CHAPTER FIVE

Heterogeneity and function of macrophages in the breast during homeostasis and cancer

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Abstract

Macrophages are diverse immune cells populating all tissues and adopting a unique tissue-specific identity. Breast macrophages play an essential role in the development and function of the mammary gland over one's lifetime. In the recent years, with the development of fate-mapping, imaging and scRNA-seq technologies we grew a better understanding of the origin, heterogeneity and function of mammary macrophages in homeostasis but also during breast cancer development. Here, we aim to provide a comprehensive review of the latest improvements in studying the macrophage heterogeneity in healthy mammary tissues and breast cancer.

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Abbreviations

BM	bone marrow
DM	ductal macrophages
ECM	extracellular matrix
ER	estrogen receptor
HA	hyaluron
PR	progesterone receptor
SM	stromal macrophages
TAM	tumor-associated macrophages
TDLU	terminal duct lobular unit
TEB	terminal end buds
TNBC	triple negative breast cancer
TRM	tissue resident macrophages

1. Introduction

Macrophages are innate immune cells seeded all over the body with essential roles in tissue homeostasis and immune defense mechanisms. Over the last decade it became clear that most tissue macrophages have an embryonic origin. Throughout adulthood, at steady state, these resident cells either maintain their abundance by proliferating or get partially replenished by bone-marrow derived monocytes in an organ specific manner (Ginhoux and Guilliams, 2016; Gomez Perdiguero et al., 2015; Hoeffel and Ginhoux, 2018; Liu et al., 2019).

Macrophages have been implicated in all stages of mammary gland development and functions (Gouon-Evans et al., 2000; Pollard and Hennighausen, 1994; Van Nguyen and Pollard, 2002). As such, macrophages are essential for morphogenesis of the ductal epithelial tree and modulate normal stem/progenitor cell activity, proliferation, alveolar budding and collagen matrix organization in the developing mammary gland (Gouon-Evans et al., 2002; Gyorki et al., 2009; Ingman et al., 2006). In addition, macrophages contribute to tissue clearance during the hormonal cycle or following damage and tissue remodeling during involution (Chua et al., 2010; Dawson et al., 2020).

Recent studies provided deeper insights into the localization and potential roles of distinct macrophage populations at steady state and in breast tumors (Dawson et al., 2020; Ramos et al., 2021; Wang et al., 2020). Tumor-associated macrophages (TAMs) are important components of the breast cancer microenvironment and their density positively correlates with

worst patient prognosis (Zhang et al., 2012; Zhao et al., 2017). TAMs are heterogenous and can originate from both tissue resident and infiltrating monocytes. These highly plastic cells can acquire distinct phenotypic and functional profiles in response to localized stimuli and tissue specific microenvironment (Cassetta et al., 2019; Wu et al., 2020). They can exert both pro-tumor (e.g., promotion of cancer cell proliferation/metastasis, stimulation of angiogenesis, reduced therapy responses or interference with $CD8^+$ T cell activation) and anti-tumor (e.g., cancer cell killing, antigen presentation or activation of adaptive immune responses) actions, however, in developing tumors TAMs have a predominantly pro-tumoral behavior (Larionova et al., 2020; Noy and Pollard, 2014). Therefore there is an increasing need to understand the existing TAM phenotypes and their functional characteristics to improve and develop novel macrophage-targeting therapies (Bercovici et al., 2019; Cassetta and Pollard, 2018; Xiang et al., 2021).

Here, we review the latest improvements and current understanding of the heterogeneity and function of mammary gland macrophages in tissue homeostasis and tumor progression.

2. Mammary gland macrophages

2.1 Development and structure of the mammary gland

The mammary gland is an exocrine gland present in mammals that can secrete milk for the feeding of young offspring. It is composed of a dynamic tissue that consists of an epithelial structure which is surrounded by a stromal microenvironment containing adipocytes, endothelial cells, fibroblasts and immune cells, including macrophages. This unique tissue undergoes profound morphological changes throughout lifetime. Mammary organogenesis starts during embryonic development, but its development predominantly occurs in the postnatal period (Cardiff and Wellings, 1999; Javed and Lteif, 2013; Macias and Hinck, 2012). During fetal development, a rudimentary ductal system develops, which after birth remains quiescent until puberty, except for an isometric growth and branching. However, while the embryonic development process of the murine mammary gland is highly conserved across mammals (Oftedal, 2002), there are marked structural differences. In the mouse mammary gland, with the onset of puberty, the hormonal changes induce an increase in the fibrous and fatty tissue of the stroma followed by the formation of proliferative terminal end buds (TEBs) (Fig. 1A). This process is accompanied with an extensive ductal branching

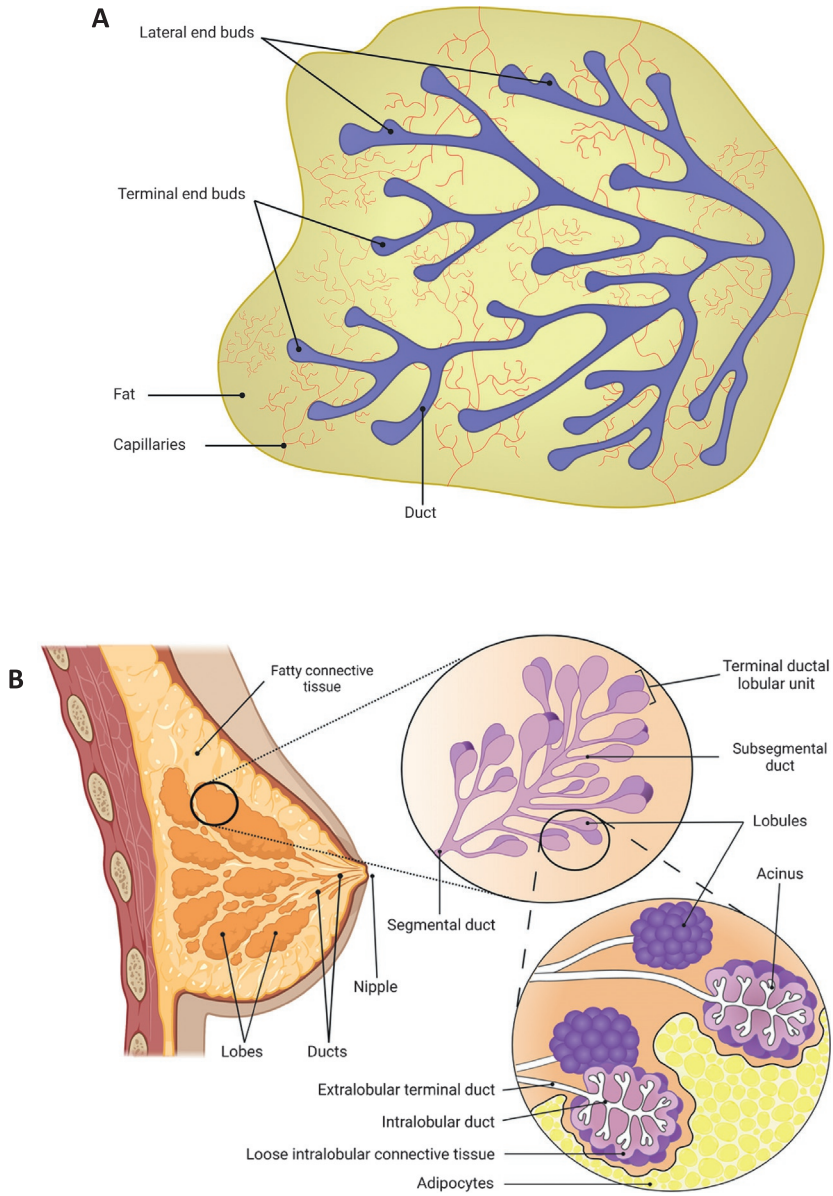

Fig. 1 Organization of the mice (A) and human (B) mammary gland. Diagram of the terminal ductal lobular unit. *This figure was created using BioRender.com.*

