UNIVERSITY OF CALIFORNIA,

IRVINE

Inflammatory macrophages promote tumor initiation in BRCA1-associated breast cancer

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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DEDICATION

To my beloved husband Sam - without whom this PhD journey would never have been possible.

To my beloved Antony family - Amma, Aswin, Anni and Maya.

To my beloved mother-in-law and father-in-law.

To my beloved daddy - who died of cancer in 2002.

To all my friends and other family members

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LIST OF ABBREVIATIONS

BRCA1: Breast cancer gene 1

ER: Estrogen Receptor

PR: Progesterone Receptor

HER2: Human Epidermal Growth Factor Receptor 2

HR: Hormone Receptor

TNBC: Triple Negative Breast Cancer

PARP: Poly (ADP-ribose) Polymerase

GEMM: Genetically Engineered Mouse Model

RNA: Ribonucleic Acid

DNA: Deoxyribonucleic Acid

OSM: Oncostatin M

OSMR: Oncostatin M Receptor

TNF: Tumor Necrosis Factor

IL6: Interleukin-6

JAK: Janus Kinase

STAT: Signal Transducer and Activator of Transcription

MAPK: Mitogen Activated Protein Kinase

PI3K: Phosphoinositide 3 Kinase

gp130: Glycoprotein 130 (IL6 signal-transducing subunit)

scRNA-seq: Single-Cell RNA-Sequencing

UMAP: Uniform Manifold Approximation and Projection

CD45: Cluster of Differentiation 45

CD11b: Cluster of Differentiation 11b

CD163: Cluster of Differentiation 163

CD206: Cluster of Differentiation 206

WT: Wild Type

CODEX: CO-Detection by indexing

ROI: Region of Interest

SOCS3: Suppressor of Cytokine Signaling 3

FBS: Fetal Bovine Serum

SNN: Shared Nearest Neighbor

FFPE: Formalin Fixed Paraffin Embedded

H&E: Hematoxylin and Eosin

ACK: Ammonium Chloride Potassium (red blood cell lysis buffer)

EGF: Epidermal Growth Factor

FGF: Fibroblast Growth Factor

ROCK inhibitor: Rho Associated Coiled-Coil Containing Protein Kinase Inhibitor

TAM: Tumor Associated Macrophages

TIL: Tumor Infiltrating Lymphocytes

EMT: Epithelial Mesenchymal Transition

CSF1R: Colony Stimulating Factor 1 Receptor

Wap-Cre: mouse model which has Cre recombinase conditionally expressed under the *Wap* promoter. *Wap* = whey acidic protein

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Role of macrophages in BRCA1-mutant breast cancer initiation

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2. **Angela Lincy Prem Antony Samy,** Mohamed Ali Hussein, Gnanasekar Munirathinam. Eprinomectin: a derivative of ivermectin suppresses growth and metastatic phenotypes of prostate cancer cells by targeting the β-catenin signaling pathway. *Journal of Cancer Research and Clinical Oncology, 2023.*

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ABSTRACT OF THE DISSERTATION

Inflammatory macrophages promote tumor initiation in BRCA1-associated breast cancer

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Women with germline pathogenic variants in BRCA1 are predisposed to developing aggressive, early onset breast cancers, most commonly of the triple negative subtype. While defective DNA repair has long been considered the primary driver of BRCA1-associated tumorigenesis, accumulating evidence suggests that additional non-cell autonomous mechanisms contribute to disease initiation. In particular, BRCA1-mutant mammary glands exhibit pronounced epithelial hyperplasia and aberrant branching prior to tumor formation, raising the possibility that interactions between epithelial cells and the immune microenvironment play a causal role in early disease progression.

In this dissertation, I investigate how immune cells specifically macrophages regulate premalignant remodeling in BRCA1-mutant mammary tissue. Using a genetically engineered mouse model of BRCA1 and p53 deficiency, I combine single-cell RNA-sequencing, spatial proteomic imaging, flow cytometry, and functional organoid assays to characterize immune dynamics across early stages of disease. I find that BRCA1-mutant mammary glands display an early expansion and spatial reorganization of distinct macrophage subsets, including CX3CR1 high inflammatory macrophages enriched for cytokine signaling pathways such as Oncostatin M.

Spatial analyses reveal that these macrophage populations occupy defined niches relative to epithelial structures and hyperplastic lesions, suggesting specialized functional roles. Functional

co-culture assays demonstrate that macrophages from BRCA1-mutant tissue are critical to drive exaggerated epithelial growth and branching in mammary organoids, implicating macrophage-derived signals as drivers of aberrant morphogenesis. Together, these findings support a model in which immune epithelial crosstalk contributes to early tumor promoting remodeling in genetically predisposed tissue.

This work reframes BRCA1-associated breast cancer initiation as a process shaped not only by intrinsic epithelial defects, but also by immune regulation of tissue architecture. Understanding these early interactions may enable the development of immune based prevention strategies for individuals at high genetic risk.

Chapter 1

BRCA1-associated breast cancer: immune context and early disease

1.1 Opportunities for impact in BRCA1-associated breast cancer prevention

Germline pathogenic variants in the *BRCA1* gene confer one of the highest known lifetime risks for breast cancer, with cumulative incidence estimates ranging from approximately 60-80% by age 70 [1,5]. In addition to elevated lifetime risk, *BRCA1* mutation carriers tend to develop breast cancer at a younger age and are disproportionately diagnosed with aggressive disease subtypes, most notably triple negative breast cancer (TNBC). TNBC is defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, which excludes patients from the most effective targeted therapies currently available for breast cancer. As a result, treatment options for *BRCA1*-associated TNBC remain limited, and clinical outcomes are highly heterogeneous despite advances in chemotherapy, PARP inhibition, and immunotherapy.

The strong genetic penetrance and early identifiability of *BRCA1* mutations present a rare and compelling opportunity for cancer prevention. Unlike sporadic breast cancer, in which risk is influenced by a complex and often poorly defined interplay of genetic, hormonal, and environmental factors, *BRCA1* mutation carriers can be identified years or even decades before disease onset through genetic testing. This long latency period between mutation identification and tumor development creates a critical window during which preventive or interceptive strategies could be deployed.

At present, risk reduction for *BRCA1* mutation carriers relies largely on surgical interventions, including prophylactic bilateral mastectomy and salpingo-oophorectomy. While these approaches substantially reduce breast and ovarian cancer risk, they are associated with profound physical, psychological, and reproductive consequences. The irreversible nature of these procedures, coupled with their impact on body image, fertility, and hormonal health, underscores the urgent need for less invasive, mechanism-based strategies that preserve quality of life while meaningfully reducing cancer risk.

Efforts to develop such strategies have been hindered by an incomplete understanding of the earliest biological events that precede malignant transformation in *BRCA1*-deficient mammary tissue. Historically, *BRCA1*-associated tumorigenesis has been conceptualized primarily through the lens of genomic instability, with cancer initiation viewed as the cumulative result of DNA damage and defective repair. While this framework has been instrumental in shaping therapeutic approaches, including the successful clinical application of PARP inhibitors, it provides a limited view of disease initiation and does not fully explain why only a subset of mutation carriers ultimately develop cancer.

Increasing evidence suggests that tumor initiation in *BRCA1* mutation carriers is not solely a cell autonomous consequence of accumulated genetic lesions. Instead, early alterations in tissue organization, epithelial differentiation, stromal composition, and cell-cell communication may play central roles in shaping the trajectory toward malignancy. These tissue level changes may emerge long before overt tumors are detectable and may act to either promote or restrain malignant progression.

Among the non-cell autonomous factors implicated in early disease, the immune microenvironment has emerged as a critical regulator of tissue homeostasis, regeneration, and disease progression. Immune cells are increasingly recognized as active participants in normal mammary development and remodeling, raising the possibility that dysregulated immune-epithelial interactions may contribute to premalignant phenotypes in genetically predisposed tissue. Understanding how immune signals intersect with *BRCA1* deficiency during the earliest stages of disease is therefore essential for identifying actionable vulnerabilities that could be targeted for cancer prevention.

This chapter synthesizes current knowledge of *BRCA1*-associated breast cancer with a focus on early disease biology, mammary gland remodeling, and the immune context in which tumor initiation occurs. In particular, it highlights gaps in understanding surrounding immune-epithelial crosstalk prior to tumor formation and establishes a conceptual framework for investigating macrophage-mediated regulation of premalignant mammary remodeling.

1.2 Canonical and emerging functions of BRCA1

The *BRCA1* gene was originally identified through linkage analysis of families with hereditary breast and ovarian cancer, marking a transformative moment in the understanding of inherited cancer susceptibility. The *BRCA1* protein is large and multifunctional, containing several conserved domains that mediate interactions with a wide array of molecular partners. Its most extensively characterized role is in the maintenance of genomic integrity, particularly through participation in the homologous recombination (HR) pathway of DNA double strand break repair [2,3].

In this canonical role, *BRCA1* facilitates DNA end resection, promotes the recruitment of HR repair machinery, and coordinates cell cycle checkpoints to ensure that damaged DNA is not propagated during cell division. Loss of *BRCA1* function forces cells to rely on error prone repair mechanisms such as non-homologous end joining, resulting in chromosomal rearrangements, copy number alterations, and widespread genomic instability. This paradigm has shaped decades of research and provided the mechanistic rationale for synthetic lethal therapeutic strategies, most notably the use of PARP inhibitors in *BRCA1*-deficient tumors [3,4].

While defects in DNA repair are a defining feature of *BRCA1*-associated cancers, this framework alone does not fully account for several key observations. First, genomic instability does not readily explain the striking tissue specificity of *BRCA1*-associated malignancies, which predominantly arise in hormone-responsive organs such as the breast and ovary despite *BRCA1* being ubiquitously expressed. Second, histological, transcriptional, and architectural abnormalities have been detected in *BRCA1*-mutant mammary tissue prior to overt tumor formation, suggesting that early phenotypic changes precede malignant transformation. Third, not all *BRCA1* mutation carriers develop cancer, even after decades of impaired DNA repair, indicating that additional modifying factors influence disease risk.

Beyond its role in DNA repair, *BRCA1* has been implicated in a broad range of cellular processes, including transcriptional regulation, chromatin remodeling, ubiquitination, and hormone receptor signaling. *BRCA1* possesses E3 ubiquitin ligase activity and has been shown to regulate the stability and activity of estrogen and progesterone receptors, thereby influencing hormone driven transcriptional programs. Given the central role of hormonal signaling in mammary gland

development and differentiation, dysregulation of these pathways in *BRCA1*-deficient cells may profoundly alter epithelial fate decisions.

BRCA1 also interacts with chromatin-modifying complexes and transcriptional regulators, linking DNA repair machinery to broader epigenetic and transcriptional landscapes. Through these interactions, *BRCA1* can influence lineage specification, cellular differentiation, and responses to developmental cues. Disruption of these functions may contribute to altered epithelial identity and plasticity in *BRCA1*-mutant mammary tissue, potentially priming cells for malignant transformation.

Collectively, these emerging functions support a model in which cancer initiation in *BRCA1* mutation carriers reflects not only the accumulation of genetic damage but also developmental mis-programming and tissue level dysregulation. In this context, *BRCA1* deficiency may sensitize mammary epithelium to aberrant responses to environmental and microenvironmental cues, including immune derived signals. Understanding how these processes intersect with immune regulation is critical for uncovering early, targetable events in disease initiation.

1.3 The mammary gland as a dynamic and immune-regulated tissue

The mammary gland is a highly dynamic organ that undergoes extensive remodeling throughout life in response to hormonal cues associated with puberty, estrous cycling, pregnancy, lactation, and involution. These processes involve tightly coordinated changes in epithelial proliferation, differentiation, extracellular matrix deposition, and stromal organization. Importantly, mammary remodeling is not driven by epithelial cells alone; it requires the active participation of immune cells, fibroblasts, endothelial cells, and other stromal components.

Immune cells are now recognized as integral regulators of normal mammary gland development. Among these, macrophages play particularly critical roles in shaping mammary architecture. During puberty, macrophages localize to terminal end buds and promote ductal elongation and branching through the secretion of growth factors, cytokines, and matrix remodeling enzymes [16,17]. Genetic or pharmacologic disruption of macrophage recruitment or function during this developmental window results in impaired ductal morphogenesis, underscoring the necessity of macrophage-derived signals for normal mammary development.

Macrophages continue to orchestrate tissue remodeling during pregnancy and involution, where they regulate epithelial expansion, apoptotic clearance, and restoration of tissue homeostasis. These context dependent roles highlight the remarkable plasticity of macrophages and their ability to adopt distinct functional states in response to local cues. Depending on the physiological context, macrophages can promote regenerative, inflammatory, or resolving programs, each of which has profound effects on epithelial behavior.

The intimate involvement of macrophages in mammary gland biology positions them as plausible mediators of aberrant remodeling in genetically predisposed tissue. Subtle alterations in macrophage number, activation state, or spatial localization could have disproportionate effects on epithelial organization, particularly in the setting of *BRCA1* deficiency. In a genetically sensitized mammary gland, immune derived signals that are normally tightly regulated during development or repair may instead drive maladaptive remodeling programs.

These observations suggest that the immune system is not merely a passive responder to epithelial transformation but an active participant in shaping epithelial fate. In the context of hereditary cancer predisposition, immune-epithelial interactions may play decisive roles in determining

whether premalignant changes resolve, persist, or progress toward malignancy. Understanding how immune regulation of mammary remodeling is altered in *BRCA1*-mutant tissue is therefore essential for elucidating the earliest stages of cancer initiation.

1.4 Premalignant phenotypes in BRCA1-mutant mammary tissue

Genetically engineered mouse models (GEMMs) have been instrumental in elucidating the biological consequences of *BRCA1* loss in the mammary gland. Most commonly, these models involve conditional deletion of *Brca1* in mammary epithelial cells, often combined with loss of *Trp53* to facilitate tumor formation [6-9]. These systems recapitulate many key features of human *BRCA1*-associated breast cancer, including basal-like or triple-negative tumor phenotypes, genomic instability, and spontaneous tumor development [6-9].

Importantly, several *BRCA1*-mutant GEMMs exhibit striking premalignant abnormalities in mammary tissue well before tumors become detectable [6-9]. These phenotypes include excessive ductal branching, alveolar like differentiation in virgin animals, epithelial hyperplasia, and aberrant expression of lineage markers [6-8]. Such changes resemble aspects of pregnancy induced mammary remodeling but occur in inappropriate developmental contexts, suggesting that *BRCA1* deficiency perturbs normal developmental programs [7,9].

These premalignant features challenge the traditional view that tumorigenesis begins abruptly with malignant transformation. Instead, they support a model in which early, tissue wide alterations create a permissive environment for cancer initiation [6-9]. In this framework, epithelial cells may undergo gradual shifts in identity, differentiation state, and spatial organization that predispose them to malignant conversion upon acquisition of additional genetic or environmental insults [8,9].

Despite consistent observations of premalignant remodeling across multiple GEMMs, the cellular drivers of these phenotypes remain incompletely understood [6-9]. Much of the existing literature has focused on epithelial cell intrinsic mechanisms, including altered proliferation, differentiation, and DNA damage responses [6-8]. However, epithelial phenotypes do not arise in isolation; they are shaped by continuous interactions with stromal and immune compartments [15-17].

Emerging evidence suggests that stromal remodeling and immune infiltration accompany epithelial abnormalities in *BRCA1*-mutant mammary tissue. Increased immune cell presence, changes in myeloid cell composition, and inflammatory gene expression signatures have been reported in both mouse models and human *BRCA1* mutation carriers without cancer [12-15,18]. These findings raise critical questions regarding causality: do immune alterations arise as a response to epithelial dysregulation, or do they actively contribute to premalignant remodeling [15-17]

Addressing this question requires moving beyond static descriptions of epithelial phenotypes toward integrated analyses of tissue ecosystems [15-17]. Understanding how epithelial, stromal, and immune compartments co-evolve in *BRCA1*-deficient mammary glands is essential for identifying early drivers of disease and potential targets for preventive intervention [18-22].

1.5 Immune infiltration and heterogeneity in BRCA1-associated breast cancer

BRCA1-deficient tumors are among the most immune infiltrated breast cancers, displaying elevated levels of tumor infiltrating lymphocytes (TILs) and myeloid cells relative to hormone receptor–positive subtypes [12-15]. This immune-rich microenvironment has fueled interest in immunotherapy approaches for TNBC, including immune checkpoint blockade [13-15]. While

some patients derive durable benefit, overall response rates remain variable, highlighting the need for improved mechanistic understanding and predictive biomarkers [14,15].

High resolution profiling approaches have revealed that immune infiltration in TNBC is not uniform but instead composed of diverse and functionally distinct populations [12-15]. Single-cell RNA-sequencing and spatial profiling studies have identified macrophages as a dominant immune population within TNBC, exhibiting extensive heterogeneity in activation state, transcriptional programs, and spatial localization [15,18-21]. These macrophages span a continuum from inflammatory and antigen-presenting states to immunosuppressive and tissue-remodeling phenotypes [18-21].

Notably, immune heterogeneity is not restricted to established tumors. Analyses of non-malignant breast tissue from *BRCA1* mutation carriers have identified increased immune cell presence and inflammatory signaling even in the absence of cancer [12,18,22]. These findings suggest that immune alterations may precede tumor formation and influence the earliest stages of disease initiation [15,18].

The functional implications of early immune infiltration remain poorly defined. Immune cells may initially serve protective roles through immune surveillance and tissue repair [15-17]. However, chronic or dysregulated immune activation can also promote epithelial plasticity, tissue remodeling, and tumor-permissive niches [16,17,19]. Distinguishing between protective and pathogenic immune functions is particularly challenging in premalignant contexts, where immune signals may have dual, context dependent effects [15-17].

Spatial organization adds an additional layer of complexity. Immune cell function is shaped not only by transcriptional state but also by proximity to epithelial structures, vasculature, and stromal niches [18-21]. Spatial profiling studies demonstrate that immune cells positioned at epithelial interfaces or within remodeling regions exert distinct effects compared to those located in stromal compartments [18,20,21]. Thus, immune function in *BRCA1*-associated disease must be understood in a spatially resolved tissue context [18-22].

1.6 Macrophages as regulators of epithelial fate and tissue architecture

Macrophages are uniquely positioned to integrate signals from epithelial cells, stromal components, and systemic cues, making them central regulators of tissue homeostasis and remodeling [15-17]. Through secretion of cytokines, growth factors, and matrix remodeling enzymes, macrophages can profoundly influence epithelial proliferation, differentiation, and plasticity [16,17,19].

In the mammary gland, macrophage-epithelial interactions are highly spatially organized. Distinct macrophage populations localize to periductal regions, stromal compartments, or within epithelial structures themselves [16,17]. These spatial niches are associated with specialized functions, suggesting that macrophage location may be as important as macrophage identity in shaping epithelial behavior [16-18].

During normal development, macrophages promote ductal elongation and branching, support epithelial survival, and coordinate tissue remodeling [16,17]. These functions are tightly regulated in time and space. However, in genetically predisposed tissue such as *BRCA1*-mutant mammary

glands, macrophage-derived signals may become dysregulated, leading to aberrant remodeling [18-20].

In cancer, macrophages have been extensively studied as tumor-associated macrophages (TAMs), where they are implicated in tumor progression, immune suppression, angiogenesis, and therapeutic resistance [15,18-21]. In contrast, far less is known about macrophage function prior to tumor initiation. Whether macrophages actively drive premalignant epithelial remodeling, respond passively to epithelial abnormalities, or attempt to restrain disease progression remains unresolved [16-19].

Evidence from other tissue systems suggests that macrophages can act as gatekeepers of epithelial fate. In the intestine, lung, and liver, macrophage-derived cytokines regulate stem cell behavior, differentiation programs, and regenerative responses [16,17,19]. Similar mechanisms may operate in the mammary gland, where macrophages could influence epithelial lineage commitment and plasticity during premalignant remodeling [18-20].

