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Abstract

Tissue-resident macrophages adapt to local signals within tissues to acquire specific functions. Neoplasia transforms the tissue, raising the question as to how the environmental perturbations contribute to tumor-associated macrophages (TAMs) identity and functions. Combining scRNAseq to spatial localization of distinct TAM subsets by imaging, we discover that TAM transcriptomic programs follow two main differentiation paths according to their localization in the stroma or in the neoplastic epithelium of the mammary duct. Furthermore, this diversity is exclusively detected in spontaneous tumor model and track the different stroma territories as well as the type of tumor lesion. These TAM subsets harbor distinct capacity to activate CD8+ T cells and to phagocyte tumor cells supporting that specific tumor regions rather than defined activation states are the major drivers of TAM plasticity and heterogeneity. The distinctions created here provide a framework to design new cancer treatment targeting specific TAM niches.

Introduction

Every adult tissue contains an abundant population of resident tissue macrophages distributed in a regular pattern with intimate interactions with other cells and extracellular matrix (Guilliams et al., 2020; Hume, 2006; Hume et al., 2019). Macrophages are also the most abundant immune cells of the tumor microenvironment (TME), composed of the stroma and the tumor parenchyma, and have been associated with bad prognosis in most cancers, including breast cancer (Condeelis and Pollard, 2006; Qian and Pollard, 2010). As a consequence, many proposed treatment modalities are directed towards suppression of tumor-associated macrophage (TAM) numbers or altering their function (Reviewed in (Cassetta and Pollard, 2018)). Macrophage adaptation to the tumor environment has commonly been discussed in terms of a spectrum of polarization states from anti-tumor M1 to pro-tumor M2 (reviewed in (Locati et al., 2020)). However, the validity of the polarization model in cancer has been widely questioned (Hume and Freeman, 2014; Mujal et al., 2022; Xue et al., 2014). Therefore, a proper understanding of TAM intrinsic diversity related to their spatial localization over time is still warranted. During tumor growth, both tissue-resident macrophages and newly recruited monocyte-derived macrophages can give rise to TAMs which instigated interrogations of their respective role in tumor progression (Franklin et al., 2014; Laviron and Boissonnas, 2019; Loyher et al., 2018; Müller et al., 2015; Zhu et al., 2017).

Within specific tissue territories, defined as the macrophage niche (Guilliams and Scott, 2017), mutual repulsion and competition for available growth factors may account for the distribution of macrophages (Guilliams et al., 2020; Hume et al., 2019). The link between tissue niche, ontogeny and macrophage polarization has been well described for different organs at steady state (Bonnardel et al., 2019; Calderon et al., 2015). In the mammary gland, at least two populations of resident macrophages have been described : stromal and ductal macrophages (SM and DM respectively), which are essential for the functions of the tissue (Dawson et al., 2020; Gouon-Evans et al., 2002; Jäppinen et al., 2019; Wang et al., 2020). Both were shown to derive from embryonic precursors but SM were slowly replaced by monocytes over life while the majority of DM were replaced in a spike at puberty and then established as long-lived cells (Dawson et al., 2020; Jäppinen et al., 2019).

Herein, we investigated the link between spatial distribution in different territories of breast cancer and the heterogeneity of TAMs. We associated single cell RNA sequencing (scRNAseq) to

spatial mapping by imaging using a multiple transgenic fluorescent reporter mouse to discriminate mammary macrophage subsets in a spontaneous and a transplanted breast tumor model (Engelhardt et al., 2012; Guy et al., 1992). Our analysis reveals that ductal and stromal TAMs follow distinct differentiation pathways. Further functional diversity of these TAM subsets was detected according to different anatomical structures of the stroma and tumor malignancy. DM associated with advanced tumor stage were potent phagocytes but did not show efficient capacity to activate CD8 T cells compared to SM. This spatial heterogeneity was lost in an orthotopic tumor model. Finally, similar subsets were identified in human breast tumor using a published scRNAseq dataset (Pal et al., 2021). Altogether, those results provide insight into the spatial heterogeneity of TAMs and the role of the local microenvironment to define their nature, which will help refining therapeutic strategies of selective TAM targeting.

Results

Breast macrophage niches evolve during tumor development

In mammary tissue, two different resident macrophage subsets were described in the literature associated with distinct locations: CD11b^{-} ductal macrophages (DM) directly in contact with the epithelium of the mammary ducts and CD11b^{+} stromal macrophages (SM) in the conjunctive and adipose mammary tissues (Bijnen and Bajénoff, 2021; Dawson et al., 2020; Jäppinen et al., 2019). We sought to determine the evolution of those niches in the MMTV-PyMT-mCherry model (named herein PyMT) which develop spontaneous multi-focal tumors from the mammary ductal epithelium at different stages recapitulating human breast tumor development (Attalla et al., 2021; Engelhardt et al., 2012; Lin et al., 2003). Up to 3 different fluorescent reporters dedicated to macrophage identification were added to the PyMT background: the MacApple transgene (Csf1r^{mApple},(Hawley et al., 2018)), the MacBlue binary transgene (ΔCsf1r-gal4/vp16/UAS-ECFP, referred to as ΔCsf1r^{ECFP}, (Ovchinnikov et al., 2008)), and the knock-in Cx3cr1$^{EGFP/+}$ (Jung et al., 2000) (**Fig. 1A**). These three different reporters discriminate distinct macrophage subsets according to the relative expression of Apple, ECFP and EGFP (Laviron et al., 2019). We used this fluorescent signature along with a panel of antibodies and performed t-distributed stochastic neighbor embedding (tSNE) reduction to monitor monocyte and macrophage accumulation in the mammary tissue from non-PyMT mice, 3-month-old PyMT with no detectable tumor at palpation, considered as pre-tumoral tissue and 6 month-old PyMT with palpable tumor nodules **(Fig. 1B)**. Healthy mammary tissue (non-PyMT and pre-tumoral) contained mainly CD11b^{+}Apple^{+} macrophages and a small proportion of Ly6C^{hi} ECFP^{+} EGFP^{low} monocytes and CD11b^{-} EGFP^{+} macrophages. The majority of CD11b^{+} Apple^{+} macrophages were also MHC-II^{+} (IA/IE) CD206^{+} and CCR2^{+} **(FigS. 1A)**. Two-photon imaging of fresh pre-tumoral mammary tissue confirmed that Apple^{+} cells with typical macrophage morphology were located in the adipose and surrounding connective tissue defining a stromal population of macrophages associated to the mammary fat (SM type I) **(Fig. 1C)**. Few round shaped ECFP^{+} cells were detected in the connective tissue, defining a second stromal population (SM type II). CD11b^{-} MHC-II^{+} EGFP^{+} macrophages corresponded to DM (Bijnen and Bajénoff, 2021; Dawson et al., 2020; Wang et al., 2020). Accordingly, these EGFP^{+} cells were intra-epithelial **(Fig. 1C, Movie 1)**. In tumoral tissue, CD11b^{-} EGFP^{+} macrophages massively accumulated along with the apparition of CD11b^{+} EGFP^{+} with lower expression of MHC-II **(Fig. 1B, FigS. 1A).** These cells did not remain only intra-epithelial but were mostly in close contact with tumor cells at all stages of tumor