and elongation until the developing tree reaches the boundaries of the mammary fat pad and occupies up to 60% of the available stroma. In the human breast, instead of a single epithelial tree, several ductal trees sprout from the mammary bud creating a more complex ductal branching system comparted to the mouse mammary gland (Cardiff and Wellings, 1999; Dontu and Ince, 2015). The primary duct branches into segmental and subsegmental ducts, which further divides into terminal ducts. Each terminal duct ends with numerous blind-ended ductulus, called acini. This group of acini derived from one terminal duct along with the surrounding intralobular stroma is called the terminal duct lobular unit (TDLU), which is the functional unit of the human mammary gland (Fig. 1B). These structural differences might partially be a result of the distinct stromal composition of the human and the mouse mammary tissue, as it has been demonstrated that the epithelial branching patterns are regulated by mesenchymal signals (Kahata et al., 2018; Parmar and Cunha, 2004). In the human mammary gland, the group of acini is embedded in loose intralobular connective tissue surrounded by the interlobular dense, fibrous stroma while the murine ductal tree is situated in an adipose-rich environment. In both species, with every ovarian cycle, the epithelium of the homeostatic female adult mammary gland undergoes additional proliferation, differentiation, and regression leading to the generation of tertiary side branches and alveolar buds (Sternlicht et al., 2006). During pregnancy, an enormous branching and alveolar budding process takes place followed by the differentiation and formation of complex milk-producing alveolar units. Following weaning of the offspring, the mammary gland returns to the homeostatic pre-pregnant resting state through massive cell death and tissue remodeling, called involution (Cardiff and Wellings, 1999; Jindal et al., 2020). The development and the characteristics of mammary gland have been reviewed in more detail elsewhere (Dawson and Visvader, 2021; Fu et al., 2020; Javed and Lteif, 2013; Parmar and Cunha, 2004b; Slepicka et al., 2021; Sternlicht, 2005).

2.2 Macrophage heterogeneity in the mammary gland

The importance of macrophages in mammary gland development and homeostasis has been well recognized for a long time (Gouon-Evans et al., 2000; O'Brien et al., 2012; Pollard and Hennighausen, 1994; Ridker et al., 2017; Van Nguyen and Pollard, 2002). However, the origin and the spatial distribution of distinct macrophage populations during different developmental states were only recently described (Dawson et al., 2020; He et al., 2020; Jäppinen et al., 2019; Stewart et al., 2019; Wang et al., 2020).

A lineage tracing study by Jäppinen and colleagues showed that macrophages colonize the murine mammary gland during embryonic development. Under homeostatic conditions, these fetal-derived tissue-resident macrophages also dominate the mammary tissue throughout adulthood (Jäppinen et al., 2019). Using macrophage depletion methods and transgenic models, this study showed the presence of two distinct tissue resident $CD45^{+}SiglecF^{-}CD11b^{+}$ macrophage populations in the adult mammary tissue being (i) fetal liver derived $CD206^{Hi}$ macrophages and (ii) bone-marrow derived $CD206^{Low/Neg}$ macrophages generated during adulthood. Interestingly, several studies also described a $CD11c^{+}CD11b^{Low}$ macrophage population localized in the mammary epithelium (Dawson et al., 2020; Plaks et al., 2015; Wang et al., 2020). Dawson and colleagues defined the $CD11c^{+}CD11b^{Low}$ macrophage population as ductal macrophages (DM) due to their specific ductal association and intra-epithelial localization in the basal-luminal interface. This DM population, in addition to their CD45, CD64 and F4/80 expression, was further characterized as $Ly6C^{-}Lyve1^{Low/Neg}CD206^{Neg}CX3CR1^{Hi}MHCII^{+}$. In parallel, this study also described $CD11b^{+}CD11c^{Low}CD206^{+}$ stromal macrophages (SM), which could be further subdivided based on their $Lyve1^{Int-Hi}$ and $MHCII^{Hi-Low}$ expression levels into $Lyve1^{Int}$ $MHCII^{Hi}$ SM1 and $Lyve1^{Hi}$ $MHCII^{Low}$ SM2 (Dawson et al., 2020). Despite the different gating strategies and limited number of cell surface markers used for the phenotypic identification in both studies, the DM and SM populations potentially correspond respectively to the $CD206^{Low/Neg}$ adult BM-derived and $CD206^{Hi}$ fetal-derived macrophages described by Jäppinen and colleagues (Table 1). However, the overlap between the DM and $CD206^{Low/Neg}$ populations is only partial since Jäppinen and colleagues pre-gated on the $CD11b^{+}$ cells, suggesting that a proportion of the $CD11b^{low}$ DMs might have been excluded from their analysis. Analysis of the Ms4a3-Cre/Rosa-tdTomato fate-mapping model, in which exclusively the monocyte-derived macrophages, but not the embryonic macrophages, are labeled (Liu et al., 2019), showed that majority of DMs in the pre-pubertal mammary glands were of embryonic origin. However, due to the rapid ductal expansion during puberty, the infiltrating BM-derived monocytes rapidly differentiated into DMs to keep up with the functional requirement of epithelial surveillance resulting in a significant drop of the proportion of DM of embryonic origin (from 80% to 46% by 6 weeks of age) (Dawson et al., 2020). Throughout adulthood, DMs similarly to SMs, showed a slow rate of replenishment by BM-derived cells, with the slowest turnover rate being observed in the $CD206^{+}Lyve1^{Hi}MHCII^{Low}$ SM1 population.

Table 1 Macrophage signatures in healthy mammary tissues and breast cancer in mouse and human.

Species	Type of macropage	Health state	Name used by authors	Platform	Defining markers	Additional highlighted markers	Reference
Mouse	Stromal/stromal-like	Naïve	Fetal-derived	CyTOF/FACS	**F4/80^{Hi} CD64^{Hi}Siglec-1^{Hi} CD206^{Hi}** (gated on CD45$^+$ SiglecF$^-$ CD11b$^+$)	CD274	Jäppinen et al. (2019)
Mouse	Stromal/stromal-like	Naïve	SM1	3D confocal imaging/bulk mRNAseq	CD11c^{Lo}CD11b$^+$CD206$^+$ **MHCII^{Hi} Lyve1^{Int}** (gated on CD45$^+$CD64$^+$F4/80$^+$)		Dawson et al. (2020)
			SM2		CD11c^{Lo}CD11b$^+$CD206$^+$ **MHCII^{Lo}Lyve1^{Hi}** (gated on CD45$^+$CD64$^+$F4/80$^+$)		
		PyMT, Neu, Wnt1 tumor	SM-like	3D confocal imaging/FACS	**Ly6C$^+$ CD11c^{Lo}** (gated on CD45$^+$Ly6G$^-$CD64$^+$CD24^{Lo})	MHCII$^{+/-}$ CX3CR1$^{+/-}$	
Mouse	Stromal/stromal-like	Naïve	F4/80$^+$Lyve-1$^+$	Bulk mRNAseq	F4/80$^+$CD11b$^+$ **Lyve1$^+$** CD206$^+$	*Mrc1, Sparc, Gas6, Igf1, Cd163, Apoe, Cd209g*	Wang et al. (2020)
			F4/80$^+$Lyve-1$^-$		F4/80$^+$CD11b$^+$ **Lyve1$^-$** CD206$^{+/-}$	*Il1b, Cd74, H2Ab1*	
Mouse	Stromal/Stromal-like	Naïve (young/aged)	Ma	sc-RNAseq/ IHC	***Cd163, Mrc1, Cd209f, Adgre1***	*Lyve1,Cd209g*	Li et al. (2020)
Human	Stromal/stromal-like	Luminal breast cancer	FOLR2$^+$	scRNA-seq/bulk mRNAseq/ CyTOF	***FOLR2***, ***SEPP1***, *SLC40A1, MRC1,* ***LYVE1*** (scRNA-seq on CD14$^+$)	*APOE, TIMD4, MAF, CD163, CXCL12*/CyTOF: LYVE1, MRC1	Ramos et al. (2021)
Mouse		PyMT	*Folr2$^+$ Mrc1$^+$*	scRNA-seq	***Folr2***, ***Mrc1***, *Slc40a1, Maf* (scRNA-seq CD45$^+$CD3/19$^-$B220$^-$NKP46$^-$)	Timd4, SpiC	
Mouse	Stromal/stromal-like	p53$^{-/-}$ PN1a lesions	MC0	scRNA-seq/IF	*Cd209g, Lyve1, Tim4d, Gas6, Mrc1*/**CD206$^+$ Lyve-1$^+$** Csfr1$^+$	*Hmox1, Pf4, Fgfr1, Nrp2*	Ibrahim et al. (2020)
			MC6		*Gas6, Axl, Tgfb1, Ccl7, Ccl2, Mrc1*/ **CD206$^+$ Lyve-1$^-$** Gas6$^+$ Csfr1$^+$	*Ccl3, Ccl8, Ptp1b, Igf*	