In *BRCA1*-mutant mammary tissue, early epithelial abnormalities may generate inflammatory cues that recruit or polarize macrophages toward distinct activation states [18-21]. Conversely, macrophages may amplify epithelial dysregulation through sustained paracrine signaling, reinforcing aberrant developmental programs [19-22]. This bidirectional relationship positions macrophages as potential drivers of tissue-level changes that precede malignant transformation [18-22].

1.7 Inflammatory macrophages as drivers of tissue remodeling and epithelial plasticity

Macrophages are highly heterogeneous cells capable of adopting diverse functional states in response to local tissue cues. Among these, inflammatory macrophages often characterized by elevated expression of cytokines, chemokines, interferon-responsive genes, and antigen presentation machinery play critical roles in shaping tissue environments during infection, injury, and chronic inflammation [15-17]. While these cells are classically studied in the context of host defense, accumulating evidence indicates that inflammatory macrophages are also potent regulators of epithelial behavior and tissue architecture [16,19].

In epithelial organs, inflammatory macrophages have been shown to regulate stem and progenitor cell behavior, promote cellular plasticity, and influence lineage decisions during both regeneration and disease [16,17,19]. In the intestine, macrophage-derived cytokines coordinate crypt regeneration following injury, while in the lung and liver, inflammatory macrophages regulate epithelial repair and remodeling through paracrine signaling [16,19,20]. These observations suggest that inflammatory macrophages act as contextual regulators of epithelial fate, capable of coupling immune activation to structural and functional changes in tissue compartments.

In the mammary gland, inflammatory macrophages expand in response to hormonal fluctuations, tissue damage, and oncogenic stress [16,17]. These cells secrete a broad repertoire of mediators, including interleukin-6 (IL-6) family cytokines, tumor necrosis factor (TNF), prostaglandins, and matrix-remodeling enzymes, all of which can influence epithelial proliferation, branching, and differentiation [16,19,20]. During normal development and regeneration, such signaling supports ductal outgrowth and repair. However, when deployed chronically or in genetically sensitized tissues, inflammatory macrophage activity may drive maladaptive remodeling [17,19].

A key feature of inflammatory macrophages is their spatial positioning within tissues. In multiple organ systems, inflammatory macrophages preferentially localize to epithelial interfaces, sites of barrier disruption, or regions undergoing active remodeling [18-21]. This spatial proximity enables direct paracrine signaling to epithelial cells and positions inflammatory macrophages as gatekeepers of epithelial fate decisions. In the mammary gland, macrophages localized to periductal or intraepithelial niches are well positioned to influence ductal architecture, epithelial plasticity, and lineage commitment [16-18].

Recent single-cell profiling studies have identified inflammatory macrophage states within breast tumors and premalignant tissue, including populations enriched for interferon stimulated genes, chemokines, and cytokines associated with chronic inflammation [15,18,21]. In established tumors, these macrophages have been implicated in promoting tumor progression, immune suppression, angiogenesis, and resistance to therapy [15,18-21]. However, their functional contribution prior to tumor formation remains poorly understood, particularly in the context of hereditary cancer predisposition.

In *BRCA1*-mutant mammary glands, early epithelial abnormalities may generate inflammatory cues that recruit or polarize macrophages toward inflammatory states [18,22]. Conversely, inflammatory macrophages may actively promote aberrant epithelial branching, altered differentiation, or the establishment of tumor permissive niches through sustained cytokine signaling [19-22]. Dissecting this bidirectional relationship is essential for understanding how immune dysregulation intersects with genetic risk to influence cancer initiation.

1.8 Oncostatin M as a macrophage-derived regulator of epithelial plasticity

Oncostatin M (OSM) is a pleiotropic cytokine belonging to the interleukin-6 (IL-6) family and is predominantly produced by activated myeloid cells, including inflammatory macrophages and neutrophils [23-25]. OSM signals through heterodimeric receptor complexes composed of the Oncostatin M receptor beta (OSMR) and the shared gp130 subunit, leading to activation of downstream signaling pathways such as JAK/STAT, MAPK, and PI3K cascades [23-25].

Through these pathways, OSM integrates inflammatory cues with transcriptional programs that regulate epithelial survival, differentiation, and plasticity. In epithelial tissues, OSM induces gene expression programs associated with wound healing, cellular dedifferentiation, and regenerative responses [26-28]. These properties position OSM as a key mediator linking immune activation to epithelial remodeling.

In the mammary gland and in breast cancer models, OSM signaling has been implicated in promoting epithelial mesenchymal transition (EMT) like states, increased migratory capacity, and resistance to therapy, particularly within basal-like and triple-negative breast cancer contexts [26-29]. Consistent with this, OSM expression is frequently enriched in tumor-associated macrophages, while OSMR expression is elevated in epithelial and tumor cells, establishing a paracrine signaling axis between immune and epithelial compartments [27-29].

Beyond cancer, OSM plays essential roles in physiological tissue remodeling. In the liver, OSM promotes hepatocyte regeneration following injury; in bone, it regulates osteoblast differentiation; and in epithelial tissues, it contributes to inflammatory repair responses [24-26]. These functions underscore the capacity of OSM to couple immune activation with structural and functional

changes in epithelial compartments. However, sustained or dysregulated OSM signaling can promote chronic inflammation, pathological remodeling, and tissue dysfunction [26-28].

Despite increasing recognition of OSM as a driver of epithelial plasticity in established tumors, its role in premalignant tissue remodeling remains largely unexplored. In genetically predisposed tissues such as *BRCA1*-mutant mammary glands, immune derived cytokines like OSM may influence epithelial organization long before malignant transformation occurs [18,22,26]. Whether OSM contributes to aberrant ductal branching, altered lineage commitment, or the establishment of tumor-permissive niches during early disease has not been directly tested.

Emerging evidence suggests that OSM signaling intersects with developmental and stress response pathways that are already perturbed in *BRCA1*-deficient epithelial cells. Activation of STAT3 and related transcriptional programs downstream of OSM may reinforce progenitor like states, suppress terminal differentiation, and promote epithelial plasticity [26-29]. In the context of *BRCA1* deficiency where epithelial differentiation and chromatin regulation are already altered such signals may have amplified or prolonged effects.

Furthermore, OSM signaling is tightly linked to spatial context. Macrophage-derived OSM acts locally, and its impact on epithelial cells is likely influenced by proximity, receptor expression levels, and co-occurring signals within the tissue microenvironment [18-21]. Spatially resolved analyses are therefore essential for understanding where and how OSM signaling operates in premalignant mammary tissue.

Together, these observations position OSM as a compelling candidate mediator of macrophage-driven epithelial remodeling in *BRCA1*-associated breast cancer initiation. Defining the sources,

targets, and functional consequences of OSM signaling in premalignant mammary glands represents a critical gap in the field.

1.9 Conceptual framework and thesis objectives

Collectively, the literature supports a model in which *BRCA1*-associated breast cancer arises from a complex interplay between epithelial genetic defects, developmental mis-programming, and immune regulation [6-9,15-22,30-51]. Premalignant mammary phenotypes emerge well before tumor formation, yet the mechanisms linking *BRCA1* deficiency to these early tissue-wide changes remain poorly defined.

This dissertation is grounded in the central hypothesis that macrophages are active contributors to premalignant mammary remodeling in *BRCA1*-mutant tissue. By shaping epithelial branching, growth, differentiation, and spatial organization through localized signaling, macrophages may create conditions that favor malignant transformation. Inflammatory macrophages, in particular, are poised to exert outsized effects through secretion of cytokines such as OSM that directly regulate epithelial plasticity.

The work presented in subsequent chapters aims to:

1. **Define immune and macrophage heterogeneity** in *BRCA1*-mutant mammary glands prior to tumor formation using single-cell profiling approaches.
2. **Map the spatial organization of macrophage subsets** relative to epithelial structures and premalignant lesions using spatial proteomics.
3. **Test the functional impact of macrophages on epithelial behavior** using reductionist and in-vivo inspired organoid and co-culture assays.

4. **Identify macrophage-epithelial communication pathways** selectively enriched in premalignant *BRCA1*-mutant tissue using cell-cell communication inference.
5. **Functionally interrogate candidate macrophage-derived signals**, including Oncostatin M, for their roles in epithelial remodeling and aberrant branching.

By integrating descriptive, spatial, and mechanistic approaches, this work seeks to establish how immune-derived signals shape epithelial fate decisions during the earliest stages of *BRCA1*-associated breast cancer initiation. By shifting the focus from established tumors to disease initiation, and from epithelial cells alone to immune–epithelial crosstalk, this dissertation aims to uncover new conceptual and therapeutic avenues for cancer prevention in *BRCA1* mutation carriers.

Chapter 2

Macrophage heterogeneity in premalignant BRCA1-mutant mammary glands

2.1 Experimental overview and definition of premalignant stages

To investigate immune regulation during the earliest stages of BRCA1-associated breast cancer initiation, we focused on mammary glands harvested prior to overt tumor formation. Using a genetically engineered mouse model with conditional loss of *Brca1* and *Trp53* in the mammary epithelium, we selected time points at which epithelial hyperplasia and aberrant ductal branching are consistently observed, but palpable tumors have not yet developed **(Figure 1a, 1b)**. Age matched WT littermates were used as controls to distinguish genotype specific effects from developmental or hormonal variation.

Mammary glands were processed for single-cell RNA-sequencing (scRNA-seq), flow cytometry, and spatial imaging, enabling integrated assessment of immune composition, transcriptional states, and spatial localization within intact mammary tissue, as established in recent mammary and breast immune atlas studies [52,53]. All analyses presented in this chapter focus on non-tumor bearing glands, hereafter referred to as premalignant BRCA1-mutant mammary tissue.

2.2 Immune landscape of premalignant BRCA1-mutant mammary glands

We first characterized the overall immune composition of premalignant mammary glands using scRNA-seq. After quality control, doublet removal, and batch correction, immune cells were identified based on expression of canonical markers and sub-clustered for downstream analysis. Unsupervised clustering revealed multiple immune populations, including T cells, B cells,

neutrophils, and myeloid cells, with myeloid cells representing the most transcriptionally diverse compartment, consistent with prior single-cell atlases of mammary and breast tissues [52,53].

Comparison of immune cell abundances between WT and BRCA1-mutant glands revealed an expansion of myeloid cells in mutant tissue **(Figure 1c)**. This shift was evident both in relative proportions within the immune compartment and in absolute immune cell counts per gland, as confirmed by flow cytometry. These findings align with emerging models of early inflammatory remodeling during cancer initiation, in which myeloid cells are preferentially expanded prior to malignant transformation [54,55]. Together, these data indicate that BRCA1 deficiency is associated with early remodeling of the immune microenvironment, characterized predominantly by alterations in myeloid cell composition.

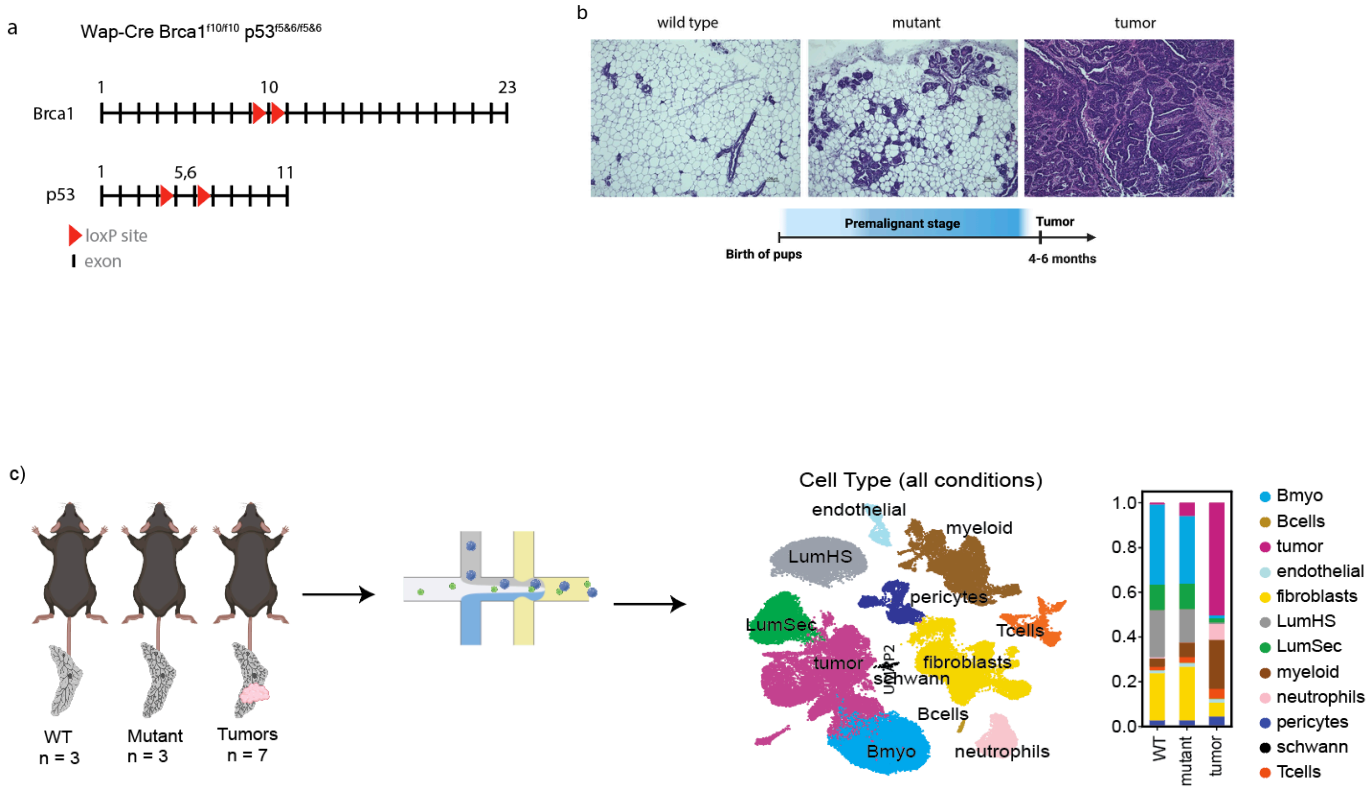
2.3 Macrophage heterogeneity emerges prior to tumor formation

Given the prominent expansion of myeloid cells, we next focused on macrophages, which constituted most of the myeloid compartment in both genotypes. Sub clustering of macrophages revealed multiple transcriptionally distinct subsets present in premalignant tissue **(Figure 1d)**. These subsets were conserved across biological replicates and could be broadly grouped into inflammatory, ductal associated, and stromal associated macrophage states based on marker gene expression, reflecting conserved macrophage activation and differentiation programs described across inflammatory and cancer associated contexts [56] **(Figure 1e)**.

Inflammatory macrophages were characterized by elevated expression of cytokines, chemokines, and interferon responsive genes, consistent with an activated inflammatory state that has been observed in both chronic inflammatory tissues and tumor associated myeloid populations [57]. In

contrast, a distinct population of macrophages expressed genes associated with tissue residency and epithelial interaction, suggesting a ductal associated identity. Additional macrophage clusters exhibited gene expression profiles consistent with extracellular matrix remodeling and stromal support.

Notably, BRCA1-mutant mammary glands displayed a preferential expansion of inflammatory macrophage subsets relative to WT controls **(Figure 1f, 1g)**. This shift was not accompanied by a uniform increase across all macrophage states, indicating selective enrichment rather than global macrophage accumulation. These findings demonstrate that macrophage heterogeneity is established early in BRCA1-mutant tissue, well before tumor initiation.



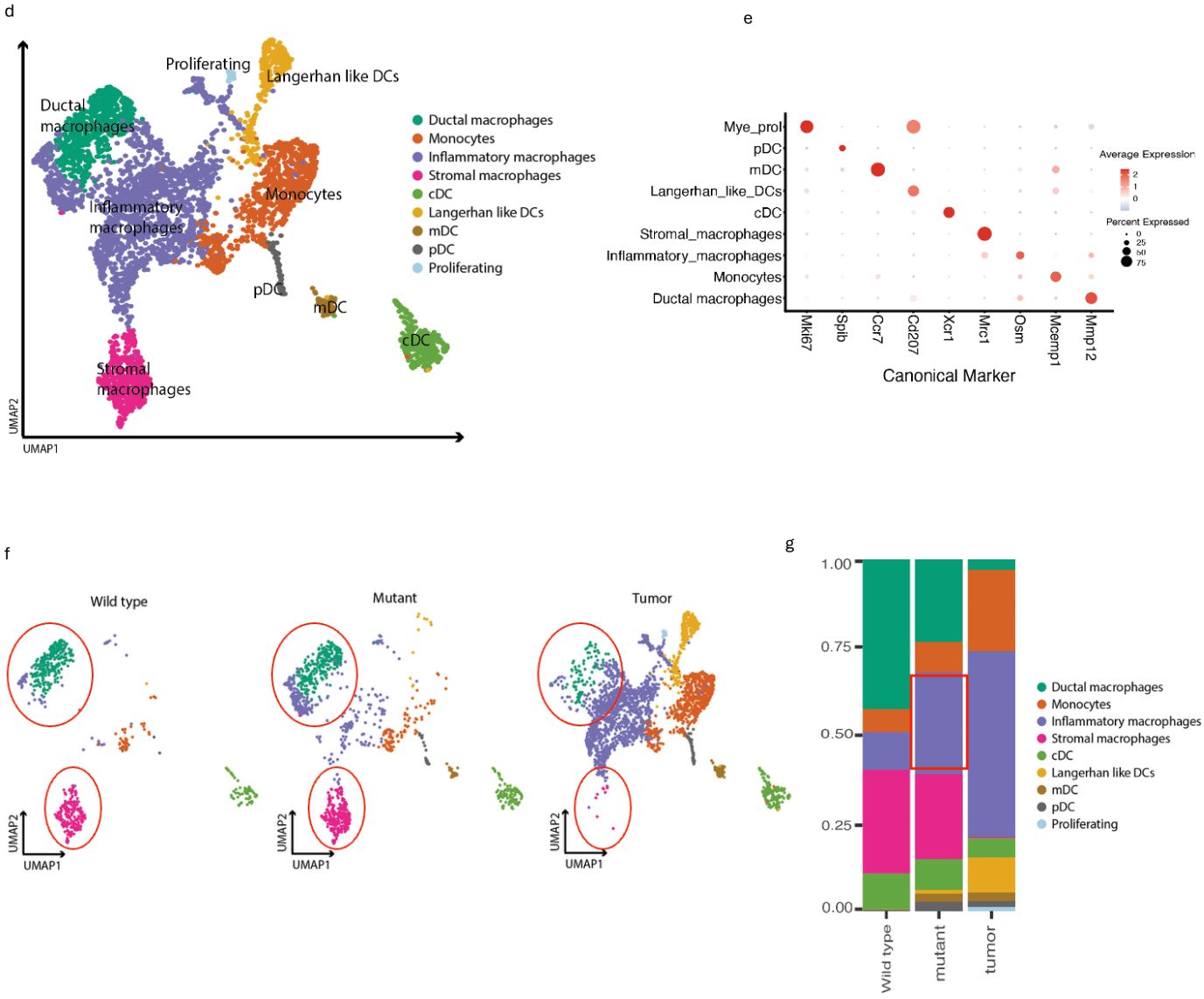
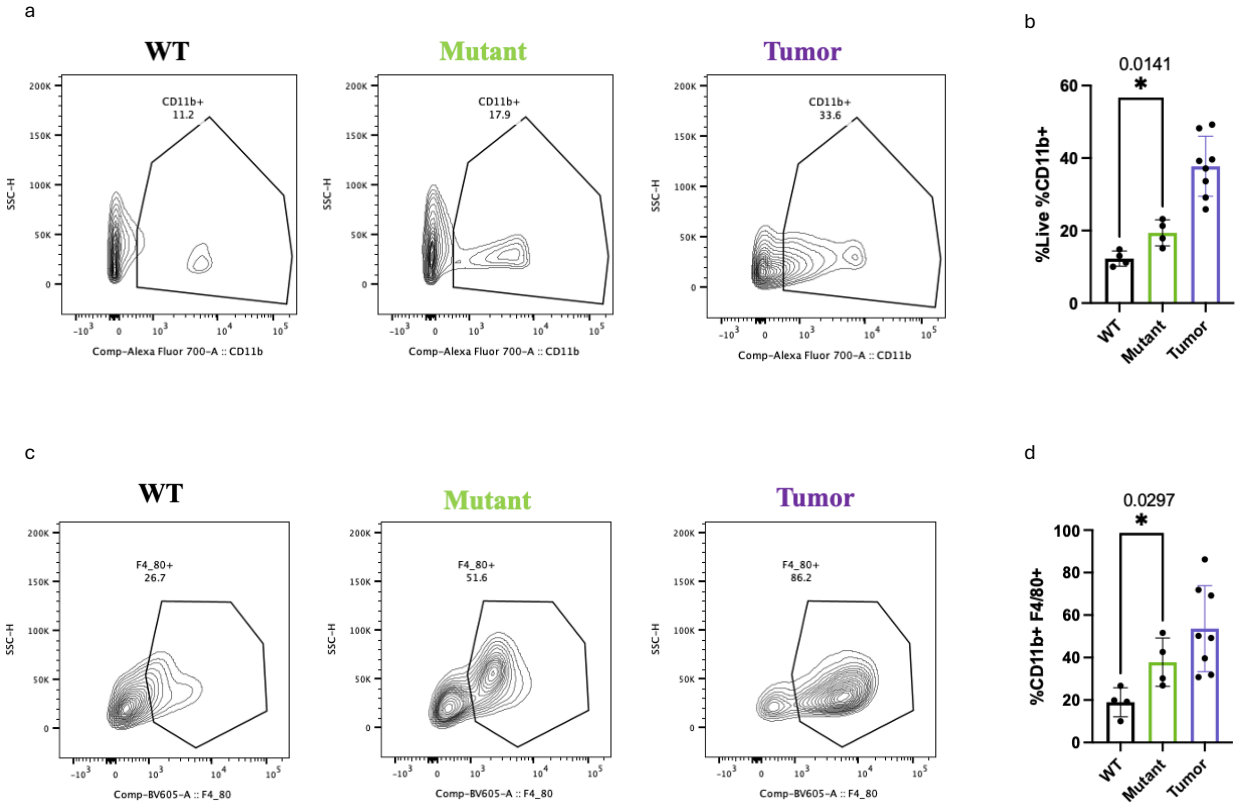