progression hence we still considered them as ductal TAMs **(Fig. 1C, D, FigS. 1B)**. Based on the description of the tumor stages by Lin and colleagues, hyperplastic lesions were characterized by increased mammary epithelium density with a detectable ductal lumen (Lin et al., 2003). The EGFP$^+$ ductal TAMs were elongated mostly intra-epithelial, parallel to the basement membrane of the mammary duct **(Fig. 1E, FigS. 1C)**. Adenoma lesions were characterized by tumor nodules with no more ductal lumen but a clear distinction of epithelial basement in which EGFP$^+$ ductal TAMs displayed stellate morphology at the periphery. Malignant lesions were characterized by a disrupted epithelial basement with invasive tumor organization where EGFP$^+$ ductal TAMs surrounded the tumor or were completely trapped within the tumor parenchyma **(Fig. 1E, FigS. 1C).** Highly phagocytic EGFP$^+$ ductal TAMs were observed located in the lumen of the mammary duct similar to the ones observed during mammary epithelium involution post weaning **(FigS. 1C)**(Dawson et al., 2020). Upon tumor development another macrophage subset defined as CD11b$^+$ ECFP$^+$ EGFP^{low} (for simplicity called ECFP$^+$) **(Fig. 1B),** appeared and was mostly detected in stromal regions and less in close contact with tumor cells **(Fig. 1C, E, FigS. 1B, D)**. These ECFP$^+$ cells were composed of both monocytes and macrophages **(Fig. 1B, C).** Numerous ECFP$^+$ cells were detected rolling and patrolling in the vasculature as expected for monocytes **(Movie 2).** They also accumulated nearby vascular regions within the tumor parenchyma and displayed a higher motility pattern compared to EGFP$^+$ cells **(Movie 3)** but exhibited decreased velocity as they were moving closer to tumor cells **(Movie 4, FigS. 1E).** We assumed that ECFP$^+$ cells represent the most recently infiltrating monocytes and macrophages that progressively migrate and differentiate in the different regions of the tumor. Finally, upon tumor expansion, the Apple$^+$ SM type I were still mostly located to the adipose tissue at the periphery of the tumor or in smaller adipose islets **(Fig. 1C, FigS. 1B)**. To summarize, EGFP$^+$ cells were considered as ductal TAMs further distinguished by the emergence of CD11b$^+$ subset. Apple$^+$ cells represented a SM type I population associated to the mammary fat. ECFP$^+$ cells represented both monocytes and a distinct tumor-induced SM type II population. In conclusion, we were able to identify in mammary tumors several subsets of TAMs exhibiting distinct localization related to the different macrophage niches existing prior to tumor development.

TAM composition shifts with tumor malignancy

EGFP$^+$ ductal TAMs comprised separable CD11b$^-$ and CD11b$^+$ subsets. To determine whether CD11b$^+$ are associated with specific location or tumor progression, we quantified by fluorescent imaging CD11b expression specifically on EGFP$^+$ cells associated to the three different stages of tumor evolution **(Fig. 2A, B)**. CD11b expression was significantly higher in EGFP$^+$ cells associated with malignant lesions as compared to hyperplastic, consistent with the CD11b$^-$ phenotype of intra-epithelial macrophages in pre-tumoral tissue **(Fig. 2B)**. Moreover, this increase was confirmed using flow cytometry **(Fig. 2C)**. The relative proportion of CD11b$^+$ and CD11b$^-$ among the whole TAM compartment strikingly correlated with tumor weight but not with the age of the mouse **(Fig. 2D)**. These results show that TAM composition shifts between hyperplastic and malignant tumor lesions.

Stromal and ductal TAMs follow distinct differentiation paths

To further characterize these different TAM subsets, three fractions of myeloid cells (total, CD11b$^+$ or CD11b$^-$ enriched) were sorted and subjected to scRNAseq. We sorted the different subsets from a pool of tumors harvested from different PyMT mice expecting to cover the different stages of tumor development **(Fig. 3A)**. The three fractions were normalized, combined and a Louvain graph-based clustering was performed which identified 14 clusters, represented using UMAP dimensional reduction method **(Fig. 3A**). Differentially expressed genes were used to annotate each cluster **(FigS. 2A)**. First, consistent with protein expression, CD11b transcript (*Itgam)* was only observed in CD11b$^+$-enriched compartment **(Fig. 3B, FigS. 2B, C, D).** The comparison of cell distribution showed that CD11b$^+$ and CD11b$^-$-enriched fractions clustered in different compartments and that the bulk overlapped both CD11b$^+$ and CD11b$^-$-enriched fractions **(FigS. 2B)**. Based upon a signature score from the combination of *Adgre1* (F4/80), *Fcgr1* (CD64) and *Csf1r* (CSF1R) expression, CD11b$^-$ and CD11b$^+$ compartments were both exhibiting macrophage signature **(Fig. 3B, FigS. 2B, C)**. As expected **(FigS. 1A)**, *H2-Ab1* encoding a class II MHC gene was more highly expressed in CD11b$^-$ ductal TAMs **(Fig. 3B, FigS. 2C, D)**. In order to support our findings based on microscopy, we first applied to our dataset the steady state DM and SM signatures as well as lactation- and weaning-associated macrophage signatures defined by Dawson and colleagues (Dawson et al., 2020)**(Fig. 3C, Table S1)**. DM signature mostly covered the CD11b$^-$ compartment including clusters 0, 4, 5, 8, 9, and 11, but also clusters 2 and

3 that belonged to a mix of CD11b^{+} and CD11b^{-} compartments. The lactation macrophage signature which reflects an active proliferation state of DM covered the same clusters than the steady state DM. However the weaning macrophage signature reflecting DM participating in the involution of the mammary tissue through phagocytosis of milk-producing cells (Dawson et al., 2020), covered mostly cluster 2 and a fraction of cluster 3 **(Fig. 3C)**. SM signature mainly covered cluster 1 **(Fig. 3C)**, but also a fraction of clusters 8 and 2 suggesting that these two clusters could be composed of both ductal and stromal macrophages or macrophages ongoing a transitory state between the stroma and the mammary duct. Dawson and colleagues also identified DC1 and DC2 signatures from sorted DC of the mammary tissue **(Table S1)**. These signatures applied to the PyMT dataset associated cluster 7 to the DC2 signature, and cluster 10 and 13 to the DC1 signature **(FigS. 2E, F)**. Finally, cluster 12 corresponded to a contamination of granulocytes.

Beyond the relationship established through the DM and SM signature overlaps, we next sought to match subsets identified by scRNAseq with the different TAM locations identified by imaging. We conclude that cluster 6 fits with ECFP^{+}Ly6C^{high} classical monocytes (Cyan circle) based on the correlation *Itgam*$^{+}$/CD11b^{+}*, Ly6c2*$^{+}$/Ly6C^{+}*, Cx3cr1*^{low}/EGFP^{low} **(Fig. 3D)** and supported by *Ccr2* expression **(FigS. 2F)**. Cluster 6 also included a small proportion of known non-classical monocyte markers *Nr4a1*$^{+}$, *Spn*$^{+}$, *Treml4*$^{+}$ **(FigS. 2F).** Clusters 0, 4, 5, 8, 9, 11 fitted with the EGFP^{+} CD11b^{-} hyperplastic ductal associated macrophages (dark green circle) based on the correspondence of expression between transcript and protein *Itgam*^{low}/CD11b^{+}*, Cx3cr1*^{high}/EGFP^{high}, *H2-Ab1*^{high}/IA-IE^{high}. Clusters 2 and 3 were linked to EGFP^{+} CD11b^{+} malignant TAMs (light green circle) based on the correlation *Itgam*$^{+/-}$/CD11b^{int}*, H2-Ab1*^{low}/IA-IE^{low}*, Cx3cr1*^{high}/EGFP^{high}. Finally, cluster 1 was linked to the stromal type I TAMs (red circle) based on the correlation *Itgam*$^{+}$/CD11b^{+}*, Mrc1*$^{+}$/CD206, *H2-Ab1*^{high}/IA-IE^{high}, *Cx3cr1*$^{-}$/EGFP^{-} **(Fig. 3D)**.