Continued

Table 1 Macrophage signatures in healthy mammary tissues and breast cancer in mouse and human.—cont'd

Species	Type of macropage	Health state	Name used by authors	Platform	Defining markers	Additional highlighted markers	Reference
Human	Stromal/stromal-like	Breast cancer (luminal and TN)	$CD206^{+}HLA\text{-}DR^{Int}$	CyTOF/IF	**$CD206^{+}$ HLA-DR^{Int}**	$CD36^{Int/Hi}$, $CD86^{Int/Hi}$	Wagner et al. (2019)
			PD-L1 + TAMs		**$CD64^{Hi}$ HLA-DR^{Hi} $CD38^{+}$** $CD169^{+}$ $CD204^{+/-}$	$CD163^{Lo}$	
Mouse	Ductal/ductal-like	Naïve	BM-derived	CyTOF/FACS	**F4/80^{Int} $CD64^{Int}$ Siglec-1^{Int} $CD206^{Lo/-}$ $CX3XR1^{Hi}$** (gated on $CD45^{+}$ $SiglecF^{-}$ $CD11b^{+}$)	$MHCII^{+}$	Jäppinen et al. (2019)
Mouse	Ductal/ductal-like	Naïve	DM	3D confocal imaging/bulk mRNAseq	$CD11c^{+}$ $CD11b^{Lo}$ $Ly6C^{-}$ **CD206-MHCII^{+} Lyve-$1^{Lo/Neg}$**	$CX3CR1^{Hi}$/*Tmem119, Vcam1, Mmp12, Mmp13, Mmp14*	Dawson et al. (2020)
		PyMT, Neu, Wnt1 tumor	DM-like	3D confocal imaging/FACS	**$Ly6C^{-}$ $CD11c^{+}$** (scRNA-seq on $CD45^{+}$ $Ly6G^{-}$ $CD64^{+}$ $CD24^{Lo}$)	$CX3CR1^{Hi}$, $TMEM119^{+}$	
Mouse	Ductal/ductal-like	Naïve (young/aged)	Mb	sc-RNAseq/ IHC	***Mmp12***, ***Mmp13***, *Spic, MHCII genes (H2-Aa, H2-Ab1, H2-Dma, H2-DMb1, H2-Eb1, H2-Oa)*	*Vcam, Cd14, Cd52, Cd63, Cd72, Cd74, Cd207, Il1a, Il1b, Il10, Il12b, Cxcl2, Cxcl16,*	Li et al. (2020)
Human	Ductal/ductal-like	Luminal breast cancer	$TREM2^{+}CADM1^{+}$	scRNA-seq/ bulk mRNAseq/ CyTOF	***TREM2***, ***SPP1***, *CADM1* (scRNA-seq on $CD14^{+}$)	*APOE, C3, ISG15, CD9, ITGAV, FN1, TLR3, MARCKS, STAT2*	Ramos et al. (2021)
Mouse		PyMT	*$Cadm1^{+}$ $Cx3cr1^{+}$*	scRNA-seq	***Cadm1***, ***Cx3cr1***, *Havcr2, Ifi44* (gated on $CD45^{+}$ $CD3/19^{-}$ $B220^{-}$ $NKP46^{-}$)	*Tgfb1, Itgav, Marcks, Tlr3, Stat2*	
Mouse	Ductal/ductal-like	$p53^{-/-}$ PN1a lesions	MC3	scRNA-seq/IF	***Itgax***, ***Cx3cr1***, ***Tmem119***/$CD206^{-}$ $Csfr11^{+}$ *$Ccr2^{+}$*	*Axl, Hexb, Cd86, Tnf, Il1b, Tgfb1, Cxcl16, Mmp12, Mmp13, Itgav, Pdgf, Vcam1*	Ibrahim et al. (2020)
			MC8		***Trem2, Fabp5, Lgals3***/$CD206^{-}$ $Csfr1^{+}$ $Ccr2^{-}$	*Cd9, Gpnmb, Cd36*	

The markers highlighted in bold were used to define or distinguish the key populations in the respective publications.

These observations confirmed that both the DM and SM populations are *bona fide* tissue resident mammary gland macrophages. Similar populations have been described in other studies (Han et al., 2018; Wang et al., 2020): Han and colleagues used unbiased scRNA-seq transcriptome analyses of naïve mammary fat pad where at least two, Lyz1^{Hi} and C1qc^{Hi} macrophage populations were identified (Han et al., 2018). Another scRNA-seq analysis comparing young *versus* aged naïve mammary glands identified two macrophages populations, referred to as Ma (marked by *Mrc1, Cd209f* and *Cd163*) and Mb (marked by *Mmp12, Mmp13* and *Spic*) (Li et al., 2020). Wang and colleagues identified two macrophage populations using scRNA-seq based on Lyve-1 expression within the CD45^{+} compartment. Furthermore, targeted bulk mRNA-seq analyses was performed on F4/80^{+}Lyve-1^{+} and F4/80^{+}Lyve-1^{-} cells (pre-gated on CD45^{+}CD11b^{+}) (Wang et al., 2020). Comparing the gene expression data derived from tissue scRNA-seq or bulk mRNA-seq analyses of sorted macrophage populations in mammary tissue suggest that the C1qc^{Hi} (Han et al., 2018), the SM (Dawson et al., 2020) and F4/80^{+}Lyve1^{+} (Wang et al., 2020) cells are remarkably similar with highly overlapping gene expression profiles (Table 1). These SMs were all enriched for additional markers like *Cd163*, *Folr2*, *Timd4*, *Cd209b*, *Cd209f*, *Cd209g* or *Pf4*, a signature that was previously associated with perivascular macrophages (Chakarov et al., 2019).

Interestingly, a recent study by Ramos and colleagues described a FOLR2^{+} *bona fide* tissue resident (TRM) macrophage population in human breast tissue and identified the CD206^{+}LYVE1^{+} stromal macrophages as their murine counterparts (Ramos et al., 2021). The comparative analysis of murine and human FOLR2^{+} macrophages showed a high alignment in their transcriptome, suggesting evolutionary conserved populations (Table 2). These SMs show periductal and perivascular localizations in both species (Dawson et al., 2020; Jäppinen et al., 2019; Ramos et al., 2021).