Figure 1. Immune landscape of BRCA1-deficient mammary tissue revealed by single-cell RNA-sequencing a) Schematic of the mouse model. b) H&E images showing the progression of the disease in this mouse model. c) Schematic of the single-cell study design, UMAP visualization of all the cells from WT, premalignant mammary glands, and tumors, colored by major immune cell type and bar plot showing the relative composition of each cell populations across conditions. d) UMAP of myeloid cells re-clustered showing all subsets. e) Dot plot showing expression of canonical markers used to annotate macrophage, monocyte, and dendritic cell populations. f) UMAP showing differences in myeloid subtypes across conditions. g) Proportional analysis of myeloid subsets across WT, premalignant, and tumor samples.

2.4 Validation of macrophage subset expansion by flow cytometry

To validate the scRNA-seq findings at the protein level, we performed flow cytometric analysis of mammary gland immune cells using markers that distinguish macrophage subsets. Macrophages were identified as $CD45^{+}CD11b^{+}F4/80^{+}$ cells and further subdivided based on tissue resident markers like CD163 and CD206.

Consistent with transcriptomic data, BRCA1-mutant glands exhibited a significant increase in the frequency of myeloid cells in general and specifically macrophages compared to WT glands **(Figure 2a, 2b, 2c, 2d)**. BRCA1-mutant glands also exhibited a significant decrease in the frequency of tissue resident macrophages compared to WT glands **(Figure 2e, 2f, 2g, 2h)**. These results confirm that macrophage subset remodeling in BRCA1-mutant mammary tissue is robust



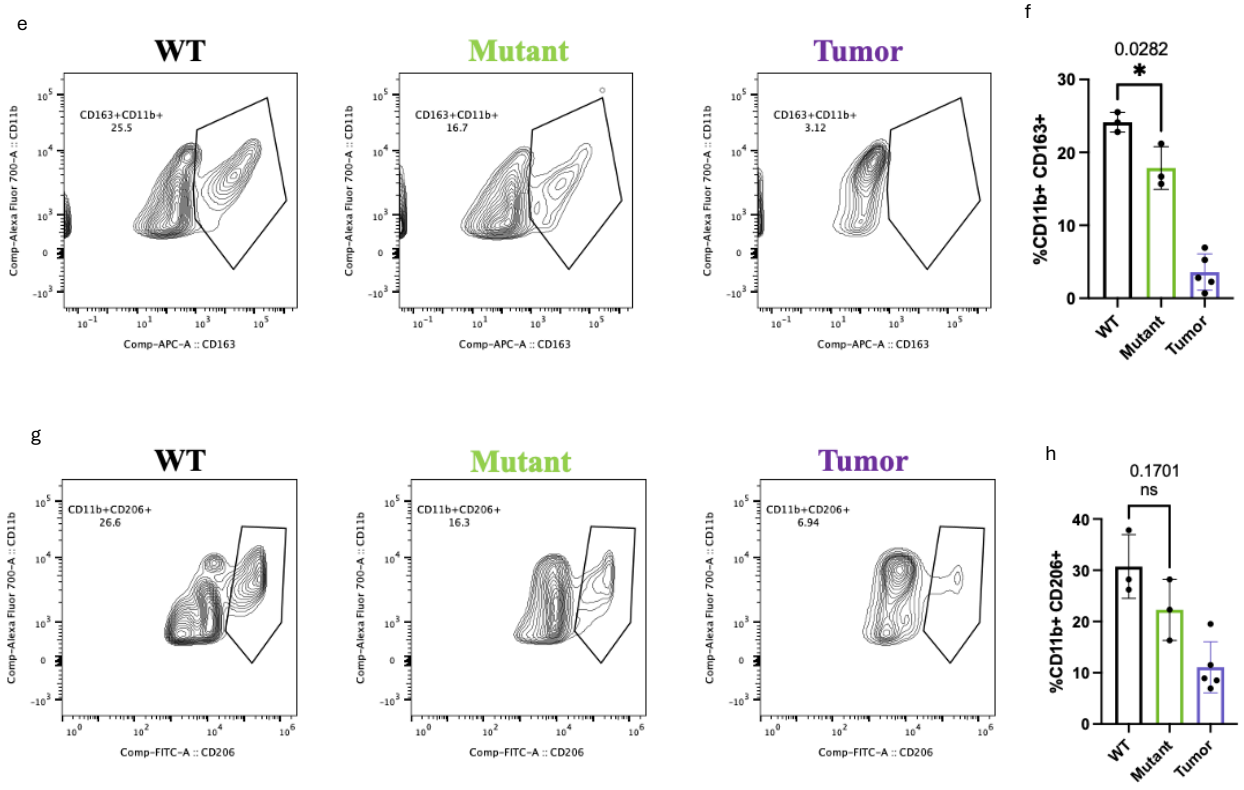


Figure 2. Flow cytometry validates expansion of macrophages in BRCA1-deficient mammary glands a) and b) Representative flow cytometry plots and quantification showing the frequency of myeloid cells in the WT, mutant and tumor samples from mice. c) and d) Representative flow cytometry plots and quantification showing the frequency of macrophages in the WT, mutant and tumor samples from mice. e) and f) Representative flow cytometry plots and quantification showing the frequency of CD163+ tissue resident macrophages in the WT, mutant and tumor samples from mice. g) and h) Representative flow cytometry plots and quantification showing the frequency of CD206+ tissue resident macrophages in the WT, mutant and tumor samples from mice.

2.5 Conservation of inflammatory macrophage expansion in human BRCA1 carriers

To assess whether the immune microenvironment alterations identified in the BRCA1-deficient mouse model are conserved in humans, we analyzed publicly available single-cell RNA-sequencing data from the Human Breast Cell Atlas, encompassing breast tissue samples from BRCA1 mutation carriers and non-carrier controls **(Figure 3a)**. Myeloid cell populations were extracted from the full dataset and visualized using uniform manifold approximation and projection (UMAP), revealing multiple transcriptionally distinct macrophage subsets across human samples **(Figure 3b)**.

Quantitative analysis of myeloid cell composition demonstrated a significant increase in inflammatory (M1-like) macrophages in breast tissue from BRCA1 mutation carriers compared to non-carrier controls **(Figure 3c, 3d)**. This shift in macrophage composition closely mirrors the selective expansion of inflammatory macrophages observed in premalignant BRCA1-deficient mouse mammary glands. Importantly, this enrichment was not accompanied by a uniform increase in all macrophage populations, indicating selective remodeling of macrophage states rather than global myeloid expansion.

Together, these findings demonstrate that inflammatory macrophage enrichment is a conserved feature of BRCA1-associated breast tissue across species. The concordance between mouse and human data supports the translational relevance of the BRCA1-driven immune remodeling identified in this study and suggests that inflammatory macrophages represent an early and conserved component of the premalignant breast microenvironment in BRCA1 mutation carriers.

a b

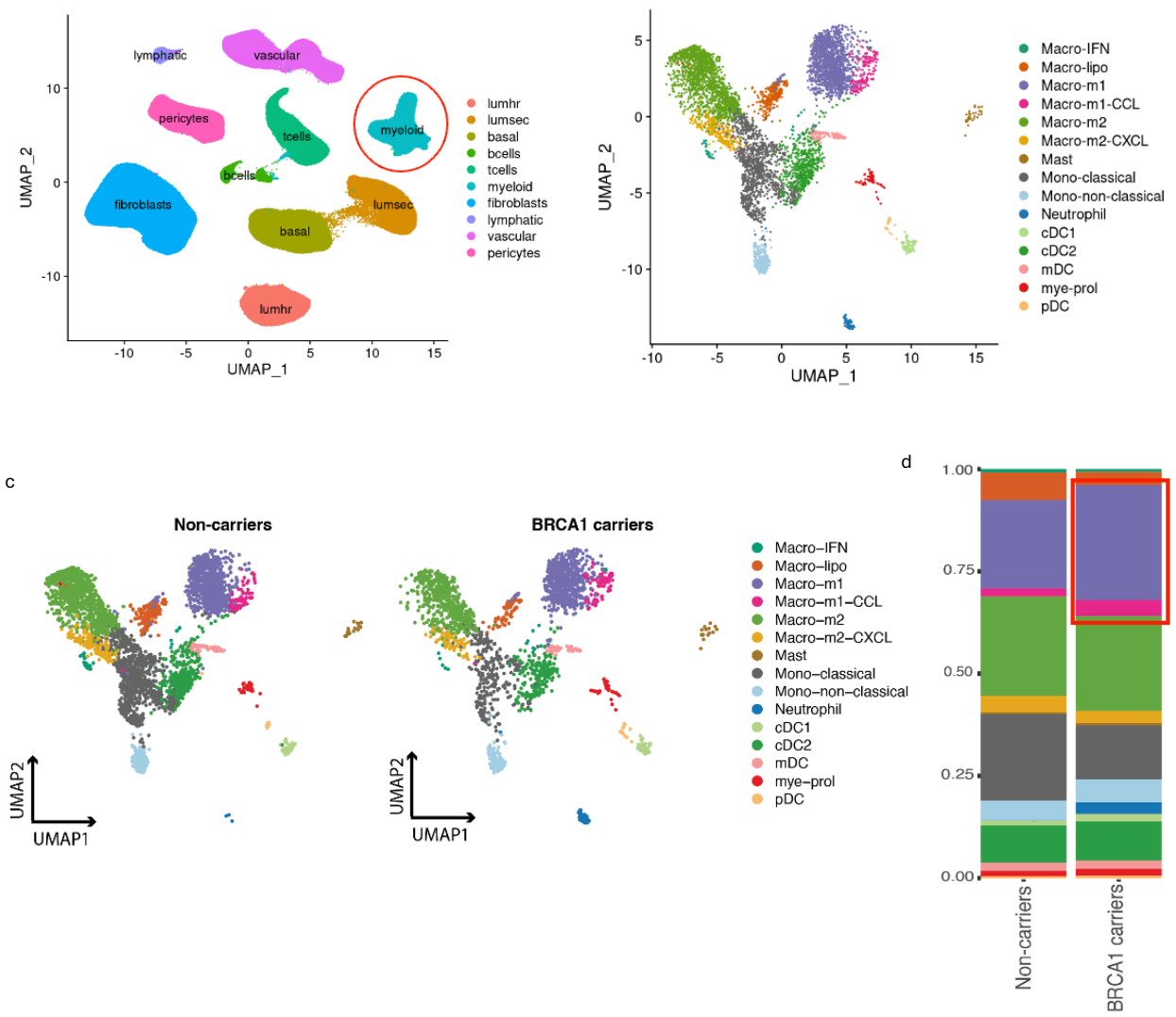


Figure 3. Conservation of inflammatory macrophage expansion in human BRCA1 mutation carriers. a) UMAP visualization of all the cells from publicly available single-cell RNA-sequencing data of human breast tissue from BRCA1 mutation carriers and non-carrier controls. b) UMAP visualization of the myeloid cells after sub-clustering analysis c) UMAP showing differences in myeloid subtypes across BRCA1 mutation carriers and non-carrier controls. d) Proportional analysis of myeloid subsets across BRCA1 mutation carriers and non-carrier controls.

2.6 Summary

Together, the results in this chapter demonstrate that BRCA1-mutant mammary glands undergo early immune remodeling characterized by selective expansion and diversification of macrophage populations prior to tumor formation. Inflammatory macrophages are preferentially enriched and exhibit transcriptional programs consistent with chronic inflammation and tissue remodeling, rather than acute immune responses. These findings are consistent with emerging paradigms in which inflammatory myeloid cells actively shape tissue states during cancer initiation rather than simply responding to established tumors [54,55,58]. Collectively, this work establishes immune and macrophage heterogeneity as an early feature of BRCA1-mutant mammary tissue and provides a foundation for subsequent spatial analyses examining how these macrophage subsets are organized relative to epithelial structures and premalignant lesions.

Chapter 3

Spatial reorganization of macrophages during tumor initiation

3.1 Rationale for spatial analysis of macrophage organization in premalignant tissue

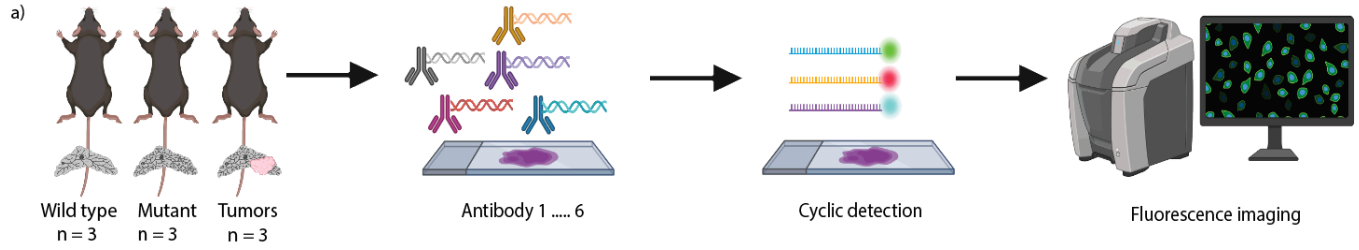
While transcriptional profiling revealed early expansion and diversification of macrophage populations in premalignant BRCA1-mutant mammary glands (Chapter 2), single-cell approaches alone cannot resolve how immune cells are positioned relative to epithelial structures within intact tissue. Immune cell function is strongly influenced by spatial context, as immune cells operate within defined cellular neighborhoods that shape cell-cell interactions, signaling gradients, and tissue remodeling outcomes [59,60]. In developmentally dynamic organs such as the mammary gland, spatial positioning of macrophages is particularly important for regulating epithelial morphogenesis and homeostasis.

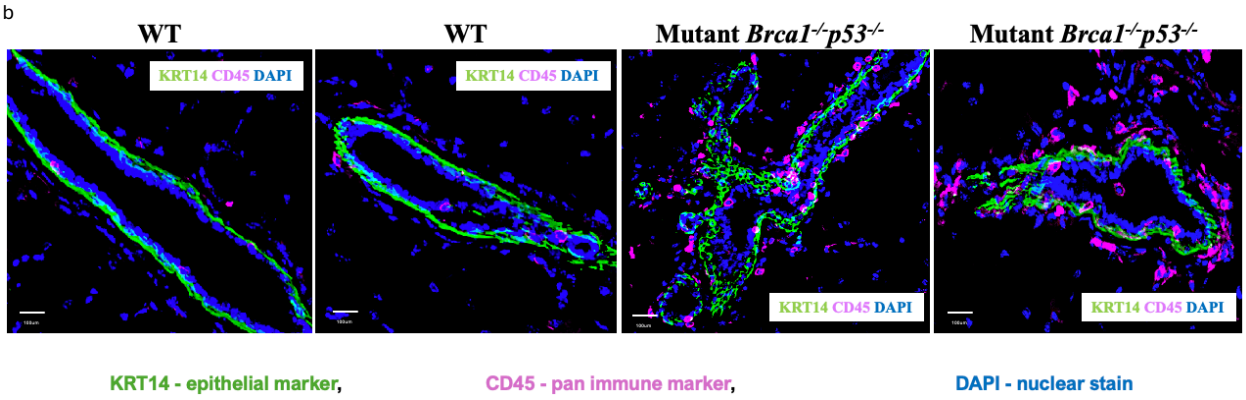
To determine whether macrophages are spatially reorganized during early stages of BRCA1-associated tumorigenesis, we performed high dimensional spatial proteomic profiling across mammary tissues spanning WT, premalignant BRCA1-mutant, and tumor bearing states.

3.2 Spatial proteomic profiling of mammary tissue using CODEX imaging

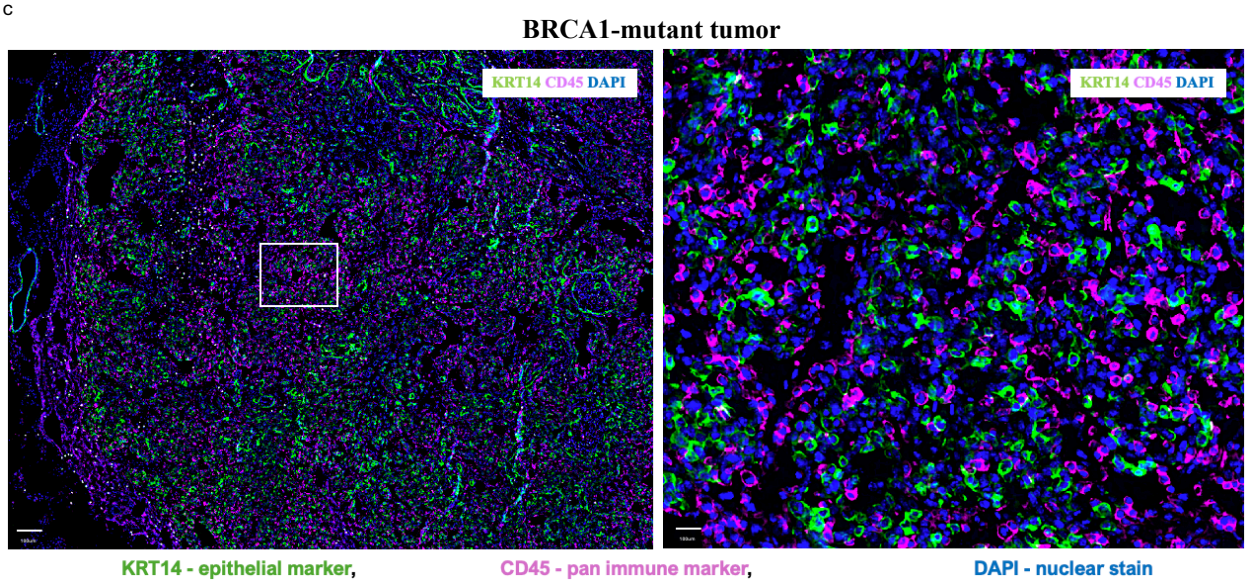
To define spatial immune remodeling during early disease, we performed CO-Detection by indEXing (CODEX) imaging on mammary tissue sections from WT, BRCA1-mutant, and tumor bearing mice **(Figure 4a)**. CODEX enables simultaneous visualization of dozens of protein markers at single-cell resolution within intact tissue sections and has emerged as a powerful approach for mapping immune-epithelial organization in situ [61].