To address the relationship between the different macrophage subsets we performed a pseudotime trajectory analysis to model the differentiation trajectories of each subset **(Fig. 3E)**. Franklin and colleagues used parabiosis experiment to confirm that peripheral monocytes contribute significantly to the accumulation of TAMs in the PyMT model (Franklin et al., 2014). Hence, we identified monocytes as the root node of the trajectory and defined two different paths evolving from this root. The stromal TAM path was linking monocytes to stromal TAMs and the ductal path linking monocytes to ductal TAMs

through cluster 8 **(Fig. 3E)**. Interestingly cluster 8 shared both stromal and ductal signature supporting this cluster as an intermediate state of stromal TAMs at the vicinity of the ductal epithelium.

While cluster 6 clearly identified monocytes, we were not able to clearly discriminate both subsets of stromal TAMs. To distinguish their transcriptomic profiles, we focused our analysis on cluster 1 and 6 corresponding to stromal TAMs and monocytes respectively **(Fig. 3C, D)** and performed a *de novo* clustering analysis to increase resolution. This sub-clustering generated 4 distinct clusters **(FigS. 3A)**. As previously, we correlated the expression of *Ly6c2, Cx3cr1, H2-Ab1* and *Mrc1* transcripts with their protein expression on the gated population. We recovered monocytes (*Ly6c2*$^{+}$/Ly6C^{+}*, H2-Ab1*^{low}/IA-IE^{low}*),* and Apple^{+} SM type I (*Mrc1*$^{+}$/CD206^{+}) and we were able to assign the two other clusters to the ECFP^{+} SM type II subset according to Cx3cr1^{+}/EGFP^{+}, *H2-Ab1*$^{+}$/IA-IE^{+} and *Mrc1*$^{+}$/CD206^{+}, fitting with the phenotype observed by flow cytometry **(FigS. 3A, B, C)**. Pseudotime analysis on the sub-clustering of the stromal TAMs suggested a linear path from monocytes to stromal type I and stromal type II TAMs suggesting that monocytes may differentiate into both type of stromal TAMs **(FigS. 3D)**. Following the ductal path, a circular relationship was shown in the region of hyperplastic ductal TAMs suggesting a contribution of local proliferation. This was supported by cell cycle analysis by flow cytometry using Topro3 to stain the DNA, showing that cycling CD11b^{-} TAMs represented the vast majority of cycling macrophages **(Fig. 3E, FigS. 3E).** In contrast, local proliferation weakly contributed to the maintenance of SM type I macrophages which might explain their relative loss along tumor evolution **(FigS. 3E).** Finally, from the hyperplastic ductal TAM clusters, an alternative path throughout cluster 3 led to malignant tumor TAMs identified in cluster 2 **(Fig. 3E)**.

Previous studies have highlighted different markers discriminating TAM subsets in different tumor types in both human and mouse. We addressed whether these markers were also linked to different populations in our model. *Hes1*, *Folr2* and *Lyve1* expression was attributed to an embryonic-like macrophage profile (Mulder et al., 2021; Sharma et al., 2020), while *Trem2*+ and *Spp1*+ macrophages were of monocytic origin, and associated with pro-tumoral functions (Cheng et al., 2021; Katzenelenbogen et al., 2020; Mulder et al., 2021; Nalio Ramos et al., 2022). We found *Folr2* and *Lyve1* specifically in *Mrc1*+ SM; *Hes1* expression was found in hyperplastic ductal TAMs, along with *Cadm1*. *Trem2* and *Spp1* were observed in malignant TAMs (cluster 2), consistent with previous observations **(Fig. 3F, FigS. 3B)**.

In conclusion, TAMs accumulate through monocyte recruitment and local proliferation and progressively acquire specific transcriptomic signatures depending on their localization in distinct territories of the tumor stroma and state of tumor malignancy.

TAM niches are associated to functional diversity.

To further investigate whether the spatial heterogeneity of TAMs is associated to a specific functional profile of these different TAM subsets, we looked at the differential gene expression (DGE) between all clusters and performed a Gene Set Enrichment Analysis (GSEA)**(Fig. 4A)**. Hyperplastic DM from cluster 4 exhibited a highly cycling score, confirming the local proliferation of CD11b^{-} TAMs. The intermediate cluster between monocytes and DM (cluster 8) was associated with inflammatory response and strong IFN responsive scores **(Fig. 4A)**. SM (cluster 1) were associated with a relatively active adipogenesis, fatty acid metabolism and response to hormones, which is consistent with their location around the adipocytes. Hyperplastic DM leading towards malignant DM presented specific TGFβ and TNFα signaling pathway scores and were also associated to the strongest hypoxic score. Finally, malignant DM were associated with biological hallmarks mostly linked to active metabolism (glycolysis, oxidative phosphorylation, protein secretion, fat metabolism), but also tumor-associated environment (EMT transition, apoptosis, angiogenesis, hypoxia, reactive oxygen species) **(Fig. 4A)**. In order to better discriminate the functions among stromal TAMs, we used the DGE among the sub-clustering obtained in **FigS. 3A** for a second GSEA **(FigS. 4A)**. Ly6C^{high} monocytes represented the most active subset with high score in IFN responses, glycolysis and oxidative phosphorylation reflecting active energetic metabolism. ECFP^{+} SM type II showed a high angiogenesis signature which might reflect their recent infiltration into the tumor.

TAM heterogeneity is commonly discussed in terms of an M1/M2 polarization spectrum. To test this model in our data, we selected a list of genes commonly related to macrophage polarization (Biswas and Mantovani, 2010; Cassetta et al., 2019; Cheng et al., 2021; Roberts et al., 2016) **(Table S2)**. Correlation of expression between these genes across all individual cells was determined. Overall, correlation scores were low and hierarchical clustering did not discriminate convincing M1- or M2-associated sets of genes, with the exception of two modules (correlation score >0.7) **(FigS. 4B)**. The first module defined by *Lyve1, Il10, Cd163 and Ccl24* identified a fraction of the *Mrc1*$^{+}$ cluster 1

corresponding to SM **(FigS. 4C)**. The second module defined by *Arg1, Arg2 and Mmp9* highlighted a fraction of malignant TAMs **(FigS. 4C)**. Individual gene expression across the different clusters confirmed the absence of a clear-cut canonical M1 or M2 profile **(FigS. 4D)** suggesting that the spatial localization of TAMs does not seem to reconcile the M1 or M2 nomenclature.

To refine the analysis and seek further markers of TAM heterogeneity, we selected a set of transcripts encoding cell surface molecules involved in homing, adhesion, and migration, obtained from the HUGO database **(Table S3)**. Hierarchical clustering of the relative expression of their transcripts confirmed that TAM subsets display specific patterns changing with tissue localization and tumor progression **(Fig. 4B)**. We sought to identify the inherent TF involved according to the transcriptomic profile of each cluster **(FigS. 4E)**. Putative role STAT1, STAT2, IRF7 and IRF9, associated to IFNγ-response (Kovarik et al., 1998) were associated with the intermediate monocyte/DM cluster (cluster 8). STAT3, associated to immune suppression (Hughes and Watson, 2018; Hughes et al., 2012), but also in mammary gland involution (Sargeant et al., 2014), was found in the cluster of hyperplastic DM leading to malignant DM. The proliferation state of hyperplastic DM from cluster 4 was also confirmed by putative role of MYC and E2F4.