The recently characterized CD11c^{+}CD11b^{Low}Cx3cr1^{+}Vcam1^{Hi} DM population (Dawson et al., 2020) does not completely align with all the similar populations identified by unbiased scRNA-seq studies (Han et al., 2018; Li et al., 2020; Wang et al., 2020), however, there are shared key gene signatures (Table 2). Potentially, this inconsistency roots partially in the depth of the analysis and in the scarcity of this population in virgin mammary fat pads compared to the stromal macrophage populations (Dawson et al., 2020; Ramos et al., 2021). In humans, this population might be identified by their CADM1 and TREM2 expression (Ramos et al., 2021), but at present these cells are poorly investigated in healthy human breast tissue. TREM2^{+}

Table 2 Comparative analysis of the list of differentially expressed genes in stromal and ductal macrophages described in the following publications: Cassetta et al. (2019), Dawson et al. (2020), Han et al. (2018), Ibrahim et al. (2020), Li et al. (2020), Mulder et al. (2021), Ramos et al. (2021), and Wang et al. (2020).

Stromal/Stromal-like macrophages	
Gene	#
Folr2	8
Ccl8	7
F13a1	7
Dab2	6
Egr1	6
Fcgrt	6
Igf1	6
Lyve1	6
Maf	6
Pla2g2d	6
Pltp	6
Pmp22	6
C4b	5
Cbr2	5
Cd163	5
Cd209d	5
Cd209f	5
Cd209g	5
Clec10a	5
Ednrb	5
Fcna	5
Fxyd2	5
Grn	5
Hmox1	5
Ier3	5
Igfbp4	5
Jun	5
Ltc4s	5
Ly6e	5
Mrc1	5
Pf4	5
Serpinb6a	5

Stromal/Stromal-like macrophages	
Gene	#
Aldh2	4
Calml4	4
Ccl2	4
Ccl24	4
Ccl6	4
Ccl9	4
Cd209b	4
Cltc	4
Ctsl	4
Gas6	4
Hal	4
Hspa1a	4
Ifi27l2a	4
Ifitm2	4
Ifitm3	4
Klf4	4
Lgals1	4
RCN3	4
Retnla	4
Rnase4	4
Smagp	4
Snx2	4
Timd4	4
Timp2	4
Txnip	4
Wfdc17	4

Ductal/Ductal-like macrophages	
Gene	#
Il1b	6
Ctss	5
Cd74	5
Hexb	5
Acp5	4
Cadm1	4
Ctsz	4
Gm2a	4
Gngt2	4
H2-EB1	4
Mpeg1	4
Tyrobp	4
Aif1	3
Ccrl2	3
Cd14	3
Cd63	3
Csf2ra	3
Cxcl16	3
Cxcl2	3
Cybb	3
H2-Aa	3
H2-Ab1	3
H2-Dma	3
Il1a	3
Itgb5	3
Mmp12	3
Ppfia4	3
Rgs1	3
Sgk1	3
Syngr2	3

Lists of differentially expressed genes were compared to the stromal and ductal gene signatures from Li et al. (2020). "#" stands for the number of studies in which the respective gene was detected as part of the stromal or ductal macrophage gene signature.

macrophages have been described in various tissues at steady state including lung (Leader et al., 2021), skin (Wang et al., 2019) or adipose tissue (Jaitin et al., 2019). However, TREM2$^+$ macrophages are more investigated in the context of cancer where TREM2 is considered as a pro-tumoral tumor-associated macrophage (TAM) marker in a wide range of tumors, including breast cancer (Chiappinelli et al., 2021; Molgora et al., 2020). The CD11b$^+$F4/80$^+$LYVE1$^-$ population, which showed lower CD206 expression compared to the LYVE1$^+$ cell, shares its key gene signature (*Cd74, Il1b, H2-Ab1* and *Cxcl16*) with the CD11c$^+$ DMs (Wang et al., 2020). This population is poorly characterized and a similar population has not yet been described in human breast.

2.3 Localization and role of macrophages in the mammary gland

In earlier studies macrophages have been shown to be localized in proximity to the neck of the TEBs during puberty, to the alveolar buds during pregnancy and they were also found dispersed in the adipose stroma (Gouon-Evans et al., 2000, 2002; Schwertfeger et al., 2006). Recent studies, using 3D imaging of cleared mammary tissue demonstrated frequent direct connections in-between mammary epithelial cells and distinct macrophage populations (Dawson et al., 2020; Jäppinen et al., 2019; Stewart et al., 2019).

With respect to macrophages subpopulations, the characterized Lyve1$^+$ CD206$^+$ macrophage population was localized in hyaluron (HA) enriched stromal regions, including the fibrous areas in the adipose tissue and the fibrous capsule surrounding the mammary fat pad (Wang et al., 2020). Interestingly, the majority of macrophages in the HA-rich region of the stroma surrounding the TEB were Lyve1$^-$, suggesting that additional factors regulate the spatial distribution of distinct macrophages subsets. The Lyve1$^+$CD206$^+$ tissue resident macrophage population has been described to negatively regulate collagen deposition in the lung and in arteries (Chakarov et al., 2019; Lim et al., 2018). As such, the absence of Lyve1$^+$ macrophages resulted in the induction of arterial stiffness (Lim et al., 2018) and exacerbated lung and heart fibrosis through enhanced collagen deposition (Chakarov et al., 2019). Similarly, macrophage depletion resulted in increased HA and collagen deposition in the mammary gland (Wang et al., 2020). In addition, the gene expression profile of the Lyve1$^+$CD206$^+$ stromal macrophages was associated with extracellular matrix (ECM) remodeling and repair functions (Dawson et al., 2020; Wang et al., 2020). Previously, HA was also shown to modulate epithelial branching during mammary development (Tolg et al., 2017). Therefore, it would be interesting to further investigate the role of CD206$^+$Lyve1$^-$ macrophages, predominantly localized in the HA-rich stroma proximity to TEBs (Wang et al., 2020) in this process. Importantly, this negative correlation between collagen deposition and the presence of CD206$^+$ macrophages has also been also described in the human breast (Huo et al., 2015).

The intra-epithelial CD11c$^+$ macrophages have been confirmed by several studies to play a unique role in ductal morphogenesis during development and lactation/involution (Dawson and Visvader, 2021; Plaks et al., 2015; Stewart et al., 2019). These uniquely duct-associated macrophages are

evenly distributed throughout the mammary epithelium with higher densities at the nipple area (Dawson and Visvader, 2021). These DMs are important for the clearance of apoptotic cells upon epithelium damage. Interestingly, intravital imaging showed that the DMs surveille the entire epithelium through dendrite movements rather than via migration (Dawson and Visvader, 2021). The phagocytic activity of DMs is also essential for mammary gland involution and these cells were shown to play a non-redundant role in tissue remodeling during the post-lactation period (Dawson and Visvader, 2021; Stewart et al., 2019). During pregnancy, the hormonal cues induce an exponential proliferation of DMs which then become the predominant macrophage population in lactating breasts. Besides regulating structural changes and monitoring the breast microenvironment, DMs showed an anti-inflammatory profile during lactation, suggesting a role in promoting an immunosuppressive/immunotolerant environment (Dawson and Visvader, 2021). Interestingly, DM-like Mb macrophages described by Li and colleagues showed an increased abundance in aged mammary glands, thereby potentially contributing to the development of a more immunosuppressive microenvironment (Li et al., 2020). Previous studies using a colony-stimulating factor 1 (CSF-1) nullizygous (*Csf1* ^{op}/*Csf1* ^{op}) mouse model have shown that total macrophage depletion resulted in reduced epithelial branching and mammary gland development (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002). However, a more directed approach using the CD11c:DTR transgenic mouse model, which results in the depletion of ductal macrophages, but spares stromal macrophage populations, suggested that these cells indirectly negatively regulate branching morphogenesis and alveolar bud formation. As such, DMs were involved in modulating the presence and activation of $CD4^+$ T helper 1 cells, which were shown to interfere with luminal differentiation via IFNγ signaling (Plaks et al., 2015). These observations suggest that the distinct macrophage populations play opposite roles in orchestrating a balanced normal mammary gland development. Based on the existing knowledge, stromal macrophages appear to be required for epithelial branching by modulating the extracellular matrix composition while the ductal macrophages ensure normal structural development by phagocytic activity. However, additional studies targeting distinct macrophage phenotypes, including spatial transcriptomics, multiplex tissue imaging, depletion studies and functional assays, will be needed to further decipher the heterogeneity and better understand the functional roles of these tissue macrophages in homeostasis and diseases.