Representative CODEX images revealed a marked increase in immune infiltration in BRCA1-mutant tissues compared to WT controls, with tumors exhibiting the highest overall immune density **(Figure 4b, 4c)**. Among immune populations, F4/80^{+} macrophages were prominently expanded in mutant samples and tumors **(Figure 4d, 4e)**. Importantly, this increase was evident in tissues harvested prior to overt tumor formation, indicating that immune infiltration and macrophage accumulation are early features of BRCA1-deficient mammary glands rather than a consequence of tumor growth.

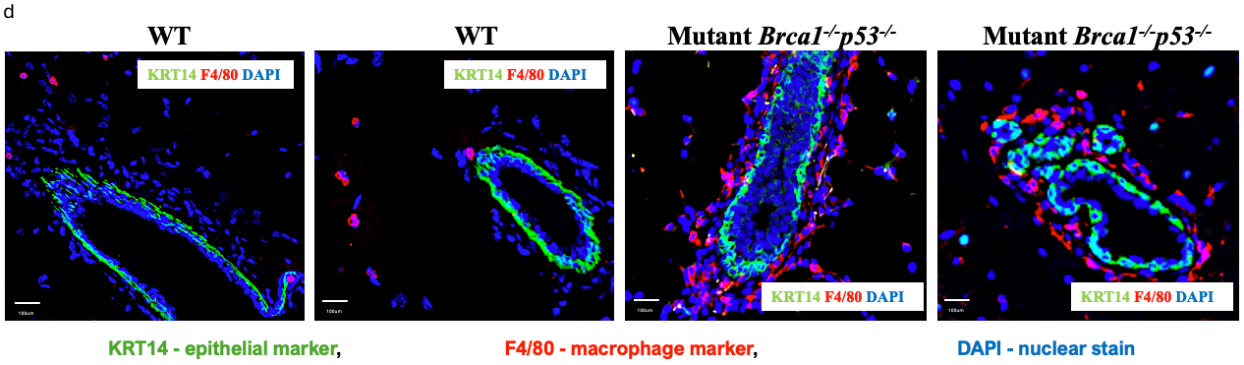




c


BRCA1-mutant tumor
KRT14 CD45 DAPI
KRT14 CD45 DAPI
KRT14 - epithelial marker,
CD45 - pan immune marker,
DAPI - nuclear stain

d


WT
WT
Mutant Brca1-/-p53-/-
Mutant Brca1-/-p53-/-
KRT14 F4/80 DAPI
KRT14 F4/80 DAPI
KRT14 F4/80 DAPI
KRT14 F4/80 DAPI
KRT14 - epithelial marker,
F4/80 - macrophage marker,
DAPI - nuclear stain

e

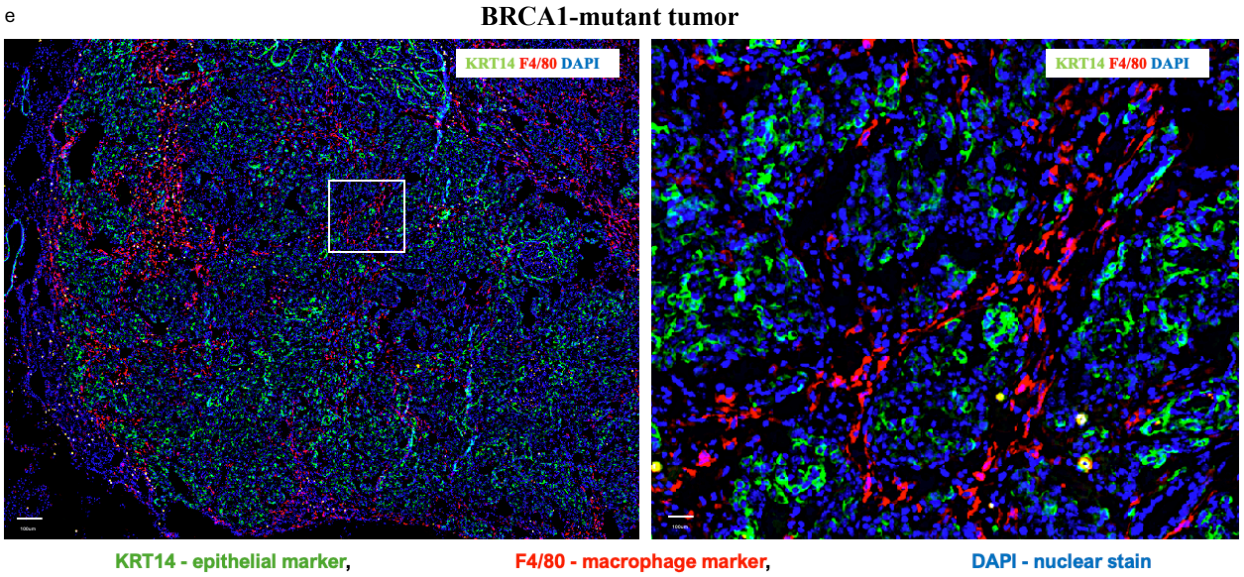

BRCA1-mutant tumor
KRT14 F4/80 DAPI
KRT14 F4/80 DAPI
KRT14 - epithelial marker,
F4/80 - macrophage marker,
DAPI - nuclear stain

Figure 4. Spatial clustering of inflammatory macrophages near hyperplastic epithelial regions. a) Schematic of the spatial proteomics study design and workflow. b) Representative high dimensional spatial proteomics (CODEX/Phenocycler) images of WT and premalignant mammary tissue stained for Keratin-14 and CD45. c) Representative high dimensional spatial proteomics (CODEX/Phenocycler) images of tumor tissue from mammary gland stained for Keratin-14 and CD45. d) Representative high dimensional spatial proteomics (CODEX/Phenocycler) images of WT and premalignant mammary tissue stained for Keratin-14 and F4/80. e) Representative high dimensional spatial proteomics (CODEX/Phenocycler) images of tumor tissue from mammary gland stained for Keratin-14 and F4/80. Spatial maps highlighting the localization of macrophage subsets relative to epithelial structures. These images reveal marked spatial reorganization of macrophages in BRCA1-deficient tissue compared to WT glands.

3.3 Region of interest analysis reveals macrophage enrichment in abnormal epithelial regions

To determine whether macrophage accumulation was spatially uniform or associated with specific epithelial features, we defined regions of interest (ROIs) within mammary sections. In BRCA1-mutant glands, ROIs were classified as mutant normal regions, which closely resembled WT glandular architecture, or mutant abnormal regions, characterized by epithelial hyperproliferation, hyperbranching, and disrupted tissue organization **(Figure 5a)**.

Cellular phenotypes across all ROIs were visualized using uniform manifold approximation and projection (UMAP), enabling comparison of immune and epithelial composition across genotypes and spatial contexts **(Figure 5b)**. Quantitative analysis revealed a significant increase in macrophage density specifically within mutant abnormal regions compared to both WT tissue and mutant normal regions **(Figure 5c)**. Notably, macrophage abundance in mutant normal regions was comparable to WT glands, indicating that macrophage expansion is not a global feature of BRCA1-mutant tissue but is instead spatially restricted to regions undergoing abnormal epithelial remodeling [59].

These findings support a model in which macrophages are selectively recruited to, or retained within, epithelial regions exhibiting premalignant architectural changes.

3.4 Voronoi tessellation reveals altered macrophage spatial organization in mutant tissue

To further examine macrophage spatial distribution across entire mammary sections, we applied Voronoi tessellation analysis to CODEX derived single cell coordinate data. This approach enables visualization of cellular neighborhoods and local cell density by partitioning tissue space into

regions defined by the nearest neighboring cell and has been widely used to characterize immune organization within tissues [60].

In WT mammary glands, macrophages were sparsely distributed and exhibited minimal clustering, consistent with a homeostatic tissue environment. In contrast, BRCA1-mutant and tumor tissues displayed dense macrophage localization with increased clustering and reduced intercellular distances. Macrophages in mutant samples were frequently positioned in close proximity to epithelial structures, particularly within regions of abnormal architecture.

These spatial features indicate a fundamental reorganization of macrophage positioning during early disease progression, consistent with emerging models in which macrophage localization within tissue niches confers functional specialization.

3.5 Premalignant spatial remodeling mirrors tumor associated immune architecture

Although tumor samples exhibited the highest overall macrophage density, several spatial features observed in tumors were already apparent in premalignant BRCA1-mutant tissue. These included localized macrophage clustering, increased macrophage-epithelial proximity, and enrichment within morphologically abnormal regions. The presence of these features prior to malignant transformation suggests that components of tumor associated immune architecture are established early during disease progression.

Importantly, the spatially restricted accumulation of macrophages in premalignant tissue argues against a purely reactive immune response to tumor growth. Instead, these data support a model in which macrophage remodeling is an early and potentially instructive event in BRCA1-associated

tumorigenesis, consistent with literature implicating macrophages in cancer initiation and early tissue remodeling [62].

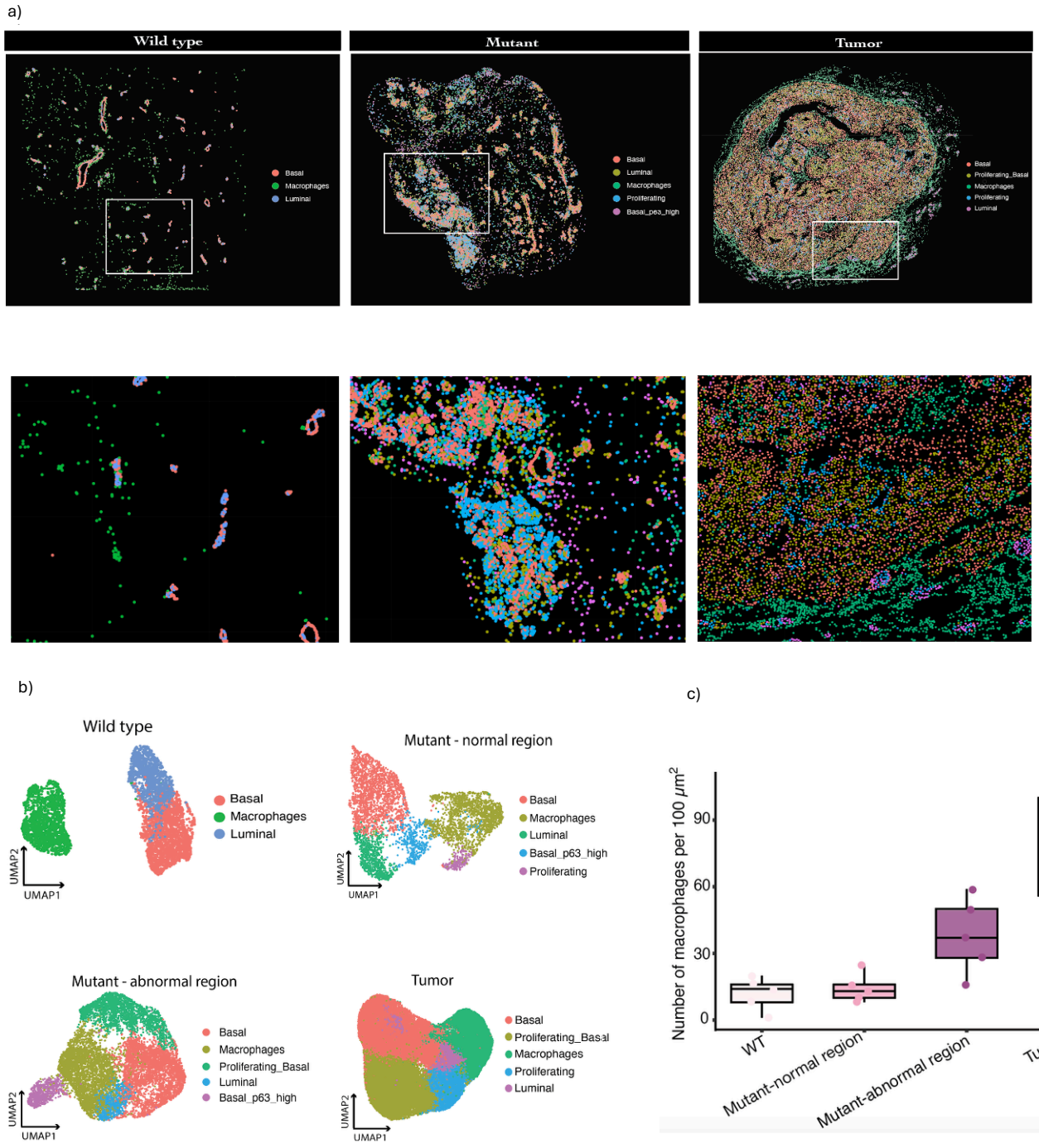


Figure 5. Quantitative analysis of macrophage density across spatial regions. a) Voronoi diagrams showing definition of regions of interest (ROIs) within hyperplastic and normal compartments used for spatial quantification. b) UMAP showing different epithelial clusters and macrophages across different spatial ROIs. c) Quantification of total macrophage density per ROI across conditions.

3.6 Summary

In this chapter, we demonstrate that macrophages are not only transcriptionally altered in premalignant BRCA1-mutant mammary glands but are also spatially reorganized in a region-specific manner. High dimensional spatial proteomic imaging reveals selective accumulation and clustering of macrophages within epithelial regions exhibiting abnormal architecture, well before tumor formation. These findings establish spatial macrophage remodeling as a defining feature of early BRCA1-associated disease and provide critical spatial context for subsequent analyses examining macrophage-epithelial communication pathways and functional mechanisms driving aberrant epithelial remodeling.

Chapter 4

Role of macrophages in BRCA1-associated tumor initiation

4.1 Functional interrogation of macrophages

Chapters 2 and 3 established that macrophage populations are transcriptionally diversified and spatially reorganized in premalignant BRCA1-mutant mammary glands, with inflammatory macrophages preferentially enriched in regions of abnormal epithelial architecture. However, whether macrophages directly contribute to epithelial remodeling during this premalignant stage, rather than serving as passive responders to epithelial dysfunction, remained unresolved. Prior work has shown that macrophages can actively regulate epithelial morphogenesis and tissue remodeling across developmental and disease contexts [63,64]. To directly test the functional impact of macrophages on epithelial behavior, we employed experimental systems designed to assess macrophage-driven effects on epithelial growth and branching.

Aberrant epithelial branching is a hallmark of premalignant mammary gland remodeling in BRCA1-deficient tissue and has been linked to increased epithelial proliferation, altered tissue architecture, and heightened susceptibility to tumor initiation [65]. Accordingly, epithelial branching provides a sensitive and quantifiable functional readout of early epithelial dysregulation.

4.2 Establishment of a macrophage-epithelial organoid co-culture system

To functionally assess macrophage-epithelial interactions in a controlled yet physiologically relevant context, we established a three-dimensional organoid co-culture system integrating primary mammary epithelial organoids with macrophages isolated from matched mouse models.

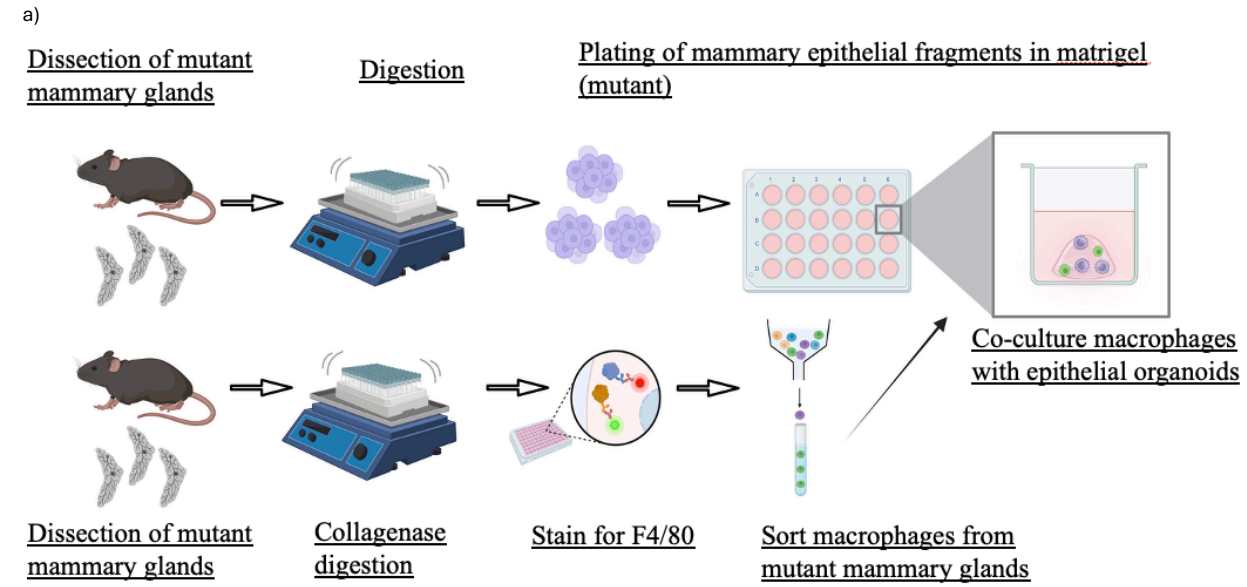
Mammary organoid systems have been widely used to model epithelial morphogenesis and branching behavior ex-vivo, while retaining key features of in-vivo tissue organization [66,67]. Macrophages were isolated from WT or BRCA1-mutant mice and introduced into organoid cultures at defined ratios, enabling direct interrogation of macrophage-driven effects on epithelial growth **(Figure 6a)**.

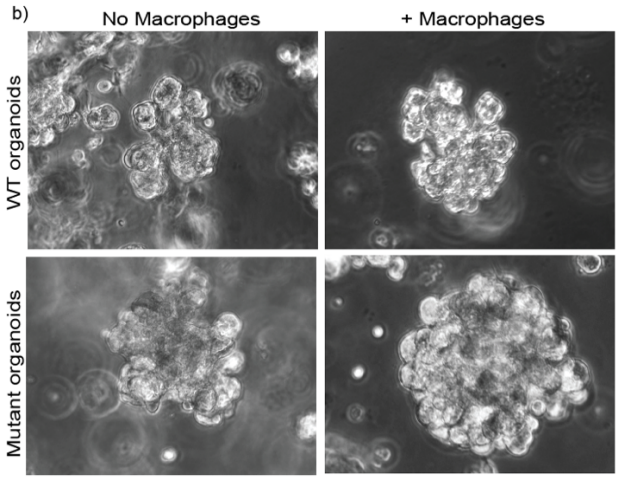
This co-culture platform allowed quantitative comparison of epithelial growth and branching outcomes across experimental groups while minimizing confounding systemic variables present in-vivo.