To gain further insights on their function beyond their different transcriptomic profile, we sorted each TAM subset from a pool of PyMT tumors loaded them with exogenous OVA peptide SIINFEKL and co-cultured them with OT-I T cells for 3 days **(Fig. 4C)**. We measured T cell proliferation and IFNγ production as a readout of their activation. In the presence of OVA peptide SM type I were the most prone to induce T cell proliferation and IFNγ production by OT-I cells. **(Fig. 4D).** Co-culture of TAMs with sorted tumor cells did not show significant impact on tumor cell proliferation or survival (data not shown). Taking advantage of mCherry expression by tumor cells, we next profiled the capacity of each TAM subset to participate in tumor cell clearance and thus exhibit mCherry fluorescence. Malignant CD11b$^+$ DM harbored the highest phagocytic potential among all subsets **(Fig. 4E)**. Imaging the phagocytic EGFP$^+$ TAMs observed previously **(FigS. 1C)** together with CD11b staining strengthened this observation **(Fig. 4F)**. Despite this high phagocytic ability of the mCherry OVA tumor cells, no proliferation of OT-I was detected in the absence of exogenous OVA peptide for any TAM subsets (data not shown). This is consistent with previous observations showing that highly phagocytic macrophages are not potent antigen-presenting cells (Broz et al., 2014; Roberts et al., 2017). Overall, we conclude

that the localization of TAM subsets drives their specific transcriptional programs and functional capacities in the tumor microenvironment.

TAM heterogeneity is reduced in orthotopic model

We next addressed whether similar TAM heterogeneity can be recovered in an orthotopic tumor model. We performed orthotopic injection of breast tumor cells into the mammary fat pad as a model of the malignant stage of tumor development. PyMT cell line derived from a spontaneous MMTV-PyMT-mCherry tumor were injected to MacApple x MacBlue x Cx3cr1$^{EGFP/+}$ mice and tumor growth and TAM composition were monitored **(Fig. 5A)**. Tumor nodules were palpable after 11 days and then grew exponentially to reach 4000 mm^3 within a further 10 days **(Fig. 5B)**. Among monocytes only ECFP$^+$EGFP^{low} signature was recovered whereas among macrophages the three fluorescent signatures, also observed in the spontaneous tumors, were present **(Fig. 5C)**. No CD11b$^-$EGFP$^+$ cells corresponding to DM were recovered in this model and ECFP$^+$TAMs and EGFP$^+$TAMs represented the most abundant subsets with a progressive accumulation with tumor growth **(Fig. 5D)**. The Apple$^+$ TAMs remained the less abundant subset and did not significantly accumulate over tumor growth **(Fig. 5D)**. CD206 did not discriminate a specific subset of stromal TAMs, as observed in the spontaneous model, as the expression was similar in all subsets **(FigS. 5A)**. We analyzed the spatial distribution of these different TAM subsets by histological analysis. The structure of the tumor appeared as a dense aggregate of tumor cells with no distinction of stroma or hyperplastic mammary epithelium as observed in the spontaneous PyMT model **(Fig. 5E)**. The tumor was homogeneously infiltrated by the three fluorescent cell subsets.

We again performed scRNAseq on sorted myeloid cells **(Fig. 5F)**. Louvain graph-based clustering identified 5 clusters **(Fig. 5G)**. Clusters 3, 4, 5 exhibited DC-associated signatures as defined by Dawson et al. **(Fig. 5G, H, FigS. 5B)** (Dawson et al., 2020). Cluster 1 corresponded to Ly6C^{hi} monocytes (*Itgam*$^+$*, Ly6c2*$^+$*, Ccr2*$^+$), while cluster 2 showed an intermediate monocyte/macrophage signature. Cluster 0 was the only cluster representing TAMs. DM and SM signatures both covered this cluster **(Fig. 5H)** suggesting that only one main TAM differentiation path occurs in the orthotopic model in accordance with the observation that all TAMs develop in a rather homogeneous TME, mainly composed of tumor cells in this model. Additionally, the expression of *Trem2, Cadm1, Folr2* and *Mrc1*

was recovered in this TAM cluster, although their level of expression seemed to identify different cells within the cluster **(Fig. 5I)**. The GSEA based on the DGE of the 5 clusters revealed that cluster 0 was highly inflammatory and metabolically active, with a proliferative signature, resembling the cluster associated to malignant TAMs in the spontaneous model (cluster 2) **(FigS. 5C)**. Consistently, the projection of the signatures extracted from clusters 0, 1 and 2 of the orthotopic model to the UMAP of the spontaneous PyMT model confirmed the similarities with the cluster of malignant TAMs and hyperplastic TAMs **(FigS. 5D)**. We conclude that orthotopic models negate the impact of specific tissue location and drive TAMs toward a functional signature representative of advanced tumor malignancy.

Human breast TAMs exhibit similar diversity

As the MMTV-PyMT model has been described to recapitulate the different stages of development of human breast cancer, we sought to determine whether the TAM heterogeneity identified in this model could be reflected in human tumors. We used scRNAseq data from Visvader's group containing a diversity of cell types sequenced from different breast tumors (Pal et al., 2021). We focused our analysis on monocytes and macrophages by selecting them based on *CSF1R*, *CD68* or *FCGR1A* expression and generated a UMAP representation of the concatenated tumor-associated myeloid cells. Louvain graph-based clustering identified 10 clusters **(Fig. 6A)**. We applied the combination of markers that were used to identify distinct TAM subsets in PyMT mouse to the human dataset **(Fig. 6A).** While those signatures were in general more heterogenous, we retrieved a similar association. Monocytes were identified by expression of *ITGAM*, *CD14* and *CCR2*. *FOLR2*$^{+}$ cells co-expressed *LYVE1* and *MRC1* as observed in the stromal/adipose TAMs in the PyMT model (**Fig. 6A, B)**. A population of *ITGAM*^{low} *HES1*$^{+}$ *CADM1*$^{+}$ was recovered, suggesting that a profile similar to Hyperplastic DM in PyMT mice can be found in human breast tumors. Finally, advanced TAMs were identified by *TREM2*, *SPP1* and *MARCO* expression. An intermediate population expressing markers from both hyperplastic-like and advanced-like TAMs, was identified (**Fig. 6A, B)**. Similar observations were recently made by Nalio Ramos and colleagues showing that *FOLR2* identify macrophages in the stroma in both healthy mammary tissue and malignant breast cancer while *TREM2* and *SPP1* expression is specific to TAMs localized in the tumor nest (Nalio Ramos et al., 2022). Stromal macrophages exhibited an androgen response-associated transcriptomic signature which is coherent with their association to the adipose

stroma of the mammary tissue **(Fig. 6C)**. As in mice, an IFN-responsive cluster was also recovered in human tumors, corresponding to advanced-like TAMs. Altogether, these observations suggest that similar niche-associated patterns are found in human breast tumors.

Discussion

Spontaneous tumors from the PyMT model generate multiple tumors from the mammary epithelium representative of the different stages of breast cancer in human tumors (Attalla et al., 2021; Lin et al., 2003). Hence, this model provides both the spatial diversity of the mammary tissue and the temporal evolution of tumor transformation. Using three different reporter transgenes, we were able to distinguish multiple subsets of TAMs, associated to distinct spatial and temporal territories. These different TAMs harbored conserved transcriptomic signatures of the two main macrophage populations isolated from the healthy mammary tissue showing that environmental perturbation induced by tumor development does not fully reprogram TAMs from their original function and that TAM heterogeneity is spatially poised.

CD11b^{-} ductal TAMs, mainly detected in early tumors were linked to monocytes through a differentiation path associated to IFN signaling signatures, known to be involved in macrophage differentiation and response to immune checkpoint blockade therapy (Benci et al., 2016; Delneste et al., 2003). This subset also exhibited lactation macrophage signature, consistent with the idea that hyperplastic lesion resembles mammary tissue in active proliferation in which DM and epithelial cells exert mutual benefits (Dawson et al., 2020; Van Nguyen and Pollard, 2002). Accordingly, the active proliferation of this subset suggests that they partially accumulate through local proliferation. We identified a secondary path leading to malignant ductal TAMs with high *Trem2* expression. Accordingly, TREM2 has been associated with bad prognosis and was suggested to regulate the immunosuppressive functions of TAMs of monocytic origin (Katzenelenbogen et al., 2020; Mulder et al., 2021). Although this subset reflects the presence of more advanced tumor lesions, these TAMs do not necessarily exert only pro-tumoral function. For instance, they harbored the most potent phagocytic capacity and similar signature to DM involved in tissue involution after weaning. TNF- and IFN-responsive clusters were identified in both human and mouse tumors suggesting that these molecular axes are important paths in TAM activation and could represent promising therapeutic targets. Additionally, both pathways were recently shown to a define a specific tumor immune archetype (Combes et al., 2022).