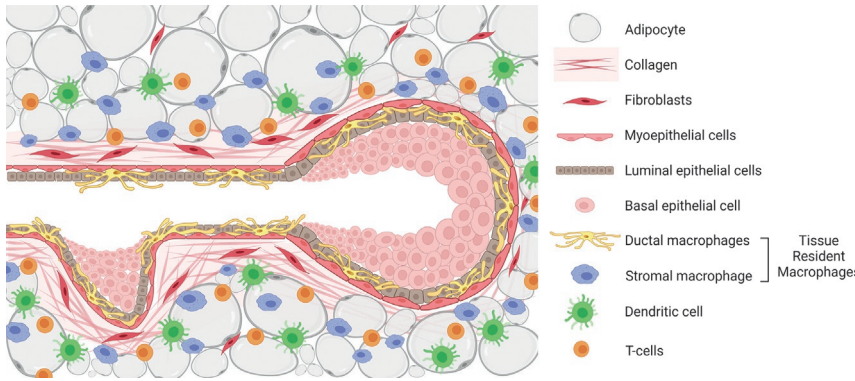

Fig. 2 Macrophages in the mice mammary gland. Representation of a terminal end bud (TEB) and stromal cellular components, surrounded by adipocytes. *This figure was created using BioRender.com.*

Altogether, based on our current knowledge we can clearly identify two spatially, phenotypically and functionally distinct tissue resident mammary gland macrophage populations: $LYVE1^{+}CD206^{+}FOLR2^{+}$ stromal and $CD11c^{+}CX3CR1^{+}VCAM1^{Hi}CADM1^{+}$ ductal $F4/80^{+}$ macrophages in mice (Fig. 2). While the phenotype of the $FOLR2^{+}$ population is highly conserved across mammals, the ductal macrophages are defined as $CADM1^{+}TREM2^{+}$ macrophages in humans. In mouse, a $CD206^{Low/+}$ $LYVE1^{-}$ poorly characterized population has been also described in association with the stroma.

3. Breast cancer

Breast cancer is the most common cancer type worldwide with 2.3 million women receiving a diagnose of breast cancer in 2020 (Sung et al., 2021). Nonetheless, even though these cancers arise in the same organ, breast cancer is a very heterogeneous disease at the molecular level. In the current clinical practice breast cancer is often classified in five molecular subtypes based on the expression of estrogen (ER) and progesterone (PR) hormone receptors, HER2 and the proliferation marker Ki67: (i) Luminal A-like cancers are positive for ER and/or PR, but are HER2 negative and show a low proliferation rate; (ii) Luminal B-like cancers express ER and/or PR, show a high proliferation, but do overexpress HER2; (iii) the HER2 subtype cancers are positive for HER2, but negative for

ER and PR; (iv) luminal HER2 cancers are positive for HER2 and ER and/or PR and (v) basal-like triple negative breast cancers (TNBC) are negative for HER2, ER and PR (Cardoso et al., 2019; Harbeck and Gnant, 2017). Nonetheless, using genomic and transcriptomic analysis, breast cancer can even further be classified in more subtypes, each impacting differently on the survival and treatment responses of patients (Ali et al., 2014; Craven et al., 2021; Curtis et al., 2012). The classification of breast cancer subtypes also defines to some extend the therapies used. Surgery, (neo)adjuvant chemotherapy and radiotherapy alone or in combination are still the mainstay of breast cancer treatment. However, in addition to the classical therapies mentioned, combinations complement approaches, including endocrine therapies, HER2 targeting therapies and immune-checkpoint (PD-L1) inhibitor therapies, are also available respectively for PR^+/ER^+, $HER2^+$ and triple-negative breast cancers. (Cardoso et al., 2019; Harbeck and Gnant, 2017; Mittendorf et al., 2020; Schmid et al., 2020). Irrespective of the type of breast cancer, when patient presents with a germline BRCA1 or BRCA2 mutation, patients can also be treated with PARP inhibitors (Cortesi et al., 2021).

The heterogeneity of breast cancer is suggested to be driven by three main factors: (i) the cell of origin in which the neoplastic mutation occurs, (ii) the multitude of oncogenic driver mutations and (iii) the stromal microenvironment (Ellis and Perou, 2013; Polyak and Kalluri, 2010; Zhang et al., 2017). Each of these factors influences the development, progression and therapy responsiveness of breast cancer and highlights the complexity of cancer treatment. In this section, we more specifically focus on the role macrophages play in these processes.

4. Macrophages in breast cancer

4.1 Macrophage heterogeneity in breast cancer

Besides contributing to the development of mammary glands and their roles in homeostasis, macrophages also play critical roles in breast cancer. Many functions modulated by macrophages, including organogenesis, angiogenesis, immune surveillance or ECM turnover, are altered during mammary tumor progression (Ingman et al., 2006; Lahmar et al., 2016; Leek et al., 1996; Lin et al., 2006; Ruffell and Coussens, 2015). Macrophages are an abundant cell population in breast cancer and their presence in general associated with a poor prognosis and more aggressive tumors (Bense et al., 2017; DeNardo et al., 2011; Mahmoud et al., 2012; Medrek et al., 2012; Yuan

et al., 2014; Zhang et al., 2012; Zhao et al., 2017). TAMs are heterogeneous showing relative variability in origin, phenotype and function based on tumor and tissue type. It is widely accepted that in breast cancer the majority of TAMs are *de novo* differentiated monocyte-derived macrophages (Bonapace et al., 2014; Franklin et al., 2014; Movahedi et al., 2010; Qian et al., 2011), however local proliferation has also been shown to contribute to the TAM pool in mouse models (Campbell et al., 2011; Tymoszuk et al., 2014).

TAM heterogeneity has been long time described (Clappaert et al., 2018; Laoui et al., 2011; Movahedi et al., 2010; Pucci et al., 2009), however, recent scRNA-seq studies revealed the existence of novel TAM subpopulations providing insight into their functional role and relation to mammary tissue-resident macrophages across species (Cheng et al., 2021; Dawson et al., 2020; Ibrahim et al., 2020; Kiss et al., 2018; Ramos et al., 2021; Wang et al., 2020; Azizi et al., 2018; Bassez et al., 2021).

