen-presenting cells (APCs) become activated and recruit other immune cells, including CD4+ T helper cells, to create an environment for tissue remodeling. However, to date, the presence and role of APCs and T cells in the development of the mammary organ have not been assessed.

Since in the mouse the MG develops from a rudimentary epithelial tree to a fully developed organ within the few weeks of puberty (Sternlicht et al., 2006), it is a powerful model system to explore whether antigen-mediated interactions between APCs and tissue T cells contribute to the normal postnatal development of non-lymphoid epithelial tissues. To explore roles for the adaptive immune system in local regulation of mammary postnatal organ development, we utilized transgenic mouse models that allowed for genetic ablation or overrepresentation of immune cell subsets and explored ductal invasion in whole-mount MGs. In parallel, we also performed live imaging and...
RESULTS

Epithelial-Associated Mammary CD11c+ Cells Negatively Regulate Branching Morphogenesis

CD11c is a selective marker for dendritic cells but also for some macrophages (Engelhardt et al., 2012; Vallon-Eberhard et al., 2006), both of which have antigen presentation capacities (Gautier et al., 2012; Miller et al., 2012). We found CD11c+ cells, which have dendritic-like features, closely associated with the mammary epithelium (Figures 1A and S1B; Movie S1). To study the role of these CD11c+ cells in mammary postnatal organogenesis, we utilized organoids cultured in fibroblast growth factor 2 (FGF2) (Figure 1B), which are well characterized ex vivo branching models to study postnatal mammary organogenesis (Ewald et al., 2008). These surrogate assays not only reflect the ductal elongation aspect of epithelial branching, which depends on cell proliferation and epithelial surface expansion (Zhang et al., 2014), but also allow the elimination of any organ non-specific or hormone-dependent effects. To assess whether these CD11c+ cells influenced MG organogenesis, we used CD11c-DTR:GFP mice (Jung et al., 2002), which express the diphtheria toxin receptor under the CD11c promoter. Utilizing organoids from CD11c-DTR:GFP MGs, we found that CD11c+ cells are closely associated with the mammary epithelium, and then we depleted them by diphtheria toxin (DTx) administration either ex vivo during organotypic culture (Figures 1C and 1D) or in vivo before organoid preparation (Figures 1E and 1F). In both cases, CD11c+ cell depletion accelerated epithelial branching (Figures 1D, 1F, and 1H–1I). These data suggest an inhibitory role for CD11c+ cells in the morphogenesis of pubertal MGs (Figure 1G).

Epithelial-Associated Mammary CD11c+ Cells Have Characteristics of APCs

We next characterized the epithelial-associated mammary CD11c+ cells. Interrogation of molecular markers using surface stains and transgenic reporters (see Supplemental Experimental Procedures) revealed that these CD11c+ cells express high levels of CX3CR1 (Figure 2A), CSF-1 receptor (CSF-1R, using the c-fms transgene) (Figure 2B), and F4/80 (Figure 2C). Most interestingly, they express high levels of major histocompatibility complex (MHC) II (Figure 2D), which is essential for antigen presentation, as well as intermediate levels of CD11b (Figure 2E). The absence of Siglec-F expression (Figure S2A) suggested that these CD11c+ cells are APCs of the monocytic lineage rather than eosinophils (Gautier et al., 2012; Gouon-Evans et al., 2000; Miller et al., 2012). In addition, we observed a macrophage-type population associated with the organoids, which is F4/80+, high for CD11b, and low for CD11c and MHCII (Figures 2C and 2E).

We examined the interactions of these CD11c+ APCs with the mammary epithelium during branching morphogenesis. Because the fluorescence of CD11c+ cells from the CD11c-DTR:GFP model is too dim for time-lapse confocal microscopy and since most CD11c+ cells are CX3CR1+, we used organoids functional analyses in primary three-dimensional (3D) organotypic mammary branching assays (i.e., organoids).
from CX3CR1-GFP/+ reporter mice, in which the GFP signal is considerably brighter (Jung et al., 2000). These experiments revealed that the CX3CR1+ APCs actively interacted with the mammary epithelium and proliferated during branching of organoids (Figures 2F and 2G; Movies S2 and S3) and mammary ductal invasion in vivo (Figure S2B). Interestingly, several studies report the in situ proliferation of both macrophages and dendritic cells (Davies et al., 2013; Veres et al., 2013; Ginhoux et al., 2009). CSF-1 expressed by mammary epithelial cells (Figure S2C) located at the neck of terminal end buds (Coussens and Pollard, 2011) could contribute to the proliferation of mammary APCs, which express CSF-1R (Figure 2B). During branching, these APCs also engulfed cellular particles (Figure 2H; Movie S4), a prerequisite for antigen presentation by monocyte-derived APCs.