A third independent path linking monocytes to stromal TAMs was identified. Stromal type I TAMs harbored potent T cell activation capacity *in vitro*, although they phagocyted less tumor cells than the CD11b$^+$ ductal TAMs. This is likely to be associated with their respective tissue localization with stromal type I TAMs restricted to the border of the tumor and in adipose islets while CD11b$^+$ ductal TAMs are found in advanced tumor lesions. Similar observations were recently made in human breast cancer where FOLR2$^+$ TAMs were positioned near blood vessels at the tumor periphery and co-localized with CD8 T cells (Nalio Ramos et al., 2022). We confirmed that the stromal type I TAMs exhibited higher capacity to activate OT-I T cells *in vitro* compared to other subsets only when adding exogenous cognate antigen. However OT-I adoptive transfer *in vivo* has been shown to ineffective at rejecting the tumor (Engelhardt et al., 2012).

scRNA-seq has some limitations as a method to detect genuine heterogeneity in populations. Even the most highly-expressed transcripts are detected in only a subset of cells (Summers et al., 2020) and clustering of cell types depends heavily on assumptions about the data structure. However, our clustering was mostly confirmed at the protein level by flow cytometry and recovered the main subsets observed by imaging. We showed that most TAM transcriptomic signatures poorly recovered the M1/M2 dichotomy, as shown recently in other mouse models (Mujal et al., 2022). CD206 is usually associated to M2-like phenotype but is widely expressed by subsets of resident tissue macrophages including those of adipose tissue (Arendt et al., 2013; Silva et al., 2019; Summers et al., 2020; Wentworth et al., 2010). Although those TAMs were not in close contact with tumor nodules, adipose-associated macrophages are known to produce several inflammatory cytokines promoting tumor development (Arendt et al., 2013; Faria et al., 2020; Picon-Ruiz et al., 2017). In the orthotopic model CD206 is expressed in a high frequency of TAMs regardless of their localization but in human tumors, *Mrc1*$^+$ stromal TAMs were present in a proportion similar to the spontaneous model. This shows that orthotopic models tend to artificially bias TAM phenotype. Indeed, TAM diversity was more restricted in the orthotopic model which we argue to be linked to the lack of niche variety and to a model representative of very progressive tumor. In the human dataset, we were able to recover a similar TAM diversity including the *HES1*$^+$*, FOLR2*$^+$ and *TREM2*$^+$ main subsets with functional hallmarks comparable to the ones present in the mouse PyMT tumor. These subsets were recently identified in human breast tumors to reside in distinct niches (Nalio Ramos et al., 2022). Those observations emphasize the importance to consider TAMs in

human tumors according to their tissue localization (Wu et al., 2021). The development of mass cytometry imaging and spatial sequencing will further address this aspect.

Taken together, our data suggest that heterogeneity derived not from any specific activation state, but from the temporal profile adaptation of monocyte and macrophage to specific locations with the growing and developing tumor. Overall, our study propose that TAM heterogeneity is directly related to the spatial diversity which is shaped by the original structure of the tissue, the histological type of the tumor and its developmental grade. Therefore, it will be key in the future to consider TAM spatial distribution to develop more tailored therapeutic strategies to precisely shape the tumor immune response.

Limitations of study

Although we were able to link spatial heterogeneity of TAMs to their transcriptomic signature, the fluorescent profiles did not fully recover the granularity of TAMs reached by scRNAseq but at least was associated to the main tissue territories. The reason why TAM subsets differentially expressed the fluorescent reporters as a result of their localization is still unclear. Further functional studies will help to determine how the surrounding signals regulate fluorescent reporter expression.

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Author contributions

Conceptualization M.L., AJ.C, C.C., MF.K., A.B.; Methodology M.L., AJ.C, MF.K., A.B.; Software M.L., M.P., AJ.C, AA.R, A.B.; Formal analysis M.L., M.P., A.B.; Investigation M.L., M.P., E. WD., S.B., T.C.; Resources AA.R, DA.H., MF.K; Data curation M.L, AJ.C, AA.R, A.B., Writing – Original draft M.L., A.B.,

Writing – Review & Editing M.L., M.P., AJ.C, DA.H., MF.K, A.B.; Supervision A.B., MF.K, Funding acquisition A.B., MF.K.

Conflict of interest

The authors declare no competing interests.

Figure 1. Breast macrophage niches evolve during tumor development.

A. MMTV-PyMT-mChOVA were crossed with *Cs1fr*-mApple, *ΔCsf1r*-ECFP and *Cx3cr1*$^{kin/GFP}$ to generate a multi-fluorescent macrophage reporter mouse (PyMTFluo). **B**. Representative opt-SNE of flow cytometry analysis of the mammary monocyte/macrophage compartment shows the expression of the different markers and fluorescent reporters over tumor development. Non-PyMT represents non tumoral mammary tissue from female fluorescent mice. Pre-tumoral tissue represents mammary tissue from < 3-month-old tumor-free female PyMTFluo mouse. Tumoral tissue represents mammary tissue from > 6-month-old tumor-bearing female PyMTFluo mice. The different macrophage subsets are color-coded based on their relative expression of ECFP, EGFP, Apple, CD11b and Ly6C. Histogram represents the percentages of each subset of fluorescent macrophages (n= 5-8 mice per group, mean ± SD are indicated). Two-way ANOVA with Turkey post test was performed. **C**. TPLSM images of the mammary tissue show the distribution of the different macrophage subsets in pre-tumoral and tumoral mammary tissue from PyMTFluo mice. Collagen was detected by second harmonic generation (SHG). Adipocytes were detected by coherent anti-stokes raman spectroscopy (CARS) (Boissonnas et al., 2020). Scale bars 20µ. **D**. TPLSM image of mammary tumor border shows the restriction of stromal Apple$^+$ cells to adipose stroma. Scale bar 20µ. **E**. 3D TPLSM reconstructions show accumulation of EGFP$^+$ and ECFP$^+$ TAMs in the different types of tumor lesions; hyperplasia, adenoma, malignant ductal, and stroma. Scale bar 20µ. All panels all representative of at least n = 4 mice. See also Figure S1.

Figure 2. Ductal TAMs express CD11b$^+$ in malignant tumor lesions.

A. Representative wide field images of hyperplastic, adenoma and malignant tumor cryo-sections from PyMTFluo mice co-stained with anti-CD11b and DAPI (left column). Zoom-in images (right columns) of each lesion type (white rectangles). Scale bar 50µ. **B**. Quantification of the percentage of CD11b$^+$ gated on EGFP$^+$ cells for each lesion (Scatter plot represents mean ± SD, n = 6 tumors processed independently; each dot is a mean of 3-6 different lesions of the same tumor. Kruskal-Wallis with Dunn's multiple comparison test was performed). **C**. Representative dot plots show the relative proportion of CD11b$^-$ and CD11b$^+$ TAMs in PyMT tumor of different weights (from left to right dot plots: no tumor, <200mg, between 200-400mg, and >400 mg). **D**. Correlation of the proportion of CD11b$^+$ TAMs with tumor weight (left panel) or mouse age at time of study. (n = 15 tumors from 10 mice. Pearson correlation coefficient is indicated).