Using the PN1a outgrowth line (generated from p53-null transgenic mice; Medina et al., 2002) transplantation model, Ibrahim and colleagues identified six distinct macrophage populations of which four were mature *Adgre1*$^{+}$ (F4/80^{+}) TAM populations both in pre-malignant and well established invasive lesions (Ibrahim et al., 2020). Interestingly, with tumor progression the proportional distribution of these populations evolved, but not the population diversity. The identified populations contained tissue resident macrophage and TAM populations previously described in normal mammary gland and tumor models, respectively. Both *bona fide* TRM, being the SM and DM populations have been identified showing well-defined gene signatures: the SM-like macrophage cluster characterized by *Cd209g, Lyve1, Tim4d, Gas6, Mrc1/Cd206, Folr2* (Ramos et al., 2021; Wang et al., 2020) while the DM-like cluster were described by *Itgax, Cx3cr1, Tmem119, Cxcl16* and *Cadm1* (Dawson et al., 2020; Ramos et al., 2021) expression. Immunostaining analysis using CD206, Lyve1 and CSFR1 marker combinations confirmed that SMs were predominantly peritumoral, while DMs showed intra-tumoral dispersion (Dawson et al., 2020; Ibrahim et al., 2020; Wang et al., 2020). It has been reported that DM-like macrophages dominate tumors (Dawson et al., 2020; Linde et al., 2018; Ramos et al., 2021), however, on the contrary Ibrahim and colleagues showed that their abundance was lower in established tumors. This discrepancy might simply derive from the difference in the experimental models (spontaneous *versus* transplantation) used, either due to the procedure or the removal of the normal residential niche (the epithelial tree

and the surrounding stroma/fat) upon mammary fat pad clearance prior to tumor transplantation. It is possible that the lack of the normal epithelial structure or the microenvironment created by the transplanted hyperplastic PN1a impairs the capacity of the newly recruited monocytes to differentiate into DMs. On the other hand, it would be also intriguing to investigate whether the proliferation and migration of DMs from the adjacent tissue into the tumor contribute to the expansion of this subset. In addition, a monocyte-derived $CD206^{+}Lyve1^{-}$ TAM population was identified with an intermediate gene expression profile sharing both SM (*Cd206, Gas6, Folr2, Ccl7, Ccl8, Pf4*) and DM (*Axl, Il1b, Cxcl2, Ccr2, Cxcr1,Cd74, Vcam1, Cxcl16*) signatures (Ibrahim et al., 2020). Immunostaining of tissue sections revealed that $CD206^{+}Lyve1^{-}$ cells are primarily localized in the invasive tumor regions which together with their inflammatory gene expression profile suggests a role in promoting tumor cell invasion (Gomes et al., 2018; Ibrahim et al., 2020). Besides the pro-tumor properties, gene ontology analyses suggest that this population also has anti-tumor characteristics by promoting T cell proliferation and activation (Ibrahim et al., 2020). At last, the previously described $Trem2^{+}$ lipid-associated TAM population (Jaitin et al., 2019) was also identified. This population, which is characterized with the signature of *Trem2, Fabp5, Lgals3, Cd36, Cd9* and *Gpnmb* expression, showed a high abundance in pre-malignant lesions but decreased in abundance in established tumors (Ibrahim et al., 2020). Previously, $CX3CR1^{+}$ and $TREM2^{+}$ tumor-associated myeloid cells were showed to reduce activated $CD8^{+}$ T cell proliferation, suggesting an immunosuppressive role for these subsets (Katzenelenbogen et al., 2020) (Fig. 3).

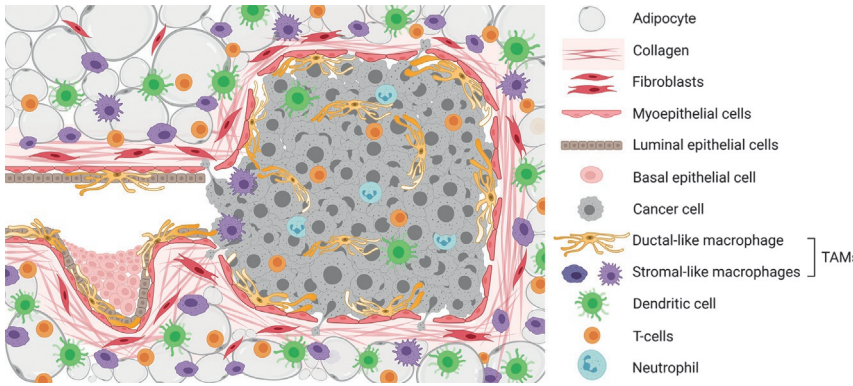

Fig. 3 Macrophages in the mice mammary tumor tissue. Representation of a terminal end bud tumor cellular microenvironment and adipocytes. *This figure was created using BioRender.com.*

TAM heterogeneity is also well documented in human breast cancer. In line with mouse studies, analysis of human breast tumors showed that TAMs are expressing gene signatures associated with both immunostimulatory ("M1-like") and immunosuppressive ("M2-like") activation states, suggesting that different phenotypes might represent distinct states on the activation spectrum (Azizi et al., 2018; Cheng et al., 2021; Wagner et al., 2019). Phenotypical characterization suggested that tissue-resident macrophages are important components of breast tumors and, so far, the described TRM subsets are not restricted to healthy tissues (Azizi et al., 2018; Ramos et al., 2021; Wagner et al., 2019). In line with this, tissue-specific signaling (Cassetta et al., 2019) or tumor cell phenotype (Wagner et al., 2019) were shown to significantly contribute to the diversity of the TAMs landscape. Comparing $CD163^{+}$ macrophages derived from healthy and tumorous breast by bulk mRNA-seq revealed a TAM-specific signature (including the expression of *FOLR2*), suggesting that TRMs and TRM-like TAMs are transcriptionally distinct, and specifically identified CD169 (Siglec1) and CCL8 as novel marker combination to discriminate TAMs associated with high tumor grade and poor clinical outcomes (Cassetta et al., 2019). Accordingly, $CCL8^{Hi}$ TAM populations have been also described in murine mammary tumors with higher frequencies in invasive tumor regions (Ibrahim et al., 2020). Several "classical" markers (including CD163, CD206, CD204, CD169, CD68, CD36, PD-L1) have been associated with TAMs, however, none of them can label a specific TAM population and even the combination of several of these markers cannot exclusively define distinct TAM subsets (Cassetta et al., 2019; Ramos et al., 2021; Wagner et al., 2019). Recent scRNA-seq analysis by Ramos and colleagues identified APOE as a TAM marker in human breast cancer and described two evolutionary conserved tissue-resident TAM populations, namely $FOLR2^{+}LYVE1^{+}MRC1\ (CD206)^{+}$ and $TREM2^{+}SPP1^{+}CADM1^{+}$ (Ramos et al., 2021) macrophages. The gene expression profile and spatial distribution of both populations showed high a correlation with their murine counterparts (Dawson et al., 2020; Ibrahim et al., 2020; Wang et al., 2020). The intra-epithelial $TREM2^{+}CADM1^{+}$ macrophages are rare in healthy tissue, but they become the prominent subset with tumor progression. $TREM2^{+}$ macrophages have been described in several cancer types in association with immunosuppression and poor prognosis (Katzenelenbogen et al., 2020; Molgora et al., 2020). On the other hand, the abundance of stromal $FOLR2^{+}LYVE1^{+}$ macrophages gradually decreases, with a low abundance in the tumor core and with the highest frequency at the peritumoral regions. The co-localization of $FOLR2^{+}$ TAMs with lymphoid

structures, their positive correlation with $CD8^+$ T cell infiltration and better survival rates suggest an active role in anti-tumor immunity (Ramos et al., 2021). Complementary, a recent cross-tissue scRNA-seq analysis identified three main macrophage populations, including $FOLR2^+$, $TREM2^+$ and $IL4I1^+$ macrophages both in healthy and diseased states, including breast cancer (Mulder et al., 2021). $FOLR2^+$ macrophages appeared to be long term resident macrophages while the $TREM2^+$ and $IL4I1^+$ macrophages were more recently differentiated monocyte-derived cells. This ontological difference was also confirmed in the murine counterparts (Sharma et al., 2020). $IL4I1^+$ macrophages are characterized by $CD38^{Hi}$ and $PD\text{-}L1^{Hi}$ expression showing a similar profile as the $PD\text{-}L1^+$ TAMs described with higher frequencies in high grade ER^+ and ER^- breast tumors (Wagner et al., 2019). Altogether, these three populations represent macrophage subsets that are conserved across tissues and species which could provide the basis for a universal annotation for future studies (Fig. 4). Interestingly, at first glance, the findings by Ramos and colleagues are in contradiction with the observations of Cassetta and