**T Cells Are Involved in the Negative Regulation of Mammary Organogenesis**

Activation of the adaptive immune system requires the presence of APCs (Hoebe et al., 2004). APCs link the innate and adaptive immune systems by capturing antigens in tissues and presenting them to T cells in lymph nodes. After activation, T cells home to non-lymphoid tissues, where they exert their effector functions (Hoebe et al., 2004). We therefore asked whether T cells are also involved, along with CD11c+ APCs, in the negative regulatory loop of mammary postnatal organogenesis (Figure 3A). Significantly, we found a reduction of mammary CD4+ and CD8+ T cell numbers after depletion of APCs, both in vivo (Figures 3B and S1C) and in organoids (Figure S3A). These data suggest that APCs may regulate epithelial morphogenesis not only by attracting T cells to or retaining T cells in MGs but also by preventing their death. We reasoned that in order to regulate mammary postnatal organogenesis, T cells would need to interact closely with the epithelium, and indeed we found CD4+ and CD8+ T cells closely associated with the mammary ducts (Figures 3C, S3B, and S3C). Finally, to test whether T cells contribute to MG organogenesis, we examined TCRα- deficient (Tcra−/−) mice, which lack CD4+ and CD8+ T cells. We observed accelerated ductal outgrowth in Tcra−/− MGs (Figures 3D and 3E) and increased branching in Tcra−/− organoids (Figure 3F), supporting an inhibitory role for CD4+ T cells, CD8+ T cells, or both in MG organogenesis.

**Antigen Presentation via MHCII to CD4+ T Cells Negatively Regulates Mammary Organogenesis**

We next sought to determine which T cell type was involved in the regulation of MG morphogenesis. For this purpose we used organoid cultures that, similar to intact MGs, also contain T cells (Figure 4A). In addition to their role in the lymph node, APCs may reactivate T cells within non-lymphoid tissues (Brez et al., 2014; Honda et al., 2014). To determine whether APCs regulate mammary organogenesis through T cells, we inhibited MHC-mediated APC-T cell activation with blocking antibodies (Banchereau and Steinman, 1998). Inhibiting MHCII-mediated CD4+ T cell stimulation resulted in accelerated branching (Figure 4B), whereas inhibiting MHCII-mediated CD8+ T cell stimulation had no effect. These data suggest that antigen presentation to CD4+ T cells may be involved in the
negative regulation of mammary organogenesis. Antigen presentation requires the close association of APCs and T cells. Indeed, CD11c+ APCs and CD4+ T cells were in close proximity and co-localized to the mammary epithelium in sections of pubertal MGs (Figure 4C). To confirm that epithelial-associated mammary APCs were capable of T cell activation via antigen presentation, we measured T cell proliferation by co-culturing organoids with ovalbumin (OVA)-specific (OT-II) CD4+ T cells. In this system, only presentation of their cognate peptide antigen (pOVA) by APCs will cause the activation and proliferation of these OT-II-naive CD4+ T cells. Indeed, upon the addition of pOVA to the culture media, OT-II T cells proliferated extensively (Figures 4D, S4A, and S4B). To further test whether the presence and activation of CD4+ T cells is important for MG organogenesis in vivo, we utilized MHCII<sup>loxx<sup>fl/fl</sup></sup>/C0<sup>3</sup>CD11c-cre mice, which are devoid of CD4+, but not CD8+ T cells, due to lack of MHCII expression on CD11c+ APCs (Figure S4C). MGs from these mice showed accelerated ductal outgrowth (Figures 4E and 4F) and increased organoid branching (Figure 4G). Taken together, these results suggest a negative regulatory role for CD4+ T cell activation in MG organogenesis (Figure 4H).

**CD4+ Th1 Cells Negatively Regulate Mammary Organogenesis**

Since CD4+ effector T cells function as distinct subsets (e.g., Th1, Th2, Th17, and T regulatory cells) specialized for specific adaptive immune responses (Zhu et al., 2010), we next asked which subset regulates MG organogenesis. By flow cytometric analysis, we observed predominantly the Th1 phenotype within MGs (Figures 5A and S5A). We therefore determined whether activation of CD4+ Th1 cells could inhibit mammary epithelial morphogenesis by adding these cells to the organoid cultures. Even though CD4+ Th2 cells are very rare in pubertal MGs, we included these cells in our analysis because they often counteract the effects of CD4+ Th1 and therefore may yield opposing results. Using time-lapse confocal microscopy, we noticed that Th1 and Th2 cells both interacted with MG APCs (Figure S5B; Movies S5 and S6), and dye-dilution experiments showed that antigen presentation induced their proliferation (Figure 5B). Interestingly, while Th1-polarized OT-II cells inhibited branching, the presence of Th2 cells increased branching (Figures 5C and S5C). Remarkably, only the addition of pOVA to the OVA-specific effector T cell-organoid co-cultures caused the significant decrease or increase in branching, suggesting that T cell activation by mammary APCs is required in situ in order to regulate MG organogenesis. However, since culturing the Th1 or Th2 cells in the presence of interleukin (IL) 2 likely promoted the production of their effector cytokines (Zhou et al., 2003), this could explain the respective initial negative or positive effect they still had on branching even without pOVA (Figure 5C). Interestingly, depletion of APCs from the T cell-organoid co-cultures rescued the effect that OVA-specific Th1 (and Th2) cells had on branching (Figure S5D), validating a role for in situ antigen presentation. The reduction in T cell numbers after depletion (Figures 3B and S3A) may further suggest that restimulation from local tissue APCs is required for T cell survival. Moreover, in support of this notion, we observed that mammary organoids from OT-II mice that are naturally enriched with OVA-specific Th1 cells exhibited decreased branching when cultured with pOVA (Figure 5D). Given that Th1 effector cells are the predominant CD4+ T cells within MGs, the accelerated branching of wild-type (WT) organoids after blockade of MHCII (Figure 4B) also suggested that in situ antigen presentation to epithelial-associated CD4+ Th1 cells plays a role in organoid branching.