Figure 3. Stromal and ductal TAMs harbor localization-dependent transcriptomic profiles and differentiation paths

A. 10x genomic processing and scRNAseq of three sorted compartments (Myeloid bulk, $CD11b^+$-enriched, $CD11b^-$-enriched) from multiple stage PyMT tumor pool (n = 4 female mice) and downstream processing following Seurat pipeline generated UMAP visualization after Louvain graph-based clustering of the overlaid samples and identify 14 clusters. **B**. UMAP visualizations show specific gene expressions or gene signatures. **C**. UMAP visualization of the transcriptomic signatures of ductal macrophages (DM), stromal macrophages (SM), lactation and weaning obtained from Dawson et al. Nat Cell Biol. **D**. scRNAseq-based UMAP (RNA) and flow cytometry-based opt-SNE (Protein) cross-analysis of selected transcripts and corresponding phenotypic markers. Color gates indicates corresponding subsets. Corresponding subsets are color coded accordingly. **E**. Trajectory analysis shows lineage relationships between the clusters. The monocyte cluster constitutes the root node of the trajectory **F**. UMAP visualization of the relative expression of indicated transcript. See also Figure S2 and S3.

Figure 4. TAM subsets exhibit distinct polarization

A. Heatmap of hierarchical clustering of the different biological hallmark k/K scores obtained from GSEA for monocyte and macrophage clusters (DC clusters were not included in the analysis). **B**. Heatmap of selected cell surface molecule-associated transcript expressions (lectins, integrins and chemokine receptors) in TAM clusters. **C**. Layout of *in vitro* co-culture experiments of TAMs and OT-I T cells. **D**. Percentage of divided and IFNγ producing T cells after 3 days of culture. Bar represent mean ± SEM pooled from three independent experiments. one-way ANOVA with multiple comparison test was performed). **E**. *In vivo* phagocytosis of $mCherry^+$ tumor debris by TAM subsets. (n=6 mice processed independently, mean ± SEM are indicated. one-way ANOVA with multiple comparison test was performed). **F**. Immunofluorescence of CD11b expression by phagocytic TAMs. Scale bar 20µ. See also Figure S4.

Figure 5. TAM heterogeneity is associated to niche diversity

A. The PyMT-derived cell line was injected orthotopically in the mammary fat pad of female Cs1fr-mApple x ΔCsf1r-ECFP x Cx3cr1$^{kin/EGFP}$ to mimic an orthotopic breast tumor model. **B**. Tumors were detectable from day 11 and grew in every mouse (n = 7). **C**. Dot plots show the fluorescent signatures among monocytes and macrophages in each subset of the orthotopic model. **D**. CD11b expression and absolute number of each subset in the whole tumor were quantified by flow cytometry at day 12, 15 and 19 post injection (mean ± SD are indicated, n = at least 3 mice per time point; representative of 3 independent experiments). Two-way ANOVA with Turkey post test was performed. **E**. Representative cryo-sections of orthotopic mammary tumor shows tumor cell organization and fluorescent TAM distribution. Scale bar 50µ. **F**. Droplet-based scRNAseq of sorted myeloid cells from the orthotopic PyMT tumors (n = 4 female mice), processed with the Seurat pipeline. **G**. UMAP visualization after Louvain graph-based clustering shows 6 clusters. **H**. UMAP visualization of the expression of indicated transcripts, the macrophage signature (composed of *Adgre1*, *Fcgr1* and *Csf1r* expression score*)*, and mammary tissue-associated signatures of DM, SM, DC1 and DC2 from Dawson et al. (*Nat Cell Biol*.). **I**. UMAP visualization of the relative expression of indicated transcript. See also Figure S5.

Figure 6. Human breast TAMs exhibit a niche-associated TAM diversity

A. UMAP visualization of relative expression of indicated genes **B.** UMAP visualization from Seurat pipeline after Louvain graph-based clustering of the human data set from Pal. et al. EMBO J (GSE161529). **C**. Heatmap shows hierarchical clustering of the different biological hallmark k/K scores obtained from GSEA.

STAR methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to Alexandre Boissonnas (alexandre.boissonnas@upmc.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Single-cell RNAseq data have been deposited at Gene Expression Omnibus and are publicly available as of the date of publication at the following accession number: GSE184096. GEO accession number is also listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECTS DETAILS

Mice

Female MMTV-PyMT-P2A-mCherry-P2A-OVA (PyMT-ChOVA)(Engelhardt et al., 2012) for the scRNAseq were bred under specific pathogen-free conditions at the University of California, San Francisco Animal Barrier Facility. For flow cytometry and imaging analyses, Cx3cr1$^{EGFP/+}$ (Jung et al., 2000), Csf1r-Gal4VP16/UAS-ECFP (MacBlue; (Ovchinnikov et al., 2008)), Csf1r-mApple (MacApple, (Hawley et al., 2018)) and PyMT-ChOVA mice were intercrossed to generate MacBlue x Cx3cr1$^{EGFP/+}$ x PyMT-ChOVA and MacBlue x Cx3cr1$^{EGFP/+}$ x MacApple x PyMT-ChOVA mouse strains. These strains were bred at the Centre d'Exploration Fonctionnelle Pitié-Salpêtrière animal facility. To monitor TAM accumulation over time, between 14 and 40-week old mice were used. All mice were maintained under SPF conditions at 22°C and used after breast tumor development. OTI-1 RAG-2$^{-/-}$ mice were kindly provided by Sebastian Amigorena (Institut Curie, France). All experiment protocols were approved by the French animal experimentation and ethics committee and validated by Service Protection et Santé

Animales, Environnement with the number #16890. Sample sizes were chosen to assure reproducibility of the experiments and according to the 3 Rs of animal ethic regulation. For scRNAseq, all mice were handled in accordance with NIH and American Association of Laboratory Animal Care standards, and experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

PyMT Tumor Cell line

The PyMT cell line was derived from primary PyMT-ChOVA female breast tumor as described (You et al.). Cells were cultured in RPMI-1640 with 10% fetal bovine serum, Na-Pyruvate, antibiotic, and anti-mycotic (GIBCO) and incubated at 37°C in 5% CO2. For orthotopic breast cancer model, 2.5×10^5 cells were injected in the mammary fat pad of 20-weeks old female.

METHOD DETAILS

Mouse Tissue Digestion and Flow Staining

Healthy mammary tissue, orthotopic and spontaneous tumors were processed the same way. Mammary tissues were harvested and enzymatically digested with 0.1 mg/ml DNase I (Sigma-Aldrich), 1.5 U/ml Dispase II (Gibco), and 200 U/ml Collagenase Type IV (Gibco) in RPMI+glutamax (Gibco) for 30 minutes at 37°C under agitation. Samples were filtered using 70µm cell strainer and washed with FACS buffer (PBS, 0.5% FCS, 0.01% azide, 2mM EDTA). Cells were then washed and non-specific binding was blocked with Fcblock (BD biosciences). Cell surface proteins were then stained at 4°C for 30 minutes in Brilliant stain buffer (BD biosciences). Anti-CD45 (30-F11), anti-CD11b (M1/70), anti-Ly6C (AL21), anti-Ly6G (1A8), anti-SiglecF (E50-2440), anti-I-A/I-E (M5/114.15.2), anti-F4/80 (T45-2342) were purchased from BD Biosciences. Anti-CD64 (X54-5/7.1), anti-CD192 (SA203G11) and anti-CD206 (C068C2) were purchased from Biolegend. Cells were then washed once in FACS buffer and analyzed directly by flow cytometry. For cell cycle analysis, cells were fixed in 1% PFA for 10min at 4°C then washed in PBS, 2%FCS, 0.01% azide, 0.1% saponin. Cells were stained 1h at RT in 500µL PBS containing 2 µM topro-3 iodide (Molecular probes) and 10 µM RNase A (Boehringer). Calculation of absolute cell number was performed by adding to each vial a fixed number (10.000) of non-fluorescent 10µm polybead carboxylate microspheres (Polysciences, Niles, IL, USA) according to the formula: Nb of cells = (Nb of acquired cells x 10.000)/(Nb of acquired beads). Number of cells obtained for each sample was normalized per mg of tissue. Flow cytometry acquisition was performed on the flow cytometer FACS LSRFortessa X-20 (BD

Biosciences, Franklin Lakes, NJ, USA) with DIVA (BD Biosciences) Flow Cytometry software. Flow cytometry data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA) or OMIQ (Omiq inc.) data analysis software for opt-SNE representations (https ://omiq.ai, Santa clara CA).