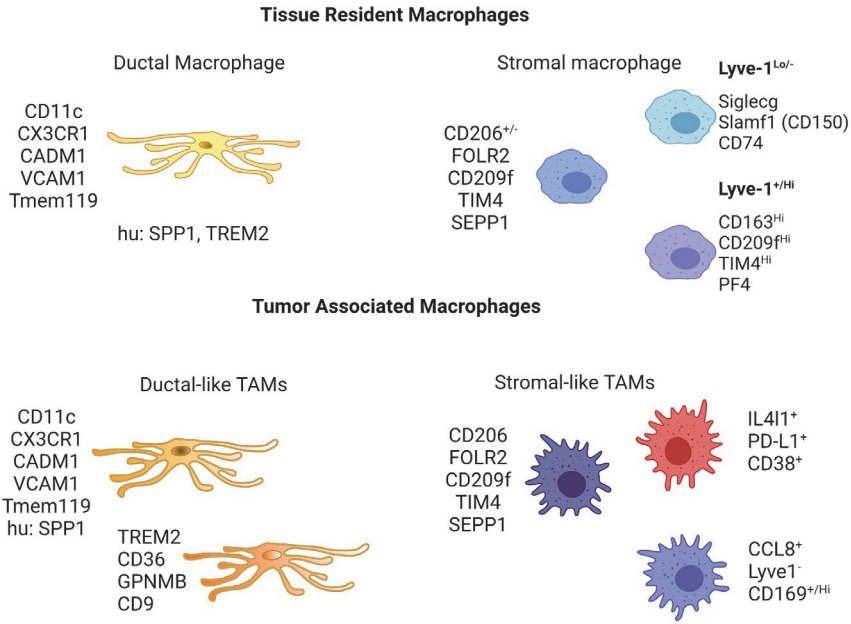

Fig. 4 Ductal and stromal tissue resident macrophages and tumor associated ductal- and stromal-like macrophages in mouse and human breast cancer. *This figure was created using BioRender.com.*

colleagues, the latter showing that the $CD163^+$ TAMs expressing high levels of *CD169, CCL8* and *FOLR2* are associated with poor survival. However, the $CD163^+$ TAMs were also enriched for markers described in distinct TRM and TAM populations including TREM2, CADM1 or CD38 (Cassetta et al., 2019; Mulder et al., 2021; Ramos et al., 2021). Altogether, this suggests that $CD163^+$ TAMs might arise from several distinct TRM or TAM subsets potentially creating a phenotypically heterogenous cluster of TAMs that might exert similar functions.

Clearly, additional studies are required to further dissect the heterogeneity, to validate the spatial distribution of TAM subsets and to clarify their exact origin and functions. An intriguing question that remains to be answered is what are the phenotypic or functional differences between tissue resident macrophage populations derived from healthy or cancerous breast tissues. Spatial transcriptomic and multiplex tissue imaging could be excellent approaches to determine subset specific unique marker combinations and to correlate existing human and murine datasets. Furthermore, systematic functional characterization will be needed to understand how much the phenotypic heterogeneity correlates with functional differences.

4.2 Impact of hypoxia on the heterogeneity and functions of TAMs in breast cancer

A tumor is not one homogenous structure, but rather presents with a high level of intratumor heterogeneity. One of the parameters causing this phenomenon is tumor hypoxia (Ramón y Cajal et al., 2020). When a tumor develops, it soon outgrows its supply of oxygen. As a consequence, a signaling cascade is activated leading to the stabilization of hypoxia-inducible factor (HIF) 1α and HIF2α transcription factors and transcription of angiogenic factors such as VEGF (Harris, 2002). Nonetheless, due to excessive production of pro-angiogenic factors, the vasculature remains immature, resulting in an abnormal and inadequate vascular network that cannot resolve the lack of oxygen (Jain, 2005). The resulting presence of hypoxia is in many cancers negatively associated with patient survival and results in therapy resistance (Walsh et al., 2014). In breast cancer specifically, a median pO_2 of 30 mmHg was observed, while normal breast tissues had a median pO_2 of 65 mmHg (Vaupel et al., 1991). In addition, the pO_2 values and anoxic fraction correlate with the degree of differentiation of breast cancer tumors with grade 1 tumors having a mean pO_2 of 59 mmHg and less than 1% anoxic fraction, while grade 4 tumors presented with a mean pO_2 of 10 mmHg and a 21.5% anoxic fraction (Hohenberger et al., 1998). Furthermore, the

surrogate hypoxia markers HIF1α, HIF2α and their target gene CAIX, have been correlated with therapy resistance, worse relapse free survival and worse overall survival (Betof et al., 2012; Bos et al., 2003; Brennan et al., 2006; Chia et al., 2001; Dales et al., 2005; Goonewardene et al., 2002; Helczynska et al., 2008; Hussain et al., 2007). TAMs play a role in mediating the detrimental effects hypoxia has on the survival of cancer patients. They can reside in both normoxic and hypoxic regions of both human and mouse breast tumors. In murine tumor models, this has been shown using immunohistochemistry and flow cytometry in spontaneous and syngeneic orthotopic breast cancer models where hypoxic TAMs are often identified as $MHCII^{Low}$ or $GLUT1^{Hi}$ TAMs (Casazza et al., 2013; Fang et al., 2009; Movahedi et al., 2010; Wenes et al., 2016). In human breast cancer, the first evidence of the presence of TAMs in hypoxic areas of breast cancer tumors was provided using immunohistochemical analysis (Murdoch and Lewis, 2005). In addition, a TAM population expressing a hypoxic gene signature has recently been identified in distinct scRNA-seq datasets of human breast cancer, suggesting their presence in hypoxic regions of the tumor (Azizi et al., 2018; Bassez et al., 2021).

TAMs are recruited into hypoxic regions of the tumor by factors such as Sema3A, Oncostatin M and Eotaxin expressed by cancer cells exposed to the hypoxic microenvironment (Casazza et al., 2013; Tripathi et al., 2014). Sema3A signaling via Nrp1-VEGFR1 complex has been shown to be responsible for recruitment of TAMs in hypoxic regions of the tumor and silencing of Sema3A, similarly to genetic deletion of its receptor Nrp1 in macrophages, resulted in an increased overall presence of TAMs, but reduced presence of pro-tumoral TAMs (Casazza et al., 2013). These results are difficult to reconcile with the recent findings that Sema3A has also been shown—in contrast to hypoxia—to be inversely correlated with the breast cancer grade (Wallerius et al., 2016). In addition, Sema3A overexpression in the cancer cells has been shown to reduce tumor growth in the 4T1 syngeneic breast cancer model by shifting the balance from the more pro-tumoral $MHCII^{Hi}$ TAMs to the anti-tumoral $MHCII^{Low}$ TAMs (Wallerius et al., 2016). The discrepancy between these results requires further investigation, but could potentially be explained by the dual role Sema3A signaling can play as in the absence of Nrp1, Sema3A via its receptors PlexinA1 and PlexinA4 has an opposite function to trap TAMs in hypoxia (Casazza et al., 2013). Therefore, experiments with overexpression of Sema3A should be taken with caution as these could disrupt the balance between ligand and receptors, though further studies are required to