**Figure 3. T Cells Are Involved in the Negative Regulation of Mammary Organogenesis**

(A) Schematic depicting the question of whether APC may negatively regulate MG postnatal organogenesis via interaction with T cells.
(B) Flow cytometry of CD4+ and CD8+ T cells (gated on CD3+), 48 hr post-PBS/DTx injections to CD11c-DTR (n = 6 MGs).
(C) Immunohistochemistry shows co-localization of CD4+ and CD8+ T cells to mammary epithelium in intact glands.
(D) Comparison of epithelial ductal outgrowths from carmine-red-stained MG whole mounts from WT and Tcra<sup>-/-</sup> mice.
(E) Quantification of ductal outgrowths in (D) (n = 8 WT and 4 Tcra<sup>-/-</sup> MGs).
(F) Increased branching in Tcra<sup>-/-</sup> organoids (n = 3 experiments).

Data in (B), (E), and (F) are represented as mean ± SEM. See also Figure S3.
We next examined the role of CD4+ Th1 cells in mammary postnatal organogenesis in vivo. Given the MG Th1 dominance, the MHCII fl/fl CD11c-cre mice lacking CD4+ T cells (Figures 4E–4G) effectively served as a model devoid of mammary Th1 cells. For a mouse model with an overrepresentation of CD4+ Th1 cells within MGs, we exploited the integrin b8fl/fl CD11c-cre mice, which have increased frequency and number of Th1 cells, while all other MG CD4+ T cell subsets are diminished (Figures 5F, S5E, and S5F). Importantly, these mice show no change in CD11c+ cell number or phenotype (Travis et al., 2007; Melton et al., 2010). The b8fl/fl CD11c-cre mice demonstrated significantly impaired MG ductal invasion, reinforcing the role of Th1 cells as the relevant negative regulatory subset within MGs (Figures 5G and 5H). We also observed blunted branching in organoids from these Th1-enhanced b8fl/fl CD11c-cre MGs (Figures 5I, S5G, and S5H). To confirm that the impaired organogenesis of b8fl/fl CD11c-cre mice depends on increased frequency and numbers of Th1 cells rather than defective APCs, we crossed b8fl/fl CD11c-cre mice into the severe combined immunodeficient (SCID) background (Figures 5 J–5L). Moreover, since the b8fl/fl CD11c-cre is the only model thus far that exhibited inhibited branching, we examined the MGs from older mice and found that the blunted branching of b8fl/fl CD11c-cre MGs persisted beyond puberty (Figure 5M). Interferon-γ (IFNγ) secreted by Th1 cells participates in driving Th1 differentiation downstream of IL-12 from naive T cells in a positive feedback loop (Magram et al., 1996). In the absence of IFNγ, most CD4+ T cells fail to differentiate into the Th1 effector phenotype (Ishii et al., 2013). We therefore crossed the b8fl/fl CD11c-cre mice into the IFNγ-deficient background (Ifng−/−). MGs from Ifng−/− b8fl/fl CD11c-cre mice revealed comparable ductal invasion and organoid branching to Ifng−/− controls (Figures S5I–S5K). These data further propose that the excess of Th1 cells in b8fl/fl CD11c-cre MGs led to their impaired development. To this end, our data suggest that tissue antigen presentation by mammary CD11c+ APCs and reactivation of CD4+ Th1 cells play roles in the negative regulation of MG organogenesis (Figure 5N).

IFNγ Mediates the Inhibitory Effect of CD4+ Th1 Cells on Mammary Organogenesis by Affecting Luminal Differentiation

IFNγ is the primary effector cytokine secreted by Th1 cells (Schroen and Wilson, 2007; Zhu et al., 2010) and is also a characteristic of mammary CD4+ Th1 cells (Figures 5A and 5B). Morphological changes of branching epithelial organs are governed by a meticulous local regulation of epithelial rearrangement (Ewald et al., 2008). To determine whether IFNγ can mediate the effects of Th1 cells on mammary organogenesis, we first tested whether it has a direct effect on mammary epithelial cells. The mammary epithelium consists of luminal and basal lineages, which are characterized by specific surface markers and cytokeratins (Figure S6A) (Rios et al., 2014; Shehata et al., 2005).
Figure 5. Antigen Presentation to CD4+Th1 Effector Cells Negatively Regulates Mammary Organogenesis

(A) CD4+ T cell subsets in pubertal MGs based on flow cytometry data. Other CD4+ cells are naive or non-activated cells.

(B) In vitro differentiated Th1- and Th2-polarized OVA-specific CD4+ (OT-II) T cells proliferate when co-cultured with organoids and pOVA (n = 3 experiments) (Movies S5 and S6). Figure shows representative histograms. Counts: Th1 no pOVA: 165; +pOVA: 1,798. Counts: Th2 no pOVA: 158; +pOVA: 451.