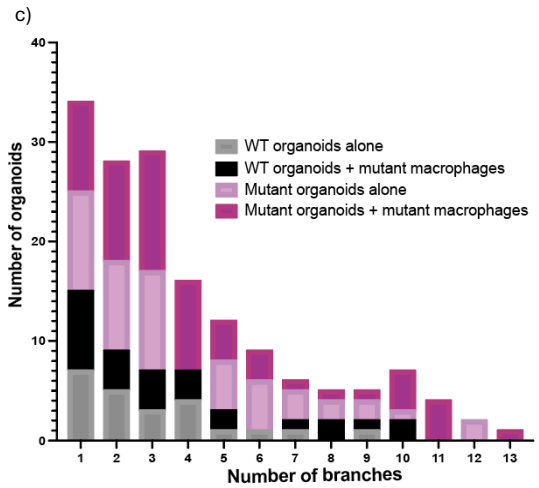
4.3 Macrophages promote aberrant epithelial growth and branching in mutant organoids

Co-culture of BRCA1-mutant mammary organoids with macrophages resulted in a pronounced increase in organoid size and branching compared to organoids cultured alone **(Figure 6b)**. Quantitative analysis demonstrated significant increases in organoid area and branching, indicating that macrophages actively promote epithelial remodeling **(Figure 6c, 6d, 6e)**.

Importantly, the magnitude of this effect depended on the origin of the macrophages. Mutant organoids co-cultured with macrophages derived from BRCA1-deficient mice exhibited the highest number of branches, exceeding those observed with macrophages isolated from WT mice. These findings are consistent with prior studies showing that macrophages exposed to chronic inflammatory or tumor prone environments acquire enhanced tissue remodeling capacity [64,68].


a)
Dissection of mutant mammary glands
Digestion
Plating of mammary epithelial fragments in matrigel (mutant)
Co-culture macrophages with epithelial organoids
Dissection of mutant mammary glands
Collagenase digestion
Stain for F4/80
Sort macrophages from mutant mammary glands


b)
No Macrophages
+ Macrophages
WT organoids
Mutant organoids


c)
Number of organoids
Number of branches
WT organoids alone
WT organoids + mutant macrophages
Mutant organoids alone
Mutant organoids + mutant macrophages

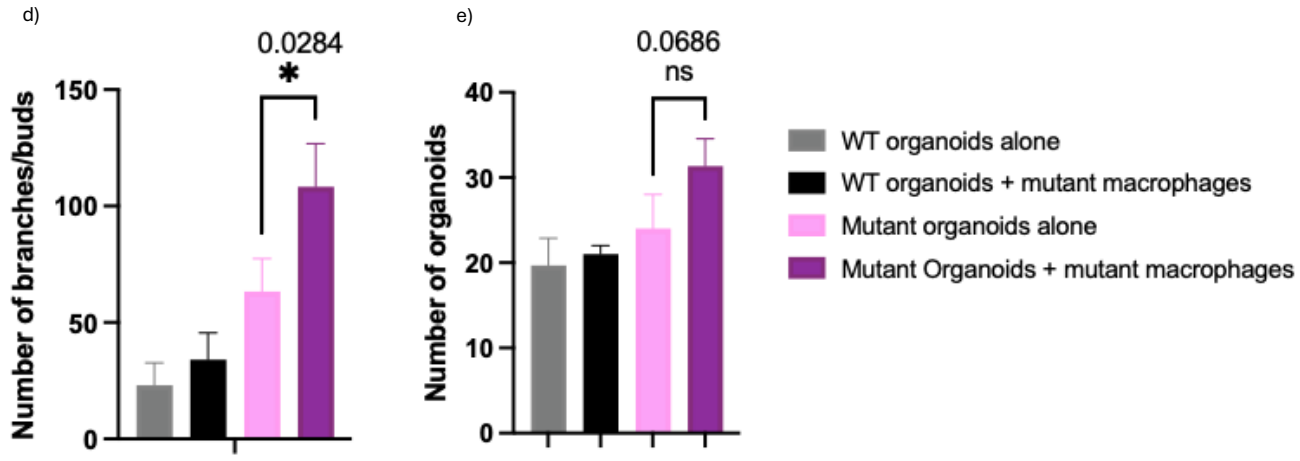

d)
0.0284
*
Number of branches/buds
e)
0.0686
ns
Number of organoids
WT organoids alone
WT organoids + mutant macrophages
Mutant organoids alone
Mutant Organoids + mutant macrophages

Figure 6. Macrophages promote aberrant growth and branching of BRCA1-deficient mammary organoids. a) Schematic of the mammary organoid co-culture system used to assess macrophage-epithelial interactions. b) Representative brightfield images of WT and BRCA1-deficient mammary organoids cultured alone or in the presence of macrophages. c) Quantification of number of organoids with specific branch numbers across the different co-culture conditions. d) Quantification of the total number of branches across the different co-culture conditions. e) Quantification of number of organoids across the different co-culture conditions.

4.4 Spatial localization of macrophage subsets identifies epithelial interacting populations

To spatially contextualize these functional effects in-vivo and identify macrophage populations most likely to directly engage the epithelium, we performed immunofluorescence staining of premalignant and tumor tissues to distinguish macrophage subsets based on CD163 and CX3CR1 expression. Previous studies have demonstrated that $CX3CR1^{+}$ macrophages localize within epithelial compartments in mammary tissue and directly interact with epithelial cells, whereas $CD163^{+}$ macrophages predominantly occupy stromal niches [69].

Consistent with these reports, in premalignant BRCA1-mutant tissues $CD163^{+}$ macrophages were largely excluded from hyperplastic epithelial structures, while $CX3CR1^{+}$ macrophages were positioned within the epithelial compartment **(Figure 7a)**. In tumor samples, $CD163^{+}$ macrophages preferentially accumulated at the tumor periphery, whereas $CX3CR1^{+}$ macrophages infiltrated the tumor mass, suggesting distinct spatial niches and functional roles during disease progression **(Figure 7b)**.

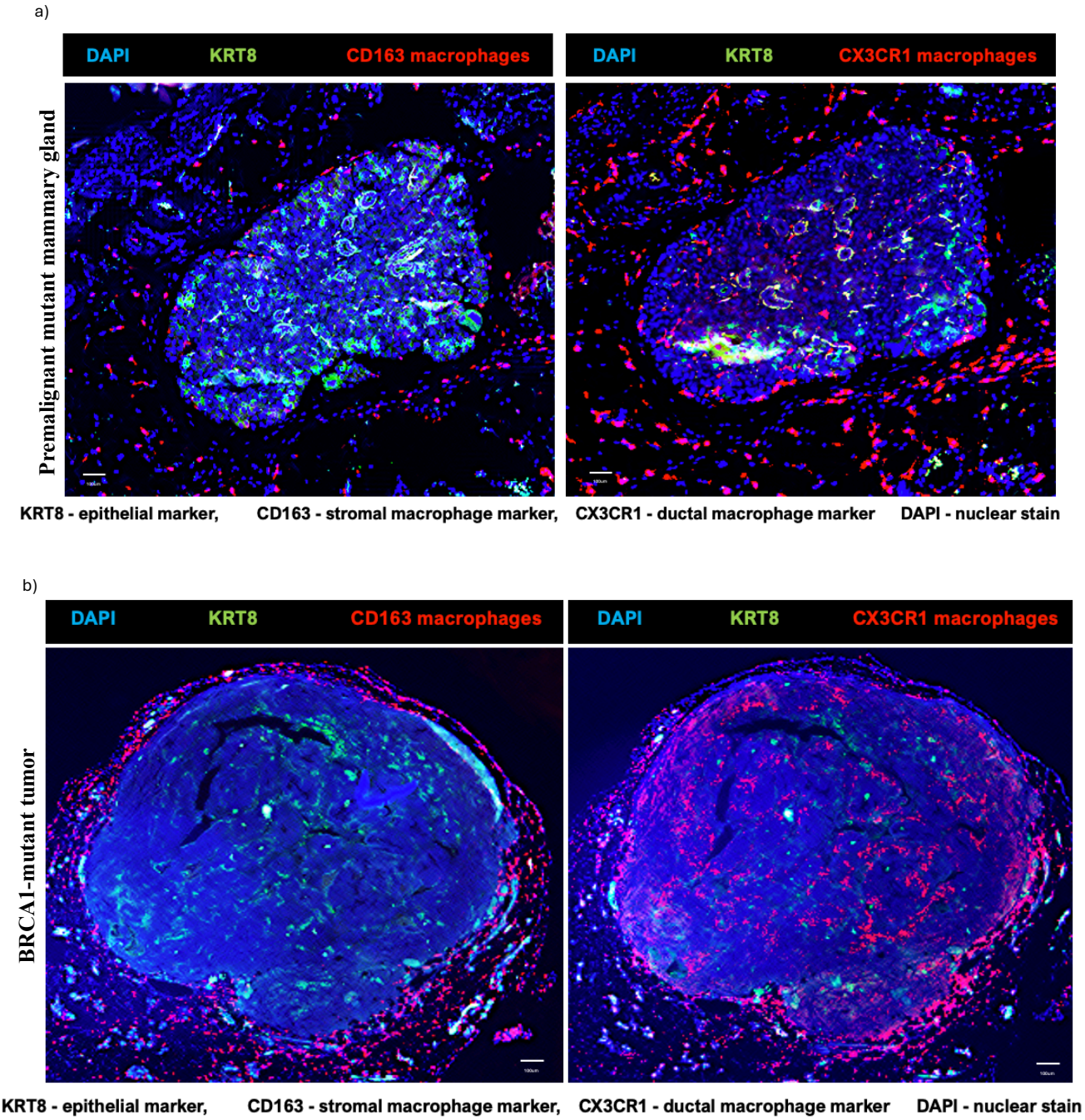


Figure 7. Distinct spatial localization of CX3CR1^{+} and CD163^{+} macrophages in-vivo. a) Representative immunofluorescence images of premalignant mammary tissue stained for CX3CR1, CD163, and Keratin-8. b) Representative immunofluorescence images of mammary gland tumor tissue stained for CX3CR1, CD163, and Keratin-8.

4.5 CX3CR1 expression defines macrophage populations with epithelial interacting potential

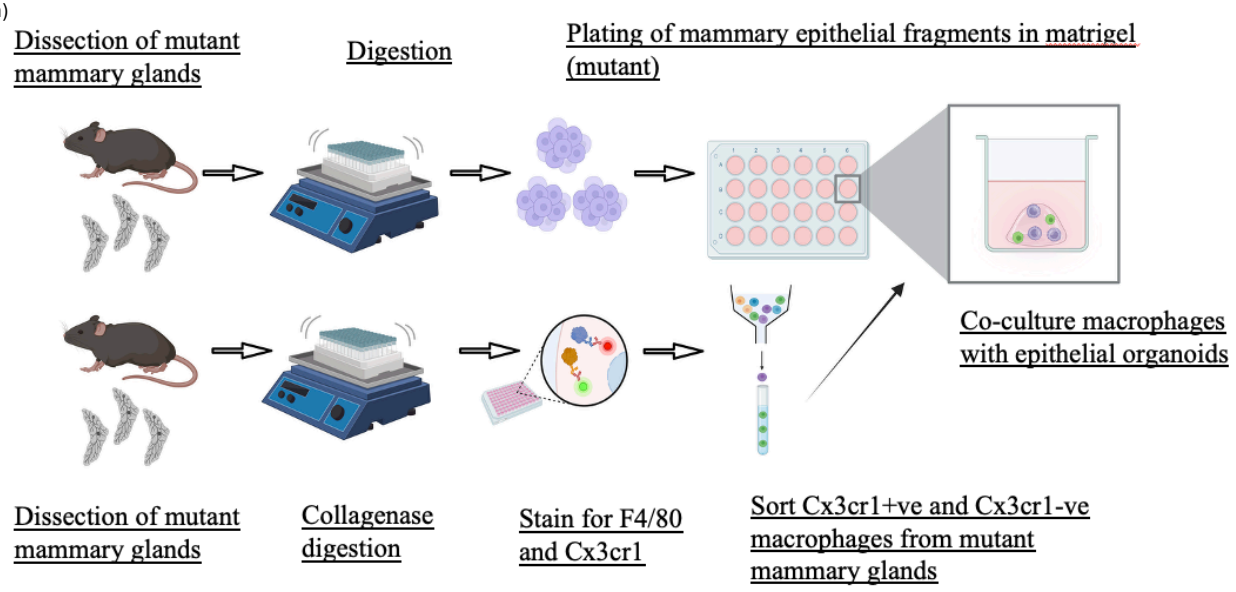
Analysis of single-cell transcriptomic data revealed that CX3CR1 expression was restricted to ductal associated and inflammatory macrophage subsets, whereas CD163 expression was enriched in stromal macrophages. CX3CR1 has been implicated in macrophage retention within epithelial niches and in facilitating direct macrophage-epithelial interactions [69,70]. This transcriptional specificity, combined with spatial localization, identified CX3CR1^{+} macrophages as the primary candidates for mediating macrophage-driven epithelial remodeling.

4.6 CX3CR1^{+} macrophages are critical to drive epithelial growth and branching

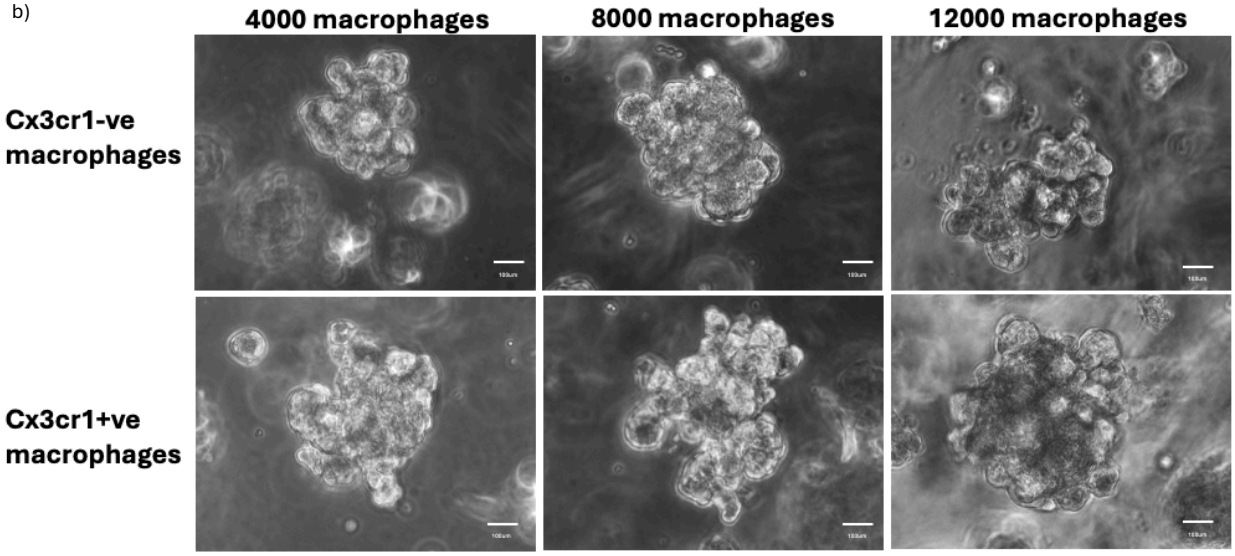
To directly test the functional contribution of CX3CR1^{+} macrophages, mutant mammary organoids were co-cultured with either CX3CR1^{+} or CX3CR1^{-} macrophages isolated from BRCA1-mutant mice **(Figure 8a)**. Organoids cultured with CX3CR1^{+} macrophages displayed markedly increased growth and branching compared to those co-cultured with CX3CR1^{-} macrophages **(Figure 8b)**.

Quantitative analyses revealed significant increases in total organoid area, total organoid number, average number of branches per organoid, and the proportion of branched organoids in the presence of CX3CR1^{+} macrophages **(Figure 8c, 8d, 8e, 8f)**. These results demonstrate that CX3CR1^{+} macrophages are functionally critical to drive epithelial growth and branching, consistent with their spatial positioning and transcriptional profile.

a)


Dissection of mutant mammary glands
Digestion
Plating of mammary epithelial fragments in matrigel (mutant)
Co-culture macrophages with epithelial organoids
Dissection of mutant mammary glands
Collagenase digestion
Stain for F4/80 and Cx3cr1
Sort Cx3cr1+ve and Cx3cr1-ve macrophages from mutant mammary glands

b)


4000 macrophages
8000 macrophages
12000 macrophages
Cx3cr1-ve macrophages
Cx3cr1+ve macrophages

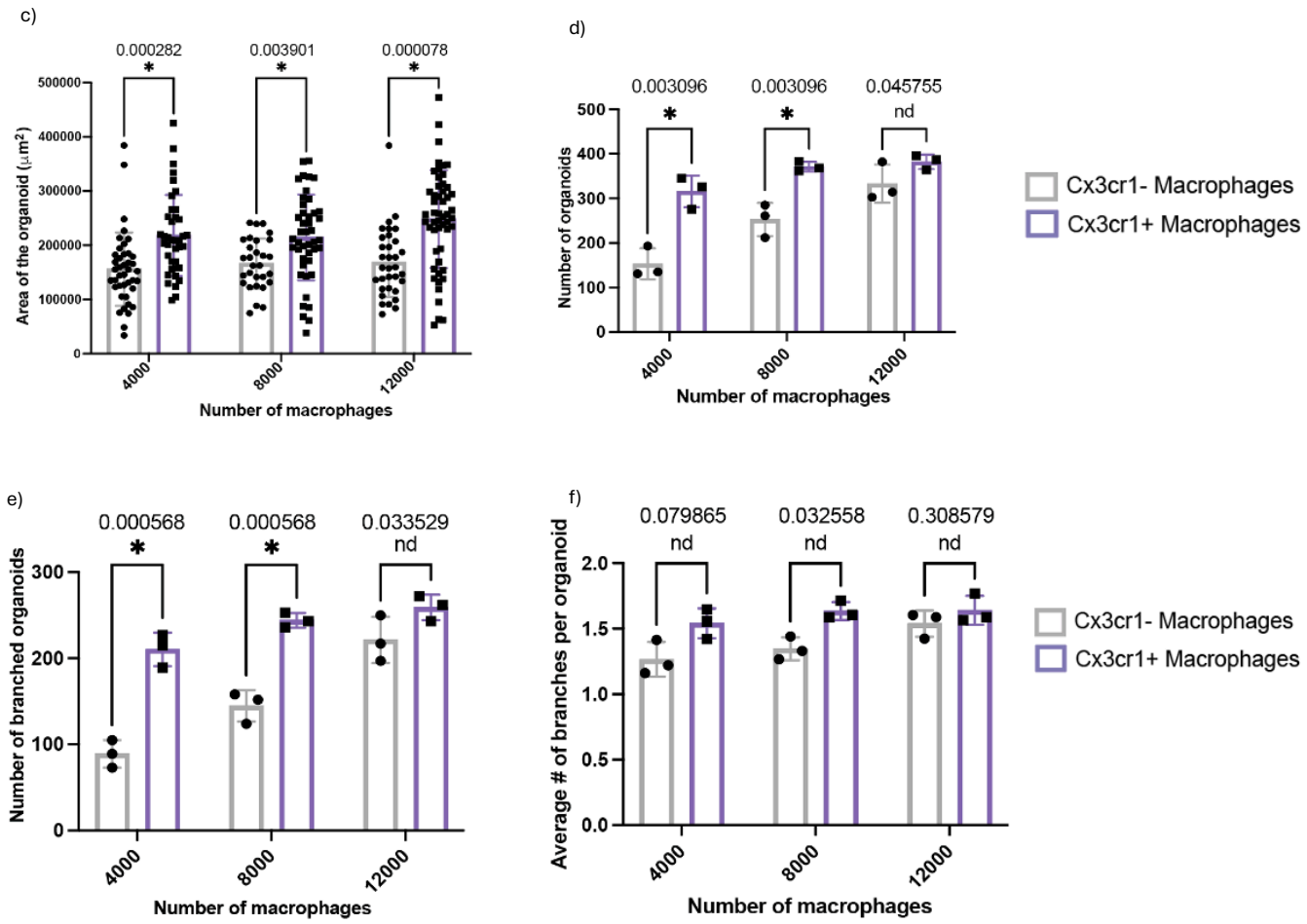


Figure 8. CX3CR1+ macrophages promote aberrant epithelial growth and branching. a) Schematic of the mammary organoid co-culture system used to assess macrophage-epithelial interactions. b) Representative images of BRCA1-deficient mammary organoids co-cultured with CX3CR1^{+} or CX3CR1^{-} macrophages at different dilutions of macrophages such as 4000, 8000 and 12000. c) Quantification of the organoid area across the different co-culture conditions. d) Quantification of the number of organoids across the different co-culture conditions. e) Quantification of the number of branched organoids across the different co-culture conditions. f) Quantification of the average number of branches per organoid across the different co-culture conditions.