Single cell RNA sequencing

Myeloid cells were sorted using a BD FACSAria Fusion (BD Biosciences, Franklin Lakes, NJ, USA), according to alive $CD45^+CD90.2^-B220^-NK1.1^-Ly6G^-CD11b^+$ or $CD11c^+$ from a mix of different tumors isolated from a pool of 4 PyMT-ChOVA mice. Among these, $CD11b^+$ and $CD11b^-$ myeloid compartment were individually sorted and processed for independent sequencing along with the bulk compartment. For the orthotopic model, only cells from the bulk gating were sorted from a pool of 5 different mice 15 days after tumor inoculation. After sorting, all cell preparations were pelleted and resuspended at $1x10^3$cells/ml in 0.04%BSA/PBS and loaded onto the Chromium Controller (10X Genomics). Samples were processed for single-cell encapsulation and cDNA library generation using the Chromium Single Cell 3'v2 Reagent Kits (10X Genomics) according to manufacturer's protocol. The library was subsequently sequenced on an Illumina HiSeq4000 (Illumina).

Single Cell Data Processing

Sequencing data was processed using 10X Genomics Cell Ranger V1.2 pipeline. The Cell Ranger subroutine mkfastq converted raw, Illumina bcl files to fastqs. Reads were then aligned with *count*, using the STAR aligner (Dobin et al., 2013) against the mm10 mouse genome. After filtering reads with redundant unique molecular identifiers (UMI), *count* generated a final gene-cellular barcode matrix. Both mkfastq and count were run with default parameters.

Cellular Identification and Clustering

For each sample, the gene/barcode matrix was passed to the R software package Seurat (v3.1.5) (Stuart et al., 2019) for all downstream analyses. We then filtered on cells that expressed a minimum of 200 genes and required that all genes be expressed in at least 3 cells. We also removed cells that contained > 9% reads associated with cell cycle genes (Kowalczyk et al., 2015; Macosko et al., 2015). Count data was then log2 transformed and scaled using each cell's proportion of cell cycle genes as a nuisance factor (implemented in Seurat ScaleData function) to correct for any remaining cell cycle effect in downstream clustering and differential expression analyses. For the spontaneous PyMT model, Bulk,

$CD11b^+$ and $CD11b^-$ enriched myeloid compartment data sets were merged for downstream analysis. Principal component (PC) analysis was performed on a set of highly variable genes defined by Seurat FindVariableGenes function. Genes associated with the resulting PCs were then used for graph-based cluster identification and subsequent dimensionality reduction using UMAP. Cluster-based marker identification and differential gene expression (DGE) were performed using Seurat FindAllMarkers for all between-cluster comparisons. Few clusters enriched for Epcam-Krt identified as tumor cells and enriched in ribosomal transcripts considered as dying cells were discarded from the analysis. Sub-clustering was performed by selecting cells from clusters of interest and the whole same processing was applied.

Gene signature overlay

Stromal macrophage, ductal macrophage, weaning, lactation, DC1 and DC2 were downloaded from (Dawson et al., 2020) (**Table S1**). We created a dataframe of gene values per cell from the input Seurat object. The calculate score was visualized on the UMAP with a saturation score between 0.5 and 0.7 for each signature. Density plot of genes and signatures were generated using the Nebulosa package (Alquicira-Hernandez and Powell, 2021).

Pseudotime analysis

Trajectory analysis was performed using the Monocle3 package (v1.0.0)(Cao et al., 2019; Qiu et al., 2017; Trapnell et al., 2014). Resolution was determined according to the clustering obtained with the Seurat package. Root node was assigned to monocytes for each analysis.

GSEA Hallmark pathway analysis

The signature of each cluster was computed into GSEA (https://www.gsea-msigdb.org) for top100 hallmark pathways with FDR q-value < 0.05. From the generated list, only the hallmark with a k/K value > 0.04 were selected. A score of 0 was set when the pathway was not found in the cluster. Heatmap was generated using the pheatmap package. k/K values of each pathway were used for the heatmap. Hierarchical clustering of centered and scaled gene expression was performed using complete linkage and Euclidean distance.

M1/M2 gene correlation

The list of commonly M1 or M2-associated genes was obtained from (Biswas and Mantovani, 2010; Cassetta et al., 2019; Cheng et al., 2021; Roberts et al., 2016) (**Table S2**). From this complete gene list (69 genes), 61 were recovered from the dataset and 50 out of the 61 were conserved using the Seurat FindVariableGenes function. Genes with lowest sct.mean (<0.02) for the dataset were discarded. We used the R package Stats (version 4.0.3) to calculate the correlation scores for each gene in individual cells. The *cor* function was used to compute gene correlation score with "spearman" as method. Resulting heatmap was generated with pheatmap. The same list was used to identify specific pattern in each cluster. The heatmap was generated using Doheatmap.

Homing molecule heatmap

Homing molecules were extracted from the HUGO database based on the manual selected lists of Regulators of G protein signaling, integrins, selectins, and chemokine receptor families (**Table S3**). The most differentially expressed genes among clusters from this list were determined using FindAllMarkers from Seurat and the heatmap was generated using Doheatmap.

Transcription factor analysis

We applied murine version of DoRothEA (version 1.2.1) package in combination with VIPER package (version 1.24.0) on single sample matrices (genes in rows and single cells in columns) containing normalized gene expression scores scaled gene-wise. Transcription factors with lowest confidence levels listed in the corresponding gene expression matrix were discarded from the analysis. VIPER provides a normalized enrichment score (NES) for each TF which we consider as a metric for the activity. Resulting heatmap was generated with pheatmap.

Human data processing

scRNAseq dataset were obtained from (Pal et al., 2021)(GSE161529). All 32 files corresponding to female tumor samples of all types were pre-processed with the same pipeline than mouse scRNAseq. After cleaning the data, myeloid cells identified by the expression of *PTPRC*, *CD68*, *CSF1R*, *FCGR1A* of each individual tumor were subsampled. Tumors containing less than 300 myeloid cells were excluded for downstream analyses. Samples were combined using anchor-based integration method from Seurat. The integrated data were used for downstream analysis, for which the same pipeline than mouse analysis was applied.

Co-culture experiment

Each TAM subset was sorted using a BD FACS Aria III according to alive $CD45^+Ly6G^-Ly6C^-CD64^+$, CD11b level and fluorescent reporter expression from pooled tumors of PyMTFluo-ChOVA mice. OT-I T cells were isolated from axillary and brachial lymph nodes and incubated in 1mM CFSE for 5min at 37°C and washed in PBS/FCS. Cells were plated at a 5:1 T cell/TAM ratio in 96 round well plate in RPMI with 10% FCS with antibiotics/antimycotics. 1µM ovalbumin peptide SIINFEKL was added to each well. Plates were incubated for 72 hours at 37°C – 5%C CO_2. For the last three hours of culture, 5µg/ml of brefeldin A was added to each well for IFNγ staining.