(C) In vitro differentiated Th1- or Th2-polarized OT-II cells decreased and increased branching, respectively, when co-cultured with organoids. The addition of pOVA alone did not affect branching as compared to IL-2 control. However, the addition of pOVA to the OVA-specific effector T-cell-organoid co-culture caused the significant decrease or increase in branching, suggesting that antigen presentation by MHCII+ CD11c+ APCs (the only MHCII-high cells associated with organoids) plays a significant role in mediating the effect of these effector T cells on organogenesis (n = 3 experiments).

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et al., 2012). Luminal cells express cytokeratin (KRT) 8 and basal cells express KRT14. In addition, in mouse MGs, both lineages are positive for the surface marker epithelial cell adhesion molecule (EpCAM). Basal cells express lower levels of EpCAM, but they are high for CD49f. We incubated mammary organoids with IFNγ and observed a transient nuclear translocation of phosphorylated STAT1 (pSTAT1), a hallmark of IFNγ receptor (IFNγR) signaling (Figures 6A and S6B). The nuclei of mammary luminal cells (marked by KRT8) were positive for pSTAT1 in confirmation of previous findings (Figure 6B) (Khalkhali-Ellis et al., 2008; Zhu et al., 2010). Corroborating the activation of IFNγR signaling in luminal cells, we also observed enriched expression of IFNγR1 in the luminal fraction of organoids (Figure 6C). This is in keeping with previous studies showing IFNγR1 and IFNγR2 expression in cultured normal human mammary epithelial cells, which represent a mixture of luminal and basal cell populations based on their cytokeratin expression (Khalkhali-Ellis et al., 2008), while proteomic profiling revealed that IFNγR1 is present only on a subset of luminal progenitors in vivo (Ji et al., 2011). Functionally, we found that incubation of organoids with IFNγ inhibited branching (Figure 6C), confirming an inhibitory role for IFNγ in mammary morphogenesis.

Given that IFNγ signaling affects mammary epithelial branching, we examined the effects of IFNγ on mammary epithelial

(D) The addition of pOVA to organoids from OT-II × CD2-RFP mice (bearing only OVA-specific Th1 cells) inhibited branching, suggesting that the effector OT-II cells associated with the organoids were reactivated with the addition of their cognate peptide antigen (n = 3 experiments).

(E) Flow cytometry of Th1 cells.

(F) Analysis of CD4+ T cell subpopulations in control and β8fl/fl × CD11c-cre mice (n = 4 control; n = 8 β8fl/fl × CD11c-cre MGs).

(G) Comparison of epithelial ductal MG outgrowths from control versus β8fl/fl × CD11c-cre mice.

(H) Quantification of ductal outgrowths from (G) (n = 6 MGs each).

(I) Decreased branching in β8fl/fl × CD11c-cre organoids (n = 3 experiments).

(J) Comparison of epithelial ductal MG outgrowths from SCID control versus SCID β8fl/fl × CD11c-cre mice.

(K) Quantification of ductal outgrowths from (J) (n = 4 control; n = 6 β8fl/fl × CD11c-cre MGs).

(L) Comparable branching of SCID control and β8fl/fl × CD11c-cre organoids (n = 3 experiments).

(M) Decreased branching in β8fl/fl × CD11c-cre MGs persists beyond puberty. These MGs showed inhibited postnatal organogenesis (versus accelerated organogenesis in all other models examined here), with blunted ductal outgrowths persisting beyond the pubertal organogenesis phase, as examined at 9 weeks of age (n = 4 MGs each).

(N) Schematic depicting antigen presentation to CD4+ Th1 cells negatively regulating mammary organogenesis.

Data in (C), (D), (F), (H), (I), (K), and (L) are represented as mean ± SEM. See also Figure S5 and Movies S5 and S6.
lineage determination. Indeed, gene profiling of mammary organoids after incubation with IFNγ and sorting into the luminal and basal cell populations (Figure S6C) confirmed that IFNγ affects the luminal lineage by downregulating the expression of some mature and progenitor luminal cell markers (Figure 6D). We found a significant reduction in the expression of the luminal progenitor markers Cd49b and Cd14 (Shehata et al., 2012). We also found a significant decrease in expression of the mature luminal markers progesterone receptor (Pgr), Gata3, and Muc1. The expression of the luminal progenitor marker Elf5 did not change. Lmo4 and Mfge8, two other luminal markers, also did not significantly change in expression following incubation with IFNγ (Shehata et al., 2012). Although the luminal marker Krt8 decreased, the change in its expression was not statistically significant, similar to Ifngr1. These results imply that branching in organoids cultured in FGF2, which reflects ductal elongation and is governed by proper luminal differentiation, is disrupted by IFNγ. Even though we detected no significant change in the overall frequency of luminal cells (Figure S6C), we found increases in both proliferation and apoptosis of cells located within the lumens of IFNγ-treated organoids (Figures S6D and S6E), reinforcing that changes occur within the luminal population. The effects on the basal lineage were not significant for the progenitor and basal markers tested (Figure S6F).