investigate these results in depth and understand the role of Sema3A in TAMs in hypoxic regions of breast cancer tumors. The other two factors described to attract macrophages to hypoxic breast cancer cells are Oncostatin M and Eotaxin. Indeed, also in breast cancer patient samples, these two molecules co-localize with HIF1α expression. Blocking of these factors in the syngeneic 4T1 breast cancer model consequently reduces tumor growth, tumor vasculature and CD206 expression, though more extensive analysis on the TAM compartment in hypoxia would be required to confirm *in vitro* experiments that Oncostatin M and Eotaxin are required for *in vivo* recruitment to and polarization of TAMs in hypoxic regions of the tumor (Shrivastava et al., 2018; Tripathi et al., 2014). Other factors suggested to play a role in the attraction of TAMs to hypoxic regions of the tumor are for example VEGF, CCL2, CCL5, EMAP-II, endothelin and SDF1α, though their role in TAM recruitment to hypoxic regions in breast cancer specifically has not been addressed yet (Henze and Mazzone, 2016).

Once TAMs arrive in hypoxic regions, they are trapped there due to the downregulation of several receptors such as Nrp1, CCR1, CCR2 and CCR5. Indeed, Nrp1 is significantly downregulated in hypoxic TAMs in a HIF2a-mediated activation of the Nf-κB pathway, thereby specifically abrogating chemoattraction to SEMA3A (Casazza et al., 2013). In addition to Nrp1, *in vitro* experiments would also suggest a role for CCR1, CCR2 and CCR5 in entrapping TAMs in hypoxic regions of the tumor as the expression of these proteins is downregulated in macrophages in hypoxia (Bosco et al., 2006; Tausendschön et al., 2011). Nonetheless, more *in vivo* studies would be required to understand the role these proteins play in entrapping TAMs in hypoxic regions of breast cancer tumors. Indeed, in contrast to the *in vitro* data, CCR1 has also been shown to be increased upon hypoxia in the monocytic THP-1 cell line suggesting a cell type/context dependent regulation of CCR1 (Scotton et al., 2020). In addition, while in the TS/A breast cancer model CCR2 levels were higher in $MHCII^{low}$ "hypoxic" TAMs compared to $MHCII^{Hi}$ "normoxic" TAMs in line with the suggested role of CCR2 in trapping TAMs in hypoxia, this was the opposite in the 3LL-R lung cancer model, again suggesting a potential context dependent regulation (Laoui et al., 2014; Movahedi et al., 2010).

Once trapped in hypoxic regions of the tumor, the phenotype of TAMs is altered significantly. More specifically, hypoxic TAMs present with a higher angiogenic potential and an increased anti-inflammatory capacity (Casazza et al., 2013; Movahedi et al., 2010). Indeed, exclusion of

macrophages from hypoxic regions of the tumor leads to a reduction in vasculature and increased anti-tumor immune response and as a consequence decreased tumor growth (Casazza et al., 2013). The factors mediating this response are plentiful and are likely the result of a combination of factors. Gene expression analysis in MHCII^{Low} and MHCII^{Hi} TAMs derived from the TS/A breast cancer model indicated differential regulation of a multitude of genes with MHCII^{Hi} TAMs expressing more angiostatic factors and lymphocyte attracting chemokines, while MHCII^{Lo} TAMs rather presented with increased expression of monocyte/macrophage chemoattractants (Movahedi et al., 2010). The pathways regulating these changes are also diverse. Deletion of HIF1α, one of the most obvious candidates to regulate TAM changes in hypoxia has been shown to delay progression of the spontaneous MMTV-PyMT breast cancer model and macrophages deficient for HIF1α caused less T-cell suppression under hypoxic conditions, potentially explaining the delayed tumor progression (Doedens et al., 2010). Nonetheless, HIF1α deletion in the myeloid cell compartment does not affect VEGF or vascularization in the MMTV-PyMT breast cancer model, confirming that multiple pathways are at play (Doedens et al., 2010). As such, hypoxia not only leads to HIF1α and HIF2α stabilization, but can also act in a HIF-independent manner on for example the Nf-κB pathway (D'Ignazio et al., 2017). In addition, hypoxia also leads to a shift in metabolism in cancer cells towards a more glycolytic metabolism, resulting in an increased lactate production. Exposure of TAMs to lactate has been shown to enhance their pro-tumoral function to increase breast cancer metastasis (Chen et al., 2017). In addition, metabolic stress caused by hypoxia can also activate the unfolded protein response, leading to mTOR inhibition, activation of ATF6, eIF2a, XBP1 and ATF4 transcription factors thereby influencing gene transcription in TAMs extensively (Díaz-Bulnes et al., 2019). In conclusion, hypoxia has a major impact on TAM activity and function, though the regulation of this phenomenon is a highly complex interplay between factors/pathways influencing each other that has not been completely unraveled yet. In addition, as likely tissue specific phenomena are taking place as well, extrapolation of data from one cancer type to another always has to be taken with caution.

5. Concluding remarks and future perspectives

Macrophages in breast and breast cancer have already been studied relatively intensively and novel technologies are further increasing our understanding about the macrophage populations present in breast and breast

cancer. Nonetheless, there are still many shortcomings to the current data that require further investigation.

Firstly, scRNA-seq has provided us with novel data on the macrophage populations present in breast and breast cancer tissues (Azizi et al., 2018; Bassez et al., 2021). Nonetheless, as the heterogeneity within breast cancer is high, it would be anticipated that also macrophage populations within these cancer types differ fundamentally. Currently however, partly due to the cost and large amount of data processing required, there has not yet been any analysis focusing on the differences in TAM populations in different breast cancer subtypes. Nevertheless, major efforts are being made to make the technology more accessible (De Rop et al., 2021) and to improve data analysis (Luecken and Theis, 2019; Saelens et al., 2019), increasing the feasibility to perform larger studies. In addition, complementing scRNA-seq with spatial transcriptomic analysis (Bassiouni et al., 2021; Chen et al., 2015; Nagasawa et al., 2021; Park et al., 2021), will allow to determine the localization of the identified TAM populations within the normal or breast cancer tissue.

This will, however, only be a starting point due to the descriptive nature of these studies without addressing the biological function of differentially expressed genes in different TAM populations. Research identifying and studying the role of novel factors determining TAM function will be required. Unfortunately, however, current studies investigating TAM ontogeny or function have been performed in a limited number of breast cancer models and therefore do not represent the diversity of breast cancer in patients. Recently however, it was shown that upon p53 deletion in breast cancer, Wnt ligand secretion was increased and induced the production of IL-1b in TAMs, thereby driving systemic inflammation and breast cancer metastasis (Wellenstein et al., 2019). This research was possible by using a panel of 16 genetically engineered breast cancer models each having their own characteristics (Wellenstein et al., 2019). Indeed, by making use of these models, it has already become clear that the origin of basal breast cancers could also be luminal (Skibinski and Kuperwasser, 2015). A further characterization of TAM subsets in these models using scRNA-seq and spatial transcriptomics could then further allow comparison to the human TAM subsets. Subsequently allowing also the functional study of TAM subsets in more clinically relevant breast cancer models.

Overall, although we are only at the start of unravelling the complexity of TAM regulation in breast and breast cancer, the recent advances in technologies will allow us to study TAMs more in depth than ever before. This will open a new era of research to discover novel potential targets to tackle

immunosuppressive TAMs in breast cancer and to develop novel therapies that can increase breast cancer survival.

Competing interests

The authors declare that they have no conflict of interest.

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