Multi-photon imaging

The two-photon laser-scanning microscopy (TPL SM) set-up used was a 7MP (Carl Zeiss) coupled to a Ti: Sapphire Crystal multiphoton laser (ChameleonU, Coherent), which provides 140-fs pulses of near-infrared light, selectively tunable between 680 and 1050 nm and an optical parametric oscillator (OPO-MPX, Coherent) selectively tunable between 1,050 and 1,600 nm. The NLO and the OPO beams were spatially aligned and temporally synchronized using a delay line (Coherent) allowing CARS imaging approach (Boissonnas et al., 2020). The excitation wavelength was 840 nm for the NLO beam and 1104 nm for the OPO beam to detect the vibrational signature of lipid rich structures at a frequency of 2846 cm^{-1} with an emission wavelength at 678 nm. The system included a set of external nondescanned detectors in reflection with a combination of a LP-600-nm followed by LP-462-nm and LP-500-nm dichroic mirrors to split the light and collect the second harmonic generation signal (SHG) with a 417-/60-nm emission filter, ECFP with a 480-/40-nm emission filter, EGFP with a 525-/50-nm emission filter, mCherry or Apple with a 624-/40-nm emission filter and CARS or blue evans signal with a LP 645nm emission filter. For live imaging, mice were anaesthetized and maintained during the imaging period with 2% isoflurane in medical air. An incision of the skin was performed at the level of the breast tumor and an imaging window was positioned to stabilize the tissue from breathing and drifting artefacts. Local temperature was monitored and maintained at 32°C using an incubation chamber. To define the tumor vasculature, Evans Blue was injected i.v. before the imaging session. Real time movies were performed by imaging every 30s by 5 consecutive 3µm z spacing image stack (total 12µm thickness). Static 3D images were performed on 100µm thick cryosections by 1µm z spacing image stack (up to 20µm

thickness). For all images the objective was a water immersion, plan apochromat ×20 (numerical aperture = 1). 3D reconstructions and mask rendering were done using Imaris software (Bitplane).

Epifluorescent imaging of tumor sections

In brief, mammary tissue and tumors were harvested and fixed in 2% PFA for 6 h and then incubated in 30% sucrose-PBS overnight at 4°C before being embedded in OCT-freezing medium (Microm microtech) and frozen at −80°C. Sectioning was completed on a HM550 Cryostat (Thermo Fisher) at −20°C; 5-µm or 100-µm sections were collected on Superfrost Plus Slides (Thermo Fisher Scientific) and stored at −20°C until use. Tissue sections were rehydrated with 0.5% Triton-PBS during 10 min. For CD11b staining, a first block step was performed with 3% BSA solution during 2h, followed by 2h incubation at room temperature with the primary antibodies (Rat anti-mouse CD11b, clone M1/70; BD Biosciences). Secondary antibody incubation was performed using anti-rat AF647-conjugated antibody 1h30 at room temperature (Jackson ImmunoResearch Laboratories). Slides were counterstained and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories). Epifluoresent images were acquired on a Zeiss Axio Z1 fluorescent microscope (Carl Zeiss, Germany) using Zen software. ECFP, EGFP, DAPI, mCherry, and AF647 signals were acquired using a combination of LED Colibri 7 and emission filters: LED 385 nm, EmBP 450/40 for DAPI; LED 430nm, EmBP 480/40 for ECFP; LED 475 nm, EmBP 525/50 for EGFP; LED 511nm, EmBP 605/70 for Apple; LED 555nm, EmBP 647/57 for mCherry; LED 630nm, EmBP 690/50 for AF674. Image quantifications were performed using histo-cytometry based approach. Briefly, after masking the cells as individual objects based on DAPI, EGFP or mCherry signal for tumor nodule contouring using Imaris software, fluorescent signals from all channels and spatial coordinates were extracted and transformed into fcs file for cytometry-based analysis using FlowJo. Distance of $EGFP^+$ and $ECFP^+$ TAMs to the tumor was calculated using the distance transformation module from Imaris software. CD11b quantification was performed by surfacing TAMs based on the EGFP channel. CD11b expression on $EGFP^+$ TAMs was measured using the sum intensity of the CD11b channel parameter gated on different regions of the tumor (hyperplastic, adenoma, malignant, according to their histological characteristics (Lin et al., 2003)). Acquisition and analysis settings were identical for both isotype and CD11b staining. Between three and six fields were chosen for each lesion type per mouse (n=6 mice). All histological quantifications are presented as a mean of the different fields for each mouse.

Quantification and Statistical Analysis

All statistical analyses were performed with Graphpad Prism 7 for flow cytometry and imaging, and R for scRNAseq analysis. Multigroup analysis of variances were performed, and one-way or two-way ANOVA tests were performed followed by Turkey post test for Gaussian distribution or Kruskall-Wallis followed by Dunn's multiple comparisons. For simple comparison analysis, Mann-Whitney was performed to compare nonparametric distribution. For correlation analysis, Pearson correlation was calculated *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Sample sizes are indicated in each figure legend.

SUPPLEMENTARY MOVIE LEGENDS

Movie 1. **Related to Figure 1. TPLSM 3D reconstruction of pre-tumoral mammary duct.** EGFP$^+$ ductal macrophages are found in the epithelial duct (green arrows), stromal type I Apple+ macrophages are associated to the collagen and the adipocytes (red arrows), and scarce stromal type II ECFP$^+$ monocytes/macrophages are found in the stroma (blue arrows).

Movie 2. **Related to Figure 1. Stromal and ductal TAMs have different motility pattern.** TPLSM intravital live imaging of PyMT tumor border shows ECFP$^+$ cells crawling in the vasculature and in the tumor border indicated by CARS imaging of the fat associated mammary tissue. EGFP$^+$ ductal TAMs are wrapped around tumor nodules and exhibit very low motility.

Movie 3. **Related to Figure 1. ECFP$^+$ stromal TAMs exhibit higher motility than ductal TAMs.** TPLSM intravital live imaging of PyMT tumor shows vasculature stained with Evans Blue (grey). Stromal type II ECFP$^+$ TAMs crawling along the vasculature and within the stroma (Cyan squares). Ductal EGFP+ TAM localizing around tumor nodules or perivascular, exhibiting low motility and phagocytic morphology.

Movie 4. Related to Figure 1. ECFP$^+$ stromal TAMs motility decreases at the vicinity of tumor cells. TPLSM intravital live imaging of PyMT tumor shows ECFP$^+$ motility behavior around tumor cells.

SUPPLEMENTARY TABLES

Table S1. Gene signatures of macrophages and DC from mammary tissue from Dawson et al., Related to Figure 3 and 5

Table S2. M1- and M2-associated gene list, Related to Figure 4

Table S3. Homing gene list obtained from HUGO database, Related to Figure 4

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45 (30-F11)	BD Biosciences	Cat#550994; RRID:AB_394003
Anti-mouse CD11b (M1/70)	BD Biosciences	Cat#563553; RRID:AB_2738276
Anti-mouse CD11b (M1/70)	BD Biosciences	Cat#564985; RRID:AB_2739033
Anti-mouse CD192 (SA203G11)	Biolegend	Cat#150605; RRID:AB_2571913
Anti-mouse CD206 (C068C2)	Biolegend	Cat#141723; RRID:AB_2562445
Anti-mouse CD64 (X54-5/7.1)	Biolegend	Cat#139314; RRID:AB_2563904
Anti-mouse Ly6C (AL21)	BD Biosciences	Cat#560596; RRID:AB_1727555
Anti-mouse Ly6G (1A8)	BD Biosciences	Cat#741813; RRID:AB_2871151
Anti-mouse I-A/I-E (M5/114.15.2)	BD Biosciences	Cat#563413; RRID:AB_2738190
Anti-mouse F4/80 (T45-2342)	BD Biosciences	