Last, we investigated how significant is IFNγ secreted by CD4+ Th1 cells to mammary epithelial branching and whether CD4+ Th1 cells could regulate the mammary epithelium through factors other than IFNγ. For this purpose we added IFNγ-neutralizing antibodies to organoid cultures with excessive numbers of CD4+ Th1 cells and found that these cultures showed significantly accelerated branching (Figure 6E). Similarly, organoids from Ifngr1−/− mice, which like Ifng−/− mice do not have properly functioning Th1 cells, showed accelerated branching (Figure 6F). Taken together, our data suggest that Th1 cells inhibit mammary organogenesis by locally inhibiting luminal differentiation through IFNγ secretion (Figure 6G).

**DISCUSSION**

In this study we have investigated whether the adaptive immune system locally contributes to postnatal mammary organogenesis. By using the postnatal MG as a model of immune-competent organ development, we have demonstrated a role for the adaptive immune system in regulating organogenesis of a non-lymphoid epithelial tissue. We provide compelling evidence that antigen-mediated interactions between mammary CD11c+ APCs and CD4+ IFNγ+ Th1 cells provide signals that negatively regulate ductal invasion. We show that mammary epithelial cells respond directly to IFNγ. These effects are reinforced by enriched expression of IfnγR1 on mammary luminal epithelial cells and activation of downstream signaling pathways upon IFNγ stimulation. This response participates in orchestrating epithelial rearrangement by locally affecting luminal epithelial differentiation.

Although the nature of the antigen or antigens involved remains to be discovered, the significance of our findings is several fold. First, we recognized an engagement or reactivation of the adaptive immune system in the regulation of normal epithelial organ architecture, under non-pathologic conditions. Second, these results have implications for understanding immune surveillance. Third, the data raise interesting questions regarding possible roles for T cell dysfunction in pathologic organ remodeling as in early stages of breast cancer transformation.

**APCs versus Other Innate Immune Cells Implicated in Mammary Organogenesis**

Several seminal papers over the last two decades have demonstrated roles for mast cells, eosinophils, and macrophages in terminal end bud elongation during postnatal MG organogenesis (CousSENS and Pollard, 2011; Gouon-Evans et al., 2000; Lilla and Werb, 2010; Van Nguyen and Pollard, 2002). Macrophages also have a role in regulating epithelial cell death during postpartum MG involution (O’Brien et al., 2012).

Csft10−/− mice and Csft1−/− mice, both lacking F4/80+ CSF1-R+ macrophages, exhibit decreased terminal end bud numbers and reduced mammary duct length, and branching during puberty (Dai et al., 2002; Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002). This phenotype is consistent with CSF-1R+ cells positively regulating ductal elongation. The mammary CD11c+ APCs that we describe here are also F4/80+ and CSF1-R+, yet these cells negatively regulate MG development. However, we also found a larger population of F4/80+ cells closely associated with the mammary epithelium, which expresses higher levels of the macrophage marker CD11b but is low for CD11c and MHCI (Figure 2E). These potentially immature or non-activated macrophages could be part of those macrophages previously described as positive regulators of mammmary pubertal development (Gouon-Evans et al., 2000).

Since the Csft10−/− study does not characterize epithelial-associated cells differentially, but rather examines the MG stroma as a whole, there may potentially be additional F4/80+ and CSF1-R+ populations located in distant stroma. Indeed, F4/80+ eosinophils, which are also lacking in Csft10−/− mice and have been identified as positive regulators of mammary ductal morphogenesis (Gouon-Evans et al., 2000), are almost exclusively located in the distant stroma fraction (Figure S2A). Therefore, it seems that our study identified a discrete population of CSF-1R+ APCs within the mammary stromal milieu, with opposing regulatory potential or location to those cell types identified in the studies on Csft10−/− MGs. The fact that very little is known about the negative regulators of mammmary postnatal organogenesis further adds to the value of this study. Since positive and negative regulators need to act in concert to orchestrate organogenesis, it will be interesting to examine how these cells strike the balance for proper MG postnatal organogenesis and whether antigen-mediated interactions participate in other stages of mammary morphogenesis such as pregnancy, lactation, and involution.

**Downstream Effects of the Th1 Effector IFNγ on Mammary Epithelial Cells**

In the present study, we focused on IFNγ, which is the cytokine characteristic of Th1 effector T cells, the predominant CD4+ effector T cell subset in the developing MG. We showed that IFNγ mediates the effect of Th1 cells on postnatal mammary organogenesis and acts directly on epithelial cells, as demonstrated by the transient nuclear translocation of pSTAT1 after IFNγ stimulation. Gene profiling of mammary organoids revealed
changes in mature and progenitor luminal cell markers, most of which exhibited decreased expression after incubation with IFNγ. Our results are reinforced by a recent study, demonstrating a role for STAT1, the downstream target of IFNγ, in MG development (Chan et al., 2014). This study showed that there is a significant increase in the number of alveolar buds in Stat1−/− mice as compared with WT mice and that excessive bud formation is observed in mature Stat1−/− mice. Although no T cell data or treatment with IFNγ was examined, the authors suggest that signaling via STAT1 negatively regulates luminal progenitor cells, as loss of STAT1 results in an expansion of CD61+ luminal progenitor cells, similar to the effects of loss of IFNγ in our study. Specifically, we show that organoids exposed to IFNγ exhibit significantly reduced expression of CD49b, a marker for mammary luminal progenitor cells. This CD49b+ population also includes IFNγR1-expressing CD61+ cells (Ji et al., 2011; Shehata et al., 2012).