4.7 Summary

In this chapter, we demonstrate that macrophages directly promote epithelial growth and aberrant branching in BRCA1-deficient mammary tissue and that this effect is driven predominantly by a specific macrophage subset. Using a three-dimensional organoid co-culture system, we show that macrophages from BRCA1-mutant tissues possess enhanced capacity to induce epithelial remodeling. Spatial and transcriptional analyses identify CX3CR1^{+} macrophages as the subset most closely associated with the epithelial compartment, and functional experiments confirm that CX3CR1^{+} macrophages are critical to drive epithelial growth and branching.

Together, these findings establish a direct functional link between macrophage heterogeneity, spatial organization, and epithelial dysregulation during the earliest stages of BRCA1-driven disease progression and provide a critical foundation for mechanistic interrogation of macrophage-derived signals in subsequent chapters.

Chapter 5

Role of macrophage-derived Oncostatin M in tumor initiation

5.1 Identification of macrophage-epithelial signaling pathways enriched in premalignant BRCA1-mutant tissue

While Chapter 4 demonstrated that CX3CR1^{+} inflammatory macrophages are functionally critical to drive epithelial growth and branching, the molecular mediators underlying this macrophage-driven epithelial remodeling remained unknown. To systematically identify candidate signaling pathways mediating macrophage-epithelial communication in BRCA1-deficient mammary tissue, we performed cell-cell communication analysis using CellChat on single-cell RNA-sequencing dataset derived from WT, premalignant BRCA1-mutant, and tumor tissues.

Global analysis revealed a progressive increase in the total number of inferred ligand-receptor interactions in mutant and tumor tissues compared to WT controls, consistent with enhanced immune-epithelial crosstalk during disease progression **(Figure 9a)**. Comparative analysis of signaling pathways between WT and mutant tissues identified marked qualitative differences in pathway usage. Among these, the Oncostatin M (OSM)-Oncostatin M receptor (OSMR) signaling axis emerged as a prominently enriched interaction in mutant samples **(Figure 9b)**. Notably, this interaction was absent or negligible in WT tissues and selectively emerged in the BRCA1-mutant condition, indicating that OSM signaling represents a disease associated communication pathway rather than a feature of normal mammary homeostasis.

5.2 Inflammatory macrophages are the dominant source of OSM in BRCA1-deficient mammary glands

To identify the cellular source of OSM, we examined Osm expression across myeloid subpopulations. Among all myeloid cells, inflammatory macrophages exhibited the highest and most specific expression of Osm **(Figure 9c)**. Furthermore, within the inflammatory macrophage population, Osm expression was significantly elevated in mutant and tumor tissues compared to WT samples, indicating amplification of OSM production in disease associated macrophages **(Figure 9d)**.

To define potential epithelial target populations, we next assessed expression of Osmr across all cell types. Dot plot analysis revealed that Osmr expression was highly enriched in basal epithelial cells, whereas Osm expression was largely restricted to myeloid cells across all conditions **(Figure 9e)**. This complementary expression pattern supports a paracrine signaling model in which inflammatory macrophage-derived OSM acts directly on epithelial cells within BRCA1-deficient mammary tissue.

Together, these findings identify OSM-OSMR signaling as a macrophage-derived, epithelial targeted communication axis that is selectively activated during premalignant BRCA1-associated disease.

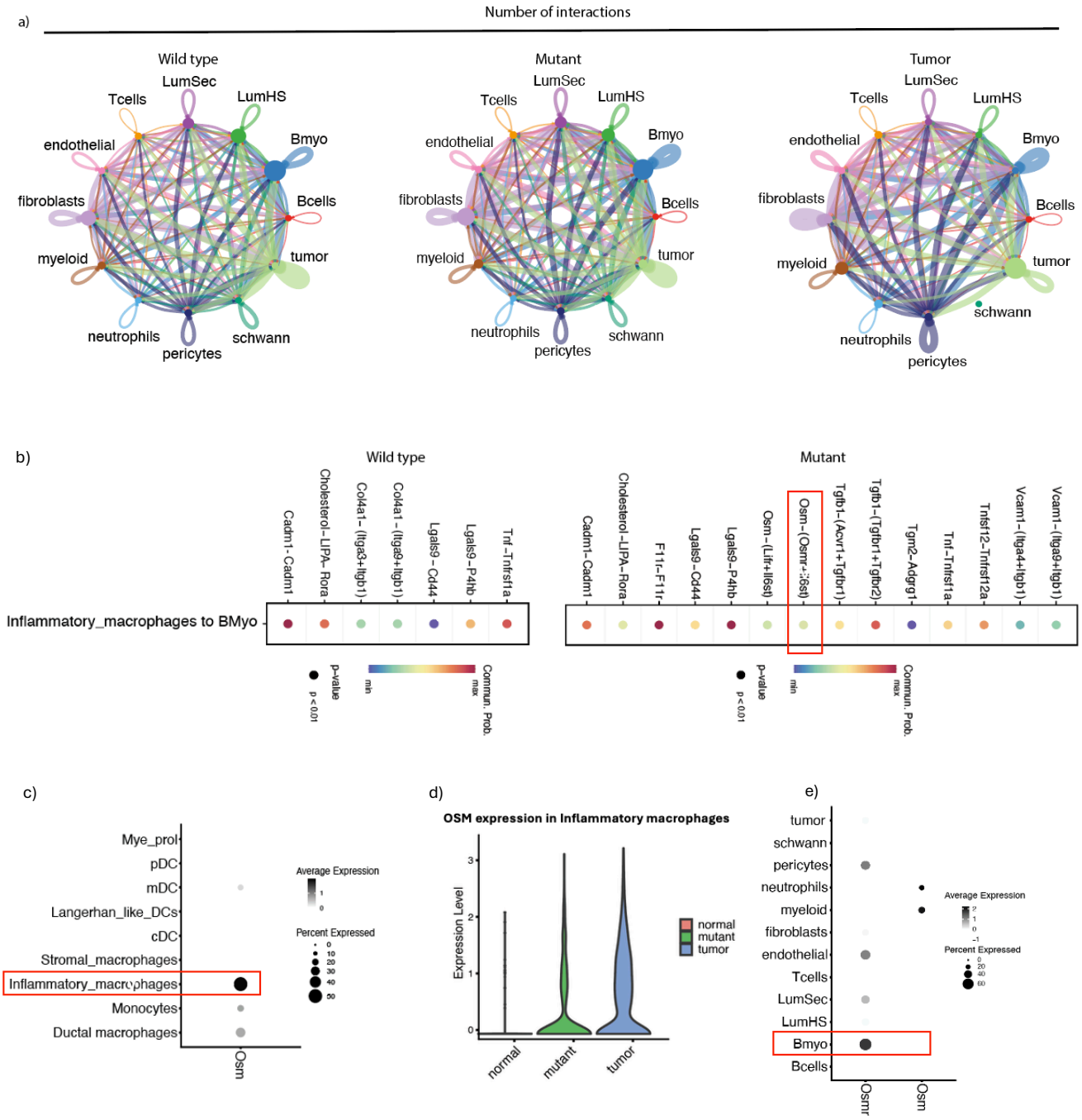


Figure 9. Cell-cell communication analysis identifies OSM signaling as a dominant macrophage-derived pathway in BRCA1-deficient mammary tissue. a) Circle plot showing the number of interactions predicted to be happening between all cell types across different conditions. b) Bubble plot highlighting the predicted significant interactions between inflammatory macrophages and basal epithelial cells in WT vs mutant mammary gland tissues. c) Dot plot showing expression of Osm across all myeloid cell populations. d) Violin plot of Osm expression levels in inflammatory macrophages across the different sample types. e) Dot plot showing expression of Osmr and Osm across all cell types in the single-cell dataset.

5.3 OSM directly promotes growth and branching of BRCA1-mutant mammary organoids

To directly test whether OSM is critical to drive epithelial growth and branching, we treated BRCA1-mutant mammary organoids with recombinant Oncostatin M (OSM) in a three-dimensional culture system **(Figure 10a)**. Mutant organoids treated with recombinant OSM exhibited a marked increase in organoid size and branching compared to untreated controls **(Figure 10b)**.

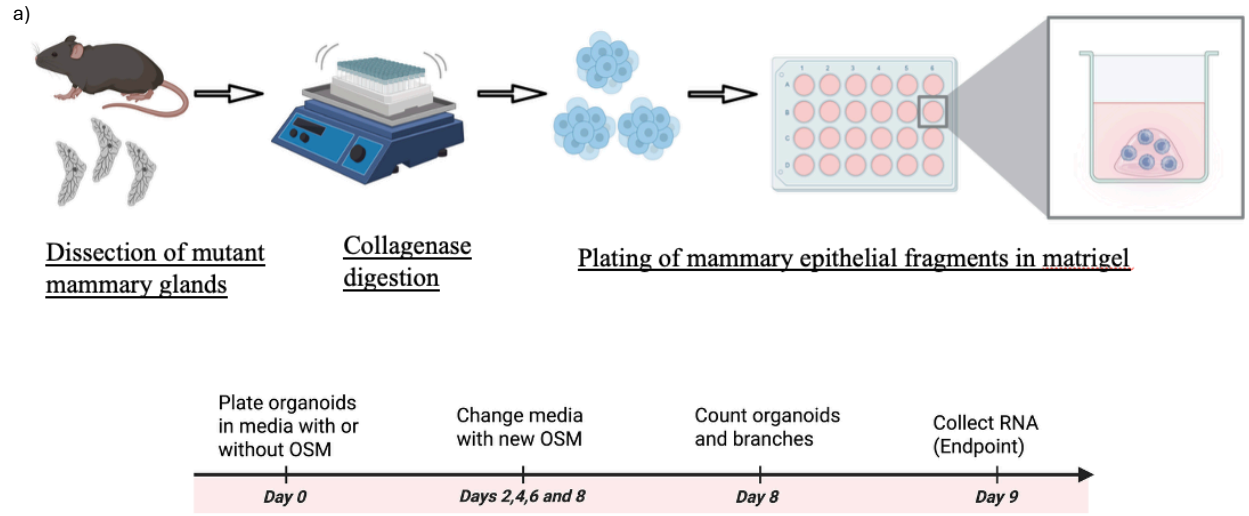
Quantitative analysis revealed significant increases in total organoid area following OSM stimulation, accompanied by an increase in the total number of organoids per well **(Figure 10c and 10d)**. In addition to promoting overall growth, OSM treatment significantly enhanced epithelial branching. OSM treated organoids displayed a higher average number of branches per organoids as well as an increased proportion of branched organoids, demonstrating that OSM directly promotes both epithelial expansion and branching in BRCA1-mutant epithelium **(Figure 10e and 10f)**.

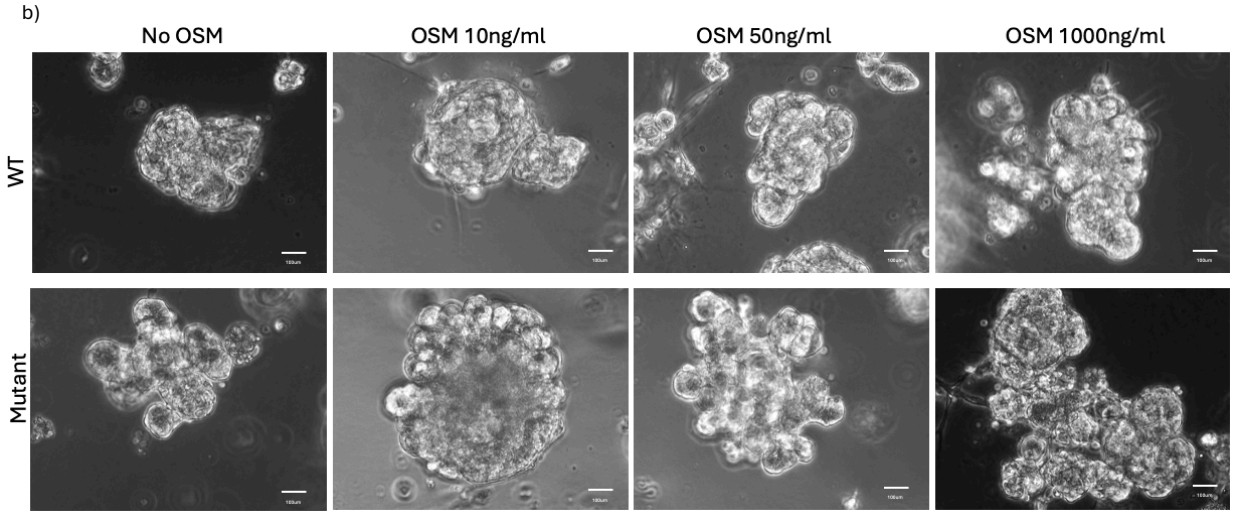
5.4 OSM activates canonical signaling pathways and promotes epithelial proliferation

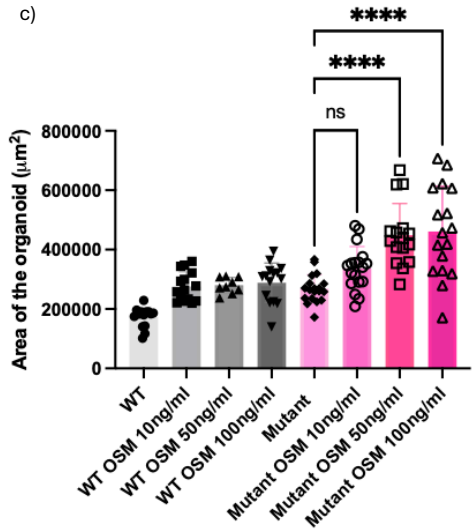
To validate activation of downstream OSM signaling pathways, we assessed expression of Suppressor of Cytokine Signaling 3 (Socs3), a canonical transcriptional target of OSM-OSMR-STAT3 signaling. Treatment with recombinant OSM induced a robust increase in *Socs3* expression, confirming activation of the OSM signaling cascade at the transcriptional level **(Figure 10g)**.

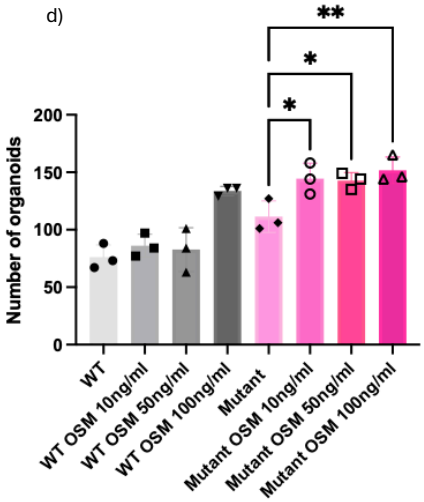
In parallel, OSM stimulation resulted in increased expression of the proliferation marker Ki67 within organoids, indicating that OSM enhances epithelial proliferative capacity in addition to

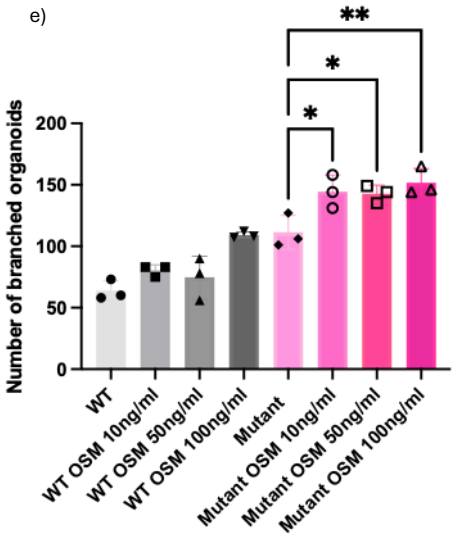
promoting branching morphogenesis **(Figure 10h)**. These data suggest that OSM drives aberrant epithelial remodeling through combined effects on proliferation and morphogenetic programs.

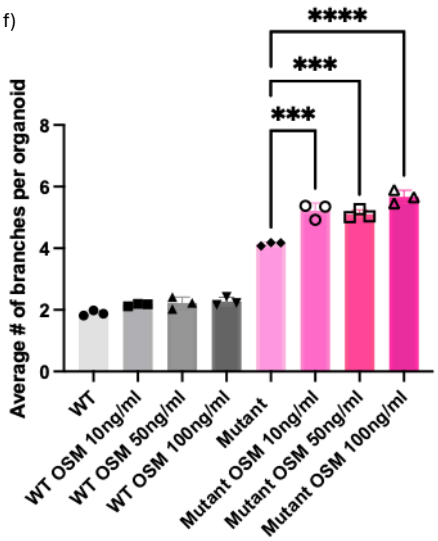





c)
Area of the organoid (μm²)
800000
600000
400000
200000
0
****
****
ns
WT
WT OSM 10ng/ml
WT OSM 50ng/ml
WT OSM 100ng/ml
Mutant
Mutant OSM 10ng/ml
Mutant OSM 50ng/ml
Mutant OSM 100ng/ml


d)
Number of organoids
200
150
100
50
0
**
*
*
WT
WT OSM 10ng/ml
WT OSM 50ng/ml
WT OSM 100ng/ml
Mutant
Mutant OSM 10ng/ml
Mutant OSM 50ng/ml
Mutant OSM 100ng/ml


e)
Number of branched organoids
200
150
100
50
0
**
*
*
WT
WT OSM 10ng/ml
WT OSM 50ng/ml
WT OSM 100ng/ml
Mutant
Mutant OSM 10ng/ml
Mutant OSM 50ng/ml
Mutant OSM 100ng/ml


f)
Average # of branches per organoid
8
6
4
2
0
****
***
***
WT
WT OSM 10ng/ml
WT OSM 50ng/ml
WT OSM 100ng/ml
Mutant
Mutant OSM 10ng/ml
Mutant OSM 50ng/ml
Mutant OSM 100ng/ml

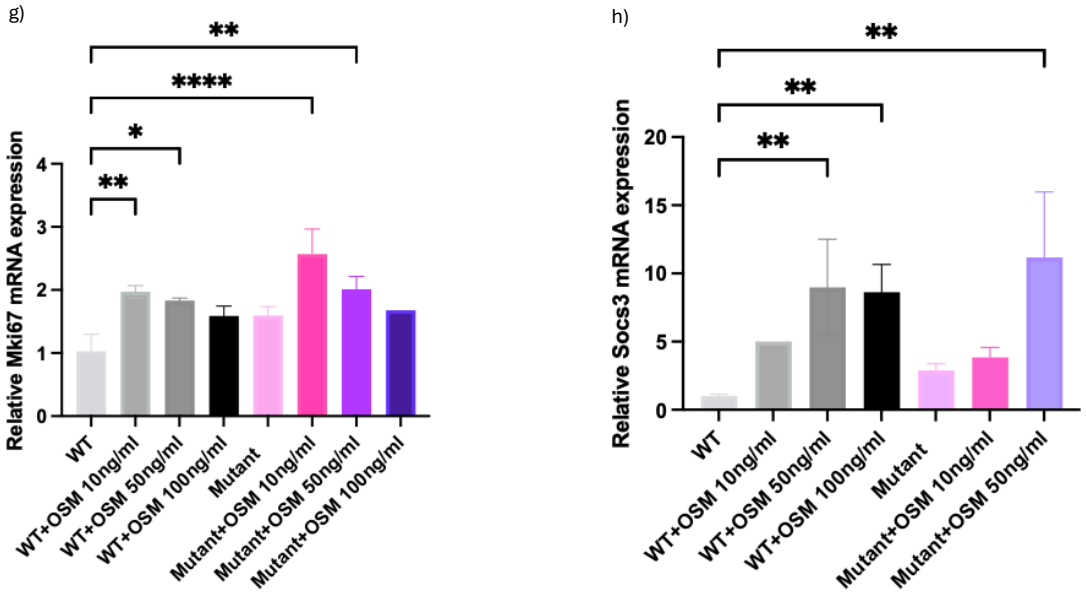


Figure 10. OSM is critical to induce aberrant growth and branching of mammary organoids. a) Schematic of the experimental design. b) Representative images of WT and mutant mammary organoids treated with recombinant human Oncostatin M (OSM) of different concentrations like 10ng/ml, 50ng/ml and 100ng/ml compared to untreated controls. c) Quantification of the organoid area across the different conditions. d) Quantification of the number of organoids across the different conditions. e) Quantification of the number of branched organoids across the different conditions. f) Quantification of the average number of branches per organoid across the different conditions. g) Gene expression of Mki67 and h) Socs3 across the different conditions.