Furthermore, in our experiments we observed that exposing organoids to activated Th2 cells induces accelerated branching (Figure 5C). This is in line with data showing that epithelial-secreted Th2 type cytokines IL-4 and IL-13, along with their downstream activators STAT6 and GATA3, promote luminal differentiation during alveolar expansion throughout gestation (Khaled et al., 2007). Together, this supports the idea that Th cell effector cytokines directly regulate mammary epithelial cells. While we did not find a role for CD8+ T cells in our study, these cells also secret IFNγ upon activation (Schoenborn and Wilson, 2007) and thus may still have roles in mammary organogenesis. We do not rule out the potential existence of pathways alternate to the one we have delineated in this study.

As a side note, our study does not argue that disrupted adaptive immune regulation would necessarily have fatal effects on mammary ductal invasion but rather suggests a role for adaptive immune regulation as an important homeostatic modulator of normal epithelial development. Since mice have ten MGs, they could potentially still nurse pups even if the epithelia in some or all glands are partially impaired or fail to cover the entire fat pad.

Implications for Cancer Progression

Immune surveillance is an important factor in preventing tumor growth, and mechanisms of normal organogenesis are frequently disrupted during cancer progression. Our data suggest that a Th1 bias in normal MGs restricts epithelial branching. By interacting with epithelial cells, Th1 type cytokines IFNγ and IL-12 induces MHCIi expression that further drives local Th1 responses (Biswas and Mantovani, 2010; Strutt et al., 2010). In contrast, breast tumor models are characterized by a Th2 bias (Czarneski et al., 2001; Jensen et al., 2003). Our observation of increased branching in Th2-organoid co-cultures echoes the observation of Th2 cells dominating mammary adenocarcinomas and promoting invasion as well as pulmonary metastasis (DeNardo et al., 2009). In fact, numerous findings in cancer research imply that survival and differentiation of mammary epithelial cells that form tumors are indeed regulated by a delicate balance between Th1/Th2 signaling.

A Th1 bias is more effective in tumor rejection. It has been shown that the combined action of the Th1 cell cytokines, IFNγ, and tumor necrosis factor (TNF) induces permanent growth arrest and drives breast and other cancers into senescence (Braumüller et al., 2013). This cytokine-induced senescence strictly requires STAT1 and TNFR1 signaling, respectively. Moreover, in an estrogen receptor (ER) α+ luminal type human breast cancer model that is heavily infiltrated by innate immune cells and T lymphocytes (Bos et al., 2013), IFNγ and CD4+ T cells (but not NK or CD8+ T cells) show anti-tumor activity and inhibit metastatic tumor progression via extensive apoptotic tumor cell death. Other studies imply that immune escape mechanisms in ERα+ breast cancer may be facilitated through an ERα suppressive mechanism on IFNγ signaling (Mostafa et al., 2014). Indeed, in a mouse model of human epidermal growth factor receptor (HER) 2-positive breast cancers, tumor cells control the outcome of tumor immune surveillance through modulation of IFNγR1 expression on tumor cells. Those that express low levels of IFNγR1 fail to be eliminated by IFNγ-produing CD8+ T cells and remain dormant and quiescent until they conceal themselves from the adaptive immune system by losing the tumor antigen, Neu (Kmieciak et al., 2013). Finally, using a Stat1 floxed model that permits tissue-specific disruption of STAT1 in mammary epithelium and a model of HER2-positive breast cancer (Neu/ERBB2-induced) allowed the investigators to identify a role for STAT1 in mammary tumor suppression separate from its role in the immune system, (Klover et al., 2010). This is also in line with the findings that Stat1-deficient mice develop ERα+ mammary tumors (Chan et al., 2014).

Our work warrants future studies examining how the adaptive immune system modulates the fate of normal epithelial and cancer cells. A follow-up study could then focus on the transition between positive and negative regulation during the different cycles of MG expansion and involution and during early mammary carcinogenesis. Understanding how this switch occurs and what impact it has on the epithelium could identify potential targets for intervention.

EXPERIMENTAL PROCEDURES

Experimental Animal Models

Experiments were approved by the UCSF Institutional Animal Use and Care Committee. Cx3cr1-GFP/+; C11c-DTR:GFP, c-fms−EGFP, Tcra−/− and MHCIIα− × CD11c-cre, OT-II × C57RFP, and integrin β8−/− × CD11c-cre (also crossed to Scid or Ilg−−−−) mice were on the C57BL/6 background.

In Vivo Depletion of CD11c+ Antigen-Presenting Cells

CD11c-DTR:GFP mice were depleted of APC by injecting diphtheria toxin (DTx; 4 ng/g weight; Sigma) intraperitoneally (i.p.) and were harvested 48 hr postinjection (see scheme in Figure S1C). Mutant DTx (4 ng/g weight) was injected as a control for possible DTx-activated epithelial growth factor (EGF) signaling.

Mammary Whole-Mount Carmine Staining and Analysis of Ductal Invasion

Inguinal (number 4) MGs excised at 7 weeks (no significant fat-pad size difference between littermates) were stained with carmine red and manually imaged with a Leica dissection scope using Nikon ACT-1 software. Data were quantified in a blind fashion.

Immunostaining of Intact Mammary Glands

MGs were fixed, and frozen sections were stained with anti-CD4 (eBioscience, 16-0041-82, 1:500), anti-CD8a (eBioscience, 16-0081-82, 1:500), and anti-GFP (CD11c-DTR:GFP; Abcam, ab5450, 1:200) antibodies and mounted with Vectashield + DAPI (Vector Laboratories) for nuclear staining.
3D Organotypic Culture and Analysis
MGs (numbers 3, 4, and 5 without lymph nodes) were digested in collagenase solution consisting of DMEM/F12, 2 μg/ml trypsin (GIBCO-BRL), 2 μg/ml collagenase IV (SIGMA), 10% fetal bovine serum (FBS), 0.5% insulin, and 0.1% gentamicin (UCSF Cell Culture Facility) and then treated with DNase (2U/μl) (SIGMA). Organoids were epithelial-enriched by differential centrifugations (pulsed to 1,500 rpm), plated (50–100 organoids/well and 3–4 wells/condition) with Growth Factor Reduced Matrigel (BD Biosciences), and cultured in DMEM/F12 with 1% Pen/Strep, 1% insulin, transferrin, sodium selenite, and supplemented with 2.5 mM FGF2 (Sigma). Ex vivo depletion of CD11c+ APCs is achieved by mixing organoids with DTx (1 μg/ml) before plating, mutant DTx (1 μg/ml) was added to control for DTx-activated EGF signaling. For some experiments, blocking antibodies were added to organoid cultures: 0.5 mg/ml anti-MHCII (14-5321-82; eBioscience), Rat IgG2b K Isotype Control (16-4031-81; eBioscience), and 1 mg/ml anti-MHCII (16-5999-82; eBioscience), anti-CD4 (16-0041-82; eBioscience) and Mouse IgG2a K Isotype Control (16-4722-81; eBioscience). For IFNγ blocking, 0.2 mg/ml of anti IFNγ (Clone XMG1.2, BioXCell) was used. IFNγ stimulation experiments were performed by adding IFNγ (PeproTech, #315-05, 5 ng/ml) on day 3 of culture. Organoids were graded by direct manual examination of the culture wells on days 5, 6, and 7. Branching quantifications are shown for day 5 of culture, unless otherwise indicated. Images were taken on a Zeiss Axios Observer A1 inverted microscope, using DFC400 camera and Leica application suite version 3 software (see additional description in Figure 1B).

Fixation and Staining of Organotypic Cultures
Matrigel drops were fixed and embedded in optimal cutting temperature (O.C.T.), Sections (20 μm) were stained with antibodies against pSTAT1 (Tyr701, Cell Signaling, 9167), GFP (for CD11c-DTR:GFP, Abcam, ab5450, 1:200), eIF2α (Clone XMG1.2), 20 ng/ml IL-4 (Peprotech), 2.5 U/ml IL-2, and 1 μg/ml OVA peptide. Th1/Th2 polarization was confirmed by intracellular Th1/Th2 cytokine stains (as in for Figure S5A) or by GFP expression indicative of Th2 cytokines, using the 4get IL-4 reporter mice. Th1/Th2 helper cells were enriched using the EasySep Mouse CD4 Enrichment Kit (StemCell Technologies), and 1 μM eFluor®70 cell proliferation dye was used for helper T proliferation assays. 10^5 naive or activated OT-II cells per well (stimulated with pOVA, 1 μg/ml, as indicated in Figure S4B) were co-cultured in organoid medium containing 10 U/ml human IL-2.

Flow Cytometry
Single cells were isolated from MGs with collagenase solution similar to organoid cultures but without trypsin in the digest. Differential centrifugations were used to isolate stroma versus epithelial-enriched fractions. For non-T-cell immune populations, samples were trypsinized and then incubated with RBC lysis buffer for red blood cell removal. Stainings were for CD11b, CD3, CD8, CD4, F4/80, MHCII, and Siglec-F (eBioscience). Intracellular cytokine analysis was performed by adding IFNγ (PeproTech, #315-05, 5 ng/ml) on day 3 of culture. Organoids were fixed and permeabilized with Fix/Perm solution (eBioscience). Intracellular cytokines were labeled with antibodies in permeabilization buffer (eBioscience). Data were acquired using BD LSRII and analyzed with FlowJo. The following antibodies (BD Biosciences or eBioscience) were used: anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-IL-17 (Th17+ CD4+ T cells, clone eBioI7B7), anti-IFNγ (Th1 effector CD4+ T cells, clone XMG1.2), anti-FoxP3 (T regulatory cells, clone FJK-16 s), and anti-IL-13 (Th2 effector CD4+ T cells, clone eBio13A).