5.5 Conservation of inflammatory macrophage-derived OSM expression in human BRCA1 carriers

To determine whether macrophage-derived OSM signaling identified in the BRCA1-deficient mouse model is conserved in humans, we extended our analysis to publicly available single-cell RNA-sequencing data from the Human Breast Cell Atlas, including breast tissue samples from BRCA1 mutation carriers and non-carrier controls. Myeloid cells were sub-clustered and analyzed to assess OSM expression across human macrophage populations.

Within the myeloid compartment, inflammatory (M1-like) macrophages exhibited the highest expression of OSM across all immune cell types **(Figure 11a)**. Importantly, OSM expression was significantly elevated in inflammatory macrophages derived from BRCA1 mutation carriers compared to non-carriers, identifying this population as the dominant source of OSM in human BRCA1-associated breast tissue **(Figure 11b)**. This selective increase was not observed across all macrophage subsets, indicating targeted upregulation of OSM within inflammatory macrophages rather than global myeloid activation.

Notably, this expression pattern closely parallels our observations in mouse models, in which inflammatory macrophages selectively expressed high levels of OSM in premalignant and tumor states. The concordance between mouse and human datasets supports a conserved role for inflammatory macrophage-derived OSM signaling in BRCA1-associated breast tissue remodeling and reinforces the translational relevance of the macrophage-epithelial communication axis identified in this study

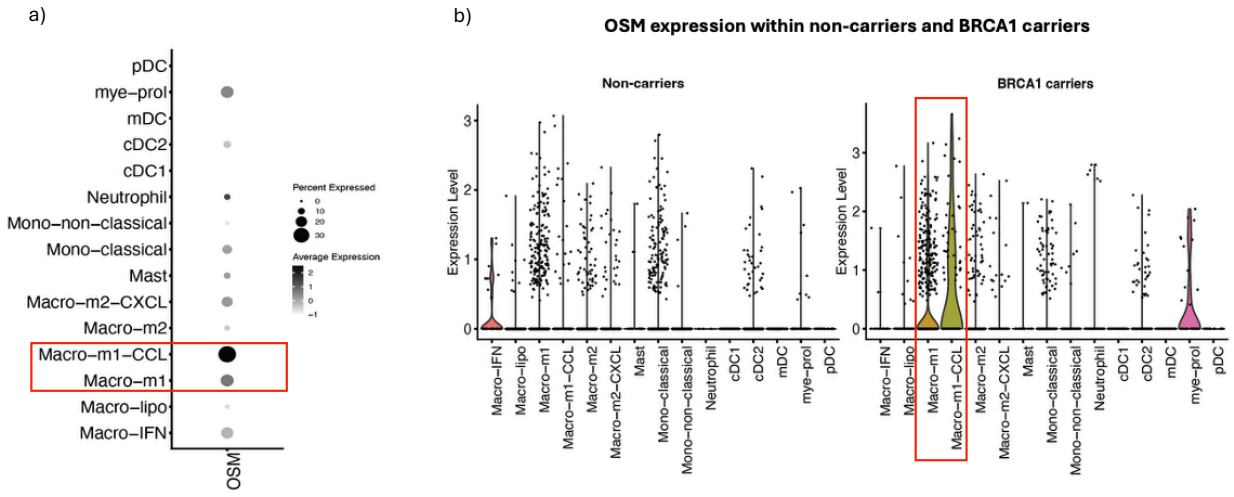


Figure 11. Conservation of high OSM expression specific to inflammatory type macrophages in human BRCA1 mutation carriers. a) Dot plot showing OSM expression across human myeloid populations. b) Violin plots highlighting OSM expression within all the myeloid cell populations in BRCA1 mutation carriers versus non-carrier controls.

5.6 Summary

In this chapter, we identify Oncostatin M as a key molecular mediator of macrophage-epithelial crosstalk in premalignant BRCA1-deficient mammary tissue. Ligand-receptor inference analysis reveals selective activation of OSM-OSMR signaling in mutant tissues, driven predominantly by inflammatory macrophages and targeted to basal epithelial cells. Functional assays demonstrate that OSM is critical to directly promote epithelial growth, proliferation and branching in BRCA1-mutant organoids and activates canonical downstream signaling pathways.

Together, these findings establish a mechanistic link between inflammatory macrophage accumulation and aberrant epithelial remodeling during early tumor initiation and position OSM

signaling as a central driver of premalignant epithelial dysregulation in BRCA1-associated breast cancer **(Figure 12)**.

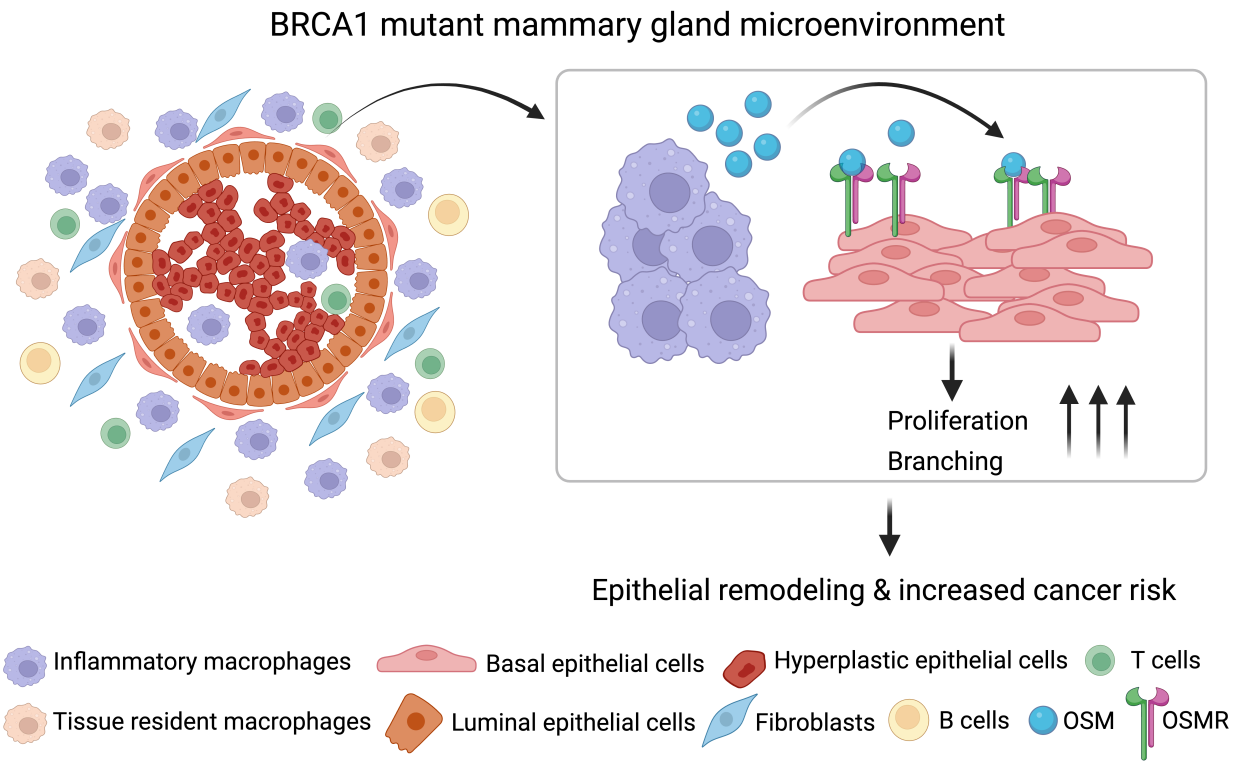


Figure 12. Integrated model of inflammatory macrophage-derived OSM signaling in BRCA1-associated breast cancer initiation. Schematic summarizing the proposed model in which inflammatory macrophages expand and spatially localize near hyperplastic epithelial structures, secrete OSM, and promote aberrant epithelial remodeling during premalignancy, thereby contributing to tumor initiation.

Discussion

BRCA1-associated breast cancer risk has traditionally been attributed to epithelial cell intrinsic defects in DNA repair and genomic stability, with tumor initiation viewed as a direct consequence of accumulated genetic damage. This paradigm has profoundly shaped the field, informing both risk assessment and therapeutic strategies such as the use of PARP inhibitors. However, this framework does not fully account for the extended premalignant phase that precedes tumor formation in BRCA1 mutation carriers, nor does it adequately explain the extensive tissue remodeling observed in BRCA1-deficient mammary glands long before malignancy becomes apparent. The work presented in this thesis expands this classical model by demonstrating that early remodeling of the immune microenvironment particularly involving macrophages is not a secondary consequence of tumorigenesis, but rather a central and functionally significant feature of BRCA1-driven disease initiation.

A key contribution of this work is the demonstration that immune alterations arise early in BRCA1-deficient mammary tissue, prior to overt tumor development. Through integrated single-cell transcriptomic and spatial analyses, this study shows that premalignant mammary glands exhibit selective expansion and diversification of macrophage populations, with inflammatory macrophages preferentially enriched, while other myeloid compartments remain comparatively stable. This pattern argues against a model of nonspecific inflammation and instead supports a model of qualitative immune reprogramming. Such selective immune remodeling is consistent with emerging concepts in cancer biology in which myeloid cells actively shape premalignant tissue states, influencing epithelial fate decisions well before malignant transformation occurs (30-37,49,50,54,55).

Importantly, similar enrichment of inflammatory macrophages was observed in breast tissue from human BRCA1 mutation carriers without cancer. This cross-species concordance strengthens the translational relevance of the mouse model and suggests that immune remodeling is a conserved response to BRCA1 loss rather than an artifact of experimental systems. These findings align with growing evidence that hereditary cancer predisposition syndromes are associated with detectable alterations in tissue microenvironments prior to tumor formation, highlighting the importance of studying cancer initiation as a tissue level process rather than a purely genetic event.

Beyond changes in immune composition, this work reveals that macrophages undergo profound spatial reorganization during premalignant progression. High dimensional spatial proteomic imaging demonstrated that macrophage accumulation is not uniform across the mammary gland but instead concentrates within epithelial regions exhibiting abnormal architecture, including hyperplastic and hyperbranched epithelium. These spatial features - macrophage clustering, close epithelial proximity, and localization within epithelial compartments are classically associated with established tumors. The observation that such tumor like immune architecture emerges during premalignancy (21,45,47,59-62) argues against a purely reactive immune response and instead supports a model in which spatial immune remodeling is an instructive event that precedes and potentially facilitates malignant transformation.

Spatial organization is increasingly recognized as a key determinant of immune function. Macrophages positioned in close proximity to epithelial cells are uniquely poised to influence epithelial behavior through paracrine signaling, extracellular matrix remodeling, and direct cell-cell interactions. In this context, the spatial enrichment of macrophages within abnormal epithelial regions suggests that immune cells may actively participate in shaping premalignant epithelial

architecture, rather than merely responding to epithelial abnormalities. This interpretation is consistent with studies in other tissues in which immune cell localization predicts functional impact more accurately than immune cell abundance alone.

A major conceptual advance of this thesis is the identification of CX3CR1^{+} inflammatory macrophages as a functionally dominant subset driving epithelial remodeling. Functional organoid co-culture assays demonstrated that macrophages are sufficient to promote epithelial growth and aberrant branching, hallmarks of premalignant BRCA1-deficient tissue. Crucially, this effect was not uniform across macrophage populations. CX3CR1^{+} macrophages exhibited a markedly enhanced capacity to induce epithelial remodeling compared to CX3CR1^{-} macrophages, underscoring the importance of macrophage heterogeneity in shaping tissue outcomes.

This functional specificity aligns with the transcriptional profile and spatial localization of CX3CR1^{+} macrophages, which are enriched for inflammatory and cytokine signaling programs and preferentially localize within epithelial compartments. These findings reinforce the idea that macrophages cannot be treated as a uniform cell type and highlight the need to define immune populations based on identity, location, and function (36-38,45,59,62). In the context of premalignant disease, such distinctions are particularly important, as subtle differences in immune cell behavior may have outsized effects on epithelial fate decisions.

Mechanistically, this work identifies Oncostatin M (OSM) as a key molecular mediator linking inflammatory macrophages to epithelial dysregulation. Cell-cell communication analysis using CellChat revealed selective activation of the OSM-OSMR signaling axis in BRCA1-mutant tissue, driven predominantly by inflammatory macrophages and targeted to basal epithelial cells (71). This computational inference was validated experimentally, as recombinant OSM was sufficient

to directly promote epithelial growth, proliferation, and branching in BRCA1-mutant organoids. These effects were accompanied by activation of canonical downstream signaling pathways, including induction of Socs3 and increased epithelial proliferation, consistent with known OSM signaling mechanisms.

These findings extend prior work implicating OSM in epithelial plasticity, wound repair, and tumor progression (51,74-77) by demonstrating a direct role for OSM in premalignant epithelial remodeling, rather than restricting its function to established cancer. This distinction is critical, as it suggests that OSM signaling contributes to disease risk at a stage when intervention may still be feasible. In this framework, OSM functions not merely as a marker of inflammation, but as an active driver of epithelial reprogramming in genetically sensitized tissue.

Notably, inflammatory macrophage-derived OSM expression was conserved in breast tissue from human BRCA1 mutation carriers, reinforcing the translational relevance of this signaling axis. The concordance between mouse and human datasets suggests that macrophage-driven cytokine signaling represents a conserved biological response to BRCA1 loss. This raises the possibility that OSM signaling contributes to disease risk long before malignant transformation and may represent a shared vulnerability across individuals with hereditary BRCA1 mutations.

The implications of these findings are particularly significant in the context of cancer prevention. Current preventive strategies for BRCA1 mutation carriers rely heavily on surgical interventions, reflecting the absence of effective biological approaches to modulate disease risk during the premalignant window. By identifying macrophage-driven signaling pathways that operate prior to tumor formation, this thesis highlights new opportunities for immune based preventive strategies. Targeting inflammatory macrophages, their spatial positioning, or downstream mediators such as

OSM could provide a means to modulate epithelial susceptibility to transformation before irreversible genetic or epigenetic changes accumulate.

Importantly, immune based prevention strategies need not involve permanent immune suppression. Instead, transient modulation of inflammatory signaling during defined windows of susceptibility may be sufficient to reset tissue architecture and reduce long term risk. This concept aligns with emerging ideas in cancer interception, which emphasize early, reversible interventions that prevent progression rather than treating established disease.

Several limitations of this work should be acknowledged. While organoid assays establish sufficiency, in-vivo perturbation studies will be required to determine whether targeting macrophages or OSM signaling can delay or prevent tumor initiation in intact organisms. Additionally, the upstream signals that recruit and activate inflammatory macrophages in BRCA1-deficient tissue remain incompletely defined. BRCA1 loss is associated with epithelial stress, altered differentiation, and architectural disruption, any of which may contribute to immune activation. Dissecting these upstream cues will be essential for understanding how immune remodeling is initiated.

Moreover, premalignant mammary tissue represents a complex ecosystem involving fibroblasts, endothelial cells, extracellular matrix, and systemic hormonal signals. Interactions between macrophages and these additional compartments were not directly addressed in this thesis but are likely to influence epithelial behavior and immune function. Future studies integrating multi compartmental analyses will be necessary to fully capture the complexity of premalignant remodeling.

In conclusion, this thesis establishes a framework in which immune microenvironment remodeling is an early, conserved, and functionally significant driver of BRCA1-associated breast cancer initiation. By linking macrophage heterogeneity, spatial organization, and cytokine mediated signaling to epithelial dysregulation, this work shifts the field away from tumor centric models toward a tissue level understanding of cancer initiation. These findings identify inflammatory macrophages and OSM signaling as potential targets for preventive intervention and underscore the broader importance of immune regulation in shaping early cancer risk in genetically high-risk populations.

Future directions

The findings presented in this thesis establish inflammatory macrophages and Oncostatin M (OSM) signaling as central regulators of epithelial remodeling during the earliest stages of *BRCA1*-associated breast cancer initiation. By integrating single-cell profiling, spatial analysis, and functional assays, this work defines macrophage heterogeneity, spatial organization, and epithelial impact in premalignant mammary tissue. However, these discoveries also raise important new questions that extend beyond the scope of this dissertation and provide a rich foundation for future investigation. Addressing these questions will be essential for translating mechanistic insight into prevention-oriented strategies for individuals at high genetic risk of breast cancer.

Establishing causality in-vivo: necessity of macrophages and OSM signaling during premalignant progression

A critical next step will be to directly test the necessity of macrophages and OSM signaling in-vivo during premalignant disease progression. While the organoid and co-culture assays presented in this thesis demonstrate that CX3CR1^{+} macrophages and OSM are sufficient to drive epithelial growth, branching, and remodeling, these reductionist systems cannot fully capture the complexity of intact tissue environments. Genetic and pharmacologic perturbation studies in *BRCA1*-deficient mouse models will therefore be required to determine whether disrupting macrophage function or OSM-OSMR signaling can delay, attenuate, or prevent aberrant epithelial remodeling and tumor onset.

Several complementary approaches could be pursued. Macrophage depletion strategies, including CSF1R inhibition or conditional depletion models, could be applied during defined premalignant

windows to assess the impact on ductal architecture, epithelial differentiation, and tumor latency. More targeted approaches, such as perturbation of CX3CR1 signaling, may allow selective disruption of ductal associated macrophage populations while preserving broader immune function. In parallel, genetic deletion or pharmacologic neutralization of OSM or OSMR in mammary epithelial cells would enable direct testing of whether epithelial responsiveness to macrophage-derived cytokines is required for premalignant phenotypes.

Importantly, the timing of these interventions will be critical. Premalignant remodeling unfolds gradually, and immune-epithelial interactions may have distinct roles at different stages of disease. Future studies should therefore incorporate longitudinal analyses to define when macrophage and OSM signaling are most influential. Such experiments would move the field beyond correlation and establish direct causal links between immune derived signals, epithelial remodeling, and cancer initiation in-vivo.

Defining upstream signals that recruit and polarize inflammatory macrophages

Another major unanswered question concerns the upstream signals that recruit and activate inflammatory macrophages in *BRCA1*-deficient mammary tissue. *BRCA1* loss is associated with epithelial stress, altered differentiation, genomic instability, and architectural disruption, all of which may contribute to immune activation. However, the specific epithelial or stromal derived cues that initiate macrophage recruitment and polarization remain unknown.