In vivo proliferation was assessed by BrdU incorporation: 100 μl BrdU (10 μg/ml; BD Pharmingen) was injected i.p. Single cells from MGs were isolated as described above 2 hr postinjection, stained for extracellular surface markers, fixed, and permeabilized using the BD (Becton Dickinson) Cytofix/Cytoperm buffer (15 min at RT). The next day, intracellular BrdU was stained overnight using the fluorescent isothiocyanate (FITC) BrdU Flow Kit Staining Protocol (BD Pharmingen, cat. no.: 559619). Data were acquired using BD LSRII and analyzed with FlowJo.

For flow cytometry on organoid cultures, Matrigel was digested in 1 U of dispase (Becton Dickinson) to release the organoids, followed by 0.05% trypsin, and strained through a 70-μm filter. Dead cells and lineage-specific cells (CD31+, Ter119+, and CD45+, as described previously; Plaks et al., 2013) were excluded. Luminal and basal populations were sorted using antibodies against EpCam and CD49f (Shehata et al., 2012). To examine T cells after depletion of APCs in organoids, Matrigel drops were collected in medium and left on ice before the dispase and trypsin digestion. Dead cells were excluded and antibodies against CD11c versus CD90.2, CD4, and CD8 were used to quantify APCs versus T cells, respectively.

Th1/Th2 Polarization, T Cell Proliferation Assay, and Co-culture with Organoids
Lymph node cells or splenocytes from OT-II or OT-II x CD22-RFP x 4get mice were cultured in RPMI (Life Sciences/GIBCO) with 10% fetal calf serum (FCS) (Hyclone, Thermo Scientific), Culture for Th1 differentiation: 0.5 μg/ml anti-CD28 (clone PV-1), 10 μg/ml anti-IL-4 (clone 11B11), 10 ng/ml IL-12, 2.5 U/ml IL-2 (Peprotech), and 1 μg/ml OVA peptide 323–336 (GenScript). For Th2 differentiation: 0.5 μg/ml anti-CD28, 10 μg/ml anti-IFNγ (clone XMG1.2), 20 ng/ml IL-4 (Peprotech), 2.5 U/ml IL-2, and 1 μg/ml OVA peptide. Th1/Th2 polarization was confirmed by intracellular Th1/Th2 cytokine stains (as in for Figure S5A) or by GFP expression indicative of Th2 cytokines, using the 4get IL-4 reporter mice. Th1/Th2 helper cells were enriched using the EasySep Mouse CD4 Enrichment Kit (StemCell Technologies), and 1 μM eFluor®70 cell proliferation dye was used for helper T proliferation assays. 10^5 naive or activated OT-II cells per well (stimulated with pOVA, 1 μg/ml, as indicated in Figure S4B) were co-cultured in organoid medium containing 10 U/ml human IL-2.

Spinning Disk Time-Lapse Confocal Microscopy
Imaging of 3D organotypic culture was performed as described previously (Ewald et al., 2009). CellTracker Red or Blue (Life Technologies) stained the mammary epithelium. Quantification was done using ImageJ.

RNA Preparation and qRT-PCR Analysis
Basal and luminal mammary epithelial cells sorted from organoids were extracted for RNA using TRIzol Reagent (Life Technologies). CDNA was generated using iScript Reverse Transcription Supermix of RT-qPCR (Biorad). RT-PCR reaction was performed with iTaq Universal SYBR Green Supermix (Biorad) using ABI 7900 Real Time PCR system. Analysis was normalized to the housekeeping gene Ppia. Primer sequences used are listed in Supplemental Experimental Procedures.

Statistics
Two-tailed unequal Student’s t test was performed. For comparisons between more than two groups, one-way ANOVA was used. Statistical significance was considered when p < 0.05. Experiments were repeated at least three times, as indicated in the figure legends. Pooled data are represented as mean ± SEM.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.07.015.

AUTHOR CONTRIBUTIONS
V.P. conceived the study. V.P., with the help of B.B. and A.C.M., designed the study. V.P. and K.K. performed APC depletion experiments in organoids and produced and analyzed live-imaging data. V.P. and A.C.M. performed the mammary CD4+ effector T cell characterization. V.P. and J.R.L. performed the experiments with mice deficient of CD4+ T cells. V.P. and J.R.L. performed whole-mount analyses and supporting organoid experiments with all the other transgenic mouse models. V.P. and B.B. designed and performed the organoid T cell co-culture experiments as well as analyzed the flow cytometry data throughout the study. N.H.N. performed qPCR experiments and analyses. N.H.N. and N.K. performed the organoid experiments examining the effect of IFNγ on mammary epithelium. N.K. and R.J.E.v.d.B. performed the organoid experiments examining Csf-1 expression and T cell presence after APC depletion. A.-J.C. designed and performed the APC in vivo proliferation experiments. V.P. wrote the manuscript and, with N.K., N.H.N., and
R.J.E.v.d.B., designed figures and schematics. D.S., A.C.M., M.F.K., and Z.W. guided the overall approach, experimental design, and trajectory of the study as well as assisted in manuscript completion.

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