Future studies could focus on identifying damage associated molecular patterns, cytokines, chemokines, or metabolic cues released by *BRCA1*-deficient epithelial cells that promote macrophage accumulation or inflammatory polarization. Single-cell and spatial transcriptomic

analyses of early premalignant tissue may reveal epithelial programs associated with immune recruitment, such as interferon signaling, stress response pathways, or altered secretory profiles. These data could be complemented by functional assays testing whether candidate epithelial derived signals are sufficient to induce inflammatory macrophage states in-vitro.

In addition to epithelial cues, stromal components such as fibroblasts and endothelial cells may play important roles in shaping macrophage behavior. Fibroblast derived chemokines, extracellular matrix remodeling, or vascular changes could indirectly influence macrophage localization and activation. Dissecting these multi directional interactions will be essential for understanding how immune and epithelial compartments co-evolve during premalignant progression.

Identifying the upstream drivers of inflammatory macrophage recruitment may also reveal intervention points earlier in the disease process, potentially enabling strategies that prevent immune dysregulation before maladaptive remodeling becomes established.

Integrating OSM signaling with broader stromal and hormonal networks

While this thesis focuses on macrophage-epithelial crosstalk, premalignant mammary tissue represents a complex ecosystem involving fibroblasts, endothelial cells, extracellular matrix, and systemic factors such as hormones. Future studies should investigate how OSM signaling integrates with these additional components to drive tissue remodeling and epithelial plasticity.

Fibroblasts, in particular, are key regulators of mammary architecture and may respond to OSM signaling by altering matrix composition, stiffness, or growth factor availability. OSM induced fibroblast activation could indirectly shape epithelial behavior by modifying the physical and

biochemical properties of the microenvironment. Similarly, endothelial cells may respond to inflammatory cytokines by altering vascular permeability or immune cell trafficking, further influencing tissue organization.

Hormonal signaling represents another critical axis of interaction. *BRCA1* is known to regulate hormone receptor signaling, and inflammatory cytokines such as OSM may intersect with estrogen and progesterone pathways to influence epithelial fate decisions. Exploring how OSM signaling modulates hormone responsiveness, particularly in premalignant tissue, could provide insight into why *BRCA1*-associated cancers arise preferentially in hormone responsive organs.

A systems level understanding of how immune derived signals integrate with stromal and hormonal networks will be necessary to fully capture the complexity of premalignant mammary remodeling.

Spatial and temporal dynamics of immune-epithelial interactions

The spatial analyses presented in this thesis highlight the importance of macrophage localization in determining epithelial outcomes. Future work should extend these observations by incorporating higher resolution and longitudinal spatial profiling approaches to track immune-epithelial interactions over time.

Spatial transcriptomics and multiplexed proteomics applied to premalignant mammary tissue could reveal dynamic changes in macrophage positioning, activation state, and signaling activity as disease progresses. Such approaches would enable identification of spatial niches that are particularly permissive for aberrant epithelial remodeling and could uncover early spatial biomarkers of cancer risk.

Temporal studies will be equally important. Premalignant remodeling likely involves waves of immune activation and resolution, and the persistence or dysregulation of these processes may distinguish tissues that progress to cancer from those that remain benign. Longitudinal sampling in mouse models, combined with computational trajectory analyses, could provide insight into how immune-epithelial interactions evolve over time and identify critical inflection points for intervention.

Extending findings to human BRCA1 mutation carriers

Given the conservation of inflammatory macrophage expansion and OSM expression observed in human *BRCA1* mutation carriers, an essential future direction will be to validate and extend these findings in larger and more diverse human cohorts. Access to non-malignant breast tissue from *BRCA1* carriers provides a rare opportunity to study premalignant biology directly in humans.

Future studies integrating spatial transcriptomic or proteomic analyses of human breast tissue could determine whether macrophage localization, inflammatory states, and OSM-OSMR signaling exhibit patterns similar to those observed in mouse models. Such analyses would strengthen the translational relevance of these pathways and help identify biomarkers that predict disease risk or progression.

Importantly, human studies should consider variables such as age, parity, hormonal status, and prior interventions, which may influence immune composition and epithelial behavior. Accounting for this heterogeneity will be critical for translating mechanistic insights into clinically actionable strategies.

Toward immune based prevention strategies

Perhaps the most exciting implication of this work is the possibility of immune based prevention strategies for individuals at high genetic risk of breast cancer. Current preventive approaches focus largely on eliminating at risk tissue through prophylactic surgery. While effective, these strategies are invasive and life altering.

The findings of this thesis suggest an alternative paradigm: modulating the premalignant immune microenvironment to reduce epithelial susceptibility to transformation. Targeting inflammatory macrophages, their spatial positioning, or cytokine signaling pathways such as OSM may offer a means to preserve tissue while reducing cancer risk.

Future work could explore whether transient modulation of immune signaling during defined windows of risk is sufficient to reset tissue architecture and prevent maladaptive remodeling. Such approaches would require careful balancing of immune function to avoid compromising host defense but could ultimately provide a less invasive and more acceptable option for cancer prevention.

Broader implications

Beyond *BRCA1*-associated breast cancer, the concepts developed in this thesis have broader implications for understanding cancer initiation in other hereditary and sporadic contexts. Immune-epithelial interactions likely play important roles in shaping premalignant tissue states across organ systems. By focusing on the earliest stages of disease, this work contributes to a growing recognition that cancer prevention requires an understanding of tissue ecosystems, not just transformed cells.

Conclusion

In summary, this thesis lays the groundwork for a new framework in which immune derived signals, particularly from inflammatory macrophages, are active determinants of epithelial fate during the earliest stages of *BRCA1*-associated breast cancer initiation. The future directions outlined here emphasize the importance of establishing causality, identifying upstream drivers, integrating multi compartmental signaling networks, and translating findings to human populations. By shifting attention from established tumors to the premalignant microenvironment, these studies underscore the potential of prevention-oriented strategies and highlight immune regulation as a central axis in early cancer development.

Materials and Methods:

Mouse strains:

The *Wap-Cre Brca1$^{f10/f10}$p53$^{f5\&6/f5\&6}$* mouse colony was a gift from Eva Y.-H P. Lee. Littermates or similarly aged & co-housed females were used in experiments. Ages used in experimental groups: wildtype & mutant = 4-months, tumor = 4 to 6 months old. All colonies were bred, housed, and maintained collaboratively by Lawson and Kessenbrock laboratories in accordance with UC Irvine IACUC protocol AUP-22-015. Males were not used because breast cancer mainly affects women.

Single-Cell RNA-Sequencing Data Analysis

Single-cell RNA-sequencing (scRNA-seq) data analysis was performed using Seurat in R unless otherwise stated. Raw gene by cell count matrices generated from 10x Genomics were imported into Seurat objects using the Read10X() function. Cells were filtered to remove low-quality cells and potential doublets based on the number of detected genes (nFeature_RNA), total UMI counts (nCount_RNA), and the percentage of mitochondrial gene expression. Cells with fewer than 200 detected genes, greater than 5,000 detected genes, or with >15% mitochondrial reads were excluded from downstream analysis. Genes expressed in fewer than 3 cells were removed. Quality control metrics were visualized using violin plots and scatter plots to guide threshold selection. Filtered cells from different biological conditions (e.g., wildtype, mutant, and tumor) were normalized using SCTransform, regressing out mitochondrial gene percentage to minimize technical variation. Integration across conditions and replicates was performed using Seurat's integration workflow, identifying shared biological features via SelectIntegrationFeatures() and

calculating integration anchors using FindIntegrationAnchors(). Integrated expression values were generated using IntegrateData(). Principal component analysis (PCA) was performed on the integrated dataset using the top variable features identified during SCTransform normalization. The number of principal components used for downstream analysis was determined based on elbow plots and variance explained. Cells were clustered using a shared nearest-neighbor (SNN) graph constructed with FindNeighbors(), followed by graph-based clustering using FindClusters() with resolution parameters optimized for biologically meaningful cell populations. Low dimensional embeddings were visualized using Uniform Manifold Approximation and Projection (UMAP). Cell clusters were annotated based on the expression of established lineage specific marker genes and comparison to published mammary gland and immune scRNA-seq datasets. Major compartments, including epithelial, immune, endothelial, fibroblast, and stromal populations, were identified first, followed by higher resolution annotation of immune subsets. Myeloid populations were further refined into monocytes, inflammatory macrophages, and other macrophage states based on canonical markers. Cluster identities were validated by differential gene expression and marker visualization using dot plots and feature plots.

Tissue harvesting and preparation for flow cytometry and sorting:

Mammary glands were harvested from mice at the indicated ages and conditions and processed immediately to generate single-cell suspensions for flow cytometry and cell sorting. Excised tissues were finely minced using sterile scissors and enzymatically digested in collagenase and DNAase at 37 °C with gentle agitation. Following digestion, samples were mechanically dissociated by gentle trituration and filtered through a 70 μm cell strainer to remove undigested debris. Cell suspensions were washed with ice cold PBS supplemented with 2% fetal bovine serum

(FBS) and subjected to red blood cell lysis using ammonium chloride potassium (ACK) lysis buffer. Cells were then washed, counted, and resuspended in flow cytometry buffer (PBS containing 2% FBS). All subsequent steps were performed on ice to preserve cell viability and minimize activation. Single-cell suspensions were stained with fluorophore-conjugated antibodies against surface markers. Dead cells were excluded using a fixable viability dye. Stained samples were filtered immediately prior to acquisition and analyzed or sorted using a BD FACSAria cell sorter. Sorted populations were collected directly into complete culture medium for downstream functional and molecular analyses.

Flow cytometry:

Tissue was prepared for flow cytometry analysis using 2 mg ml^{-1} Collagenase D (Milipore Sigma cat. no. 11213857001) for digestion. Cells were stained with a viability dye. Next, cells were stained with fluorescent antibodies for extracellular markers and analysed using BD Fortessa X20 and FlowJo v10 software. The following antibodies were used for flow cytometry analysis: CD45-AF700 (Biolegend, 30-F11, 1:100), CD11b-BV605 (Biolegend, M1/70, 1:100), F4/80-PE (Biolegend, BM8, 1:100), CX3CR1-BV711 (Biolegend, SA011F11, 1:100), CD163-APC (Biolegend, S15049I, 1:100), CD206-FITC (Biolegend, C068C2, 1:100).

Tissue harvesting and preparation for all in-situ analysis

Mammary glands and tumor tissues were harvested from mice at the indicated ages and experimental conditions and immediately processed for histological analysis. Excised tissues were gently trimmed to remove excess fat and fixed in 10% neutral buffered formalin at room temperature for 24 hours. Following fixation, tissues were washed in phosphate-buffered saline

and processed through graded ethanol dehydration, cleared in xylene, and embedded in paraffin according to standard protocols. Formalin fixed paraffin embedded (FFPE) tissue blocks were sectioned at 5 μm thickness using a microtome and mounted onto charged glass slides. Sections were dried overnight and stored at room temperature until further use. FFPE sections were used for downstream in situ analyses, including hematoxylin and eosin (H&E) staining and immunofluorescence (IF) as indicated.

Highly multiplexed immunostaining using CODEX:

Formalin fixed paraffin embedded (FFPE) mammary gland and tumor tissues from BRCA1 wildtype and mutant mice were analyzed using the CODEX (PhenoCycler, Akoya Biosciences) platform. All procedures followed the manufacturer's protocol. Briefly, tissues were sectioned at 5 μm thickness and mounted on 22 mm × 22 mm glass coverslips pre-coated with 0.1% poly-L-lysine. Sections were then dewaxed and stained with a mixture of oligonucleotide-barcoded PhenoCycler antibodies, followed by post-fixation as per the PhenoCycler user manual. A panel of seven antibodies, targeting proteins listed in Supplementary Table [insert table number], was used for staining. Imaging was performed on the PhenoCycler-Open system using iterative cycles of three spectrally distinct fluorescent oligo reporters. Image acquisition was carried out using the Keyence BZ-X800 fluorescence microscope equipped with a 20× PlanApo 0.75 numerical aperture lens. Raw image data were processed using CODEX Processor v1.8.

Computational analysis of CODEX data:

Cell segmentation was performed using StarDist. For each cell, the average intensity of each protein was quantified using the segmentation masks and corresponding protein images. When a

protein was localized to the nucleus (e.g., Ki-67, P63, PR), intensity values were extracted specifically from the nuclear mask generated by StarDist. In contrast, for membrane-localized proteins (e.g., SMA, Keratin-8, Keratin-14, Keratin-5), intensities were calculated using the membrane mask. The resulting average protein intensities were then z-score normalized across all cells. Unsupervised clustering was subsequently carried out using the Leiden algorithm on the normalized data to assign cluster labels to cells within each sample independently.

Immunofluorescence analysis:

Tissue sections were incubated at 65 °C and deparaffinized using Histo-Clear (National Diagnostics, #HS-200), followed by rehydration through a graded ethanol series (100% to 50%). Antigen retrieval was carried out using a microwave pressure cooker in 10 mM citric acid buffer containing 0.05% Tween-20 (Thermo Fisher Scientific, #BP337500, pH 6.0). Sections were then blocked in a solution of Blockaid (Thermo Fisher Scientific, #B10710). Primary antibodies, diluted in blocking buffer, were applied overnight at 4 °C. After PBS washes, sections were incubated with secondary antibodies (also diluted in blocking buffer) for 1 hour at room temperature. Slides were mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, #H-1200), and fluorescent images were captured using the Keyence BZ-X700 microscope. The following antibodies were used for IF analysis: CX3CR1 polyclonal antibody (Proteintech, Cat# 29819, 1:100), CD163 polyclonal antibody (Proteintech, Cat# 31024 1:100), Keratin14 (Biolegend, Clone - Poly19053, Cat# 905303, 1:100), Keratin 8 (DSHB, Hybridoma concentrate, 1:200), F4/80 (Cell Signaling Technologies, Cat# 70076, Clone – D2S9R, 1:100)

Macrophage sorting:

Cells were stained with fluorophore conjugated antibodies against surface markers to identify macrophage populations. Macrophages were defined as live, single, $CD45^+$ $CD11b^+$ $F4/80^+$ cells. For subset-specific experiments, macrophages were further subdivided based on Cx3cr1 expression, allowing isolation of $Cx3cr1^+$ and $Cx3cr1^-$ macrophage populations. Antibody staining was performed for 30 minutes on ice in the dark, followed by washing prior to sorting. Flow cytometric sorting was performed on a BD FACSAria cell sorter. Doublets were excluded based on forward and side scatter properties, and dead cells were excluded using the viability dye. Macrophage populations were sorted to high purity (>95%) directly into complete culture media for downstream functional assays. For co-culture assays, macrophages were counted immediately after sorting and added to mammary epithelial organoids at defined ratios. All sorting procedures were performed at 4°C to preserve cell viability and minimize activation.

Organoid culture:

Murine mammary organoids were generated from freshly isolated mammary glands as previously described [78]. Briefly, mammary fat pads were dissected, mechanically minced, and enzymatically digested using Collagenase (Sigma-Aldrich, Cat# C5138) and dispase (StemCell Technologies, Cat# 07913) to release mammary epithelial fragments. Red blood cells were removed by ACK lysis (Gibco ACK Lysing buffer, Fisher Scientific, Cat# A1049201), and epithelial cell aggregates were enriched by differential centrifugation. Purified epithelial fragments were resuspended in growth factor reduced Matrigel (Corning, Cat# 354230) and plated as three-dimensional domes in 24 well plates precoated with Matrigel. After polymerization at 37 °C, organoids were overlaid with organoid culture medium consisting of DMEM/F12 supplemented

with Recombinant murine EGF (PeproTech, Cat# 315-09), Recombinant murine FGF2 (PeproTech, Cat# 450-33), R-spondin-1, Insulin-Transferrin-selenium (Thermo Scientific, Cat# 41400045), and the ROCK inhibitor Y-27632 (MedChem Express, Cat# 129830-38). Organoids were maintained at 37 °C in a humidified incubator with 5 % CO_2, and media were refreshed every 2 days. For the OncostatinM organoid experiment, every two days the organoids were replenished in media containing 0, 10, 50 or 100ng/ml recombinant mouse oncostatinM (R&D Systems, Cat# 495-MO-025/CCF).

Organoid-Macrophage co-culture

For co-culture assays, flow sorted macrophages were added directly inside Matrigel to organoids to allow cell-cell interactions at defined macrophage to organoid ratios (4,000-12,000 macrophages per well, as indicated). Co-cultures were maintained for 7-10 days, with media refreshed every 2 days. Parallel organoid only cultures were maintained as controls.

Quantification of organoids:

Organoids were imaged using brightfield microscopy at defined time points. Quantitative analysis of organoid phenotypes, including organoid number**,** size**,** and branching complexity**,** was performed using ImageJ. Branching was defined as the presence of two or more epithelial protrusions extending from the organoid body. At least three biological replicates were analyzed per condition, with multiple fields quantified per replicate.

Cell-cell communication analysis:

We used CellChat [71] to analyze cell-cell communication between putative cell types due to potential ligand-receptor interactions. We analyzed each condition (wildtype, mutant, tumor) separately. We applied the "standard" CellChat workflow using the variance-stabilized gene expression counts, i.e., the log-normalized UMI counts. That is, for each sample, we performed the following steps. First, we used subsetData to subset the full gene expression matrix to only analyze signal ligand genes or signal receptor genes, reducing computation time. Second, we determined significantly expressed signal ligand genes and signal receptor genes (with respect to cell type groups) using identifyOverExpressedGenes, and, consequently, significantly expressed ligand-receptor pairs with respect to cell type pairs using identifyOverExpressedInteractions. Then, CellChat calculated 30 ligand-receptor interaction scores using the trimean (i.e., type = "trimean") to calculate the average ligand and receptor gene expression in potential sender and receiver cell type groups, as implemented in computeCommunProb. To avoid biasing of cell-cell communication results due to rare cell types, we removed ligand-receptor interactions involving cell type groups containing fewer than ten cells using filterCommunication (setting min.cells = 10). To analyze common ligand-receptor interactions between basal cells and inflammatory macrophages across all samples, we used subsetCommunication to extract the list of statistically significant ligand-receptor interactions, as determined by CellChat's permutation test applied to to cell type labels, where an interaction was denoted as significant if the computed p-value satisfied $P < 0.05$.

Quantitative PCR:

Organoids were harvested from Matrigel culture using ice cold dispase. RNA was extracted from cells using Quick-RNA Micro Prep (Zymo Research, R1050). cDNA was obtained from iScript cDNA Synthesis Kit (Bio Rad, 1708891). qPCR amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, A25742). Gene specific primers were utilized to amplify the following genes: *Socs3* (Forward primer: GGAGATTTCGCTTCGGGACT, Reverse primer: TCGCTTTTGGAGCTGAAGGT), *Osmr* (Forward primer: ATGTCTTTCAAGTGCCCGGTTA, Reverse primer: TCCTCCAAGACTTCGCTTCG), *Mki67* (Forward primer: ATAACCATCATTGACCGCTCCT, Reverse primer: CATCTGAGGCAGGGCTATCTG), *Keratin14* (Forward primer: AGTTTGAGACGGAGCAGAGC, Reverse primer: GCCACCTCCTCGTGGTTC), *Keratin8* (Forward primer: AGGACTGACCGACGAGATCA, Reverse primer: CTCGTACTGGGCACGAACTT), *Acta2* (Forward primer: TTCCTTCGTGACTACTGCCG, Reverse primer: TATAGGTGGTTTCGTGGATGCC) Primers were generated by Integrated DNA Technologies. Relative quantification of transcripts for all samples was performed in triplicates. Gene expression levels were normalized to housekeeping genes, and relative expression was calculated using the ΔΔCt method.

Statistical analysis:

Data are presented as the mean ± s.d. from two or three independent experiments, unless stated otherwise. Statistics were conducted using Student's *t* test. Statistical test results are represented on graphs. No statistical method was used to determine sample size.

Data and code availability

Data generated in this study and code used in data analysis will be published along with the final work. Reasonable requests prior to publication may be granted, on a case-by- case basis.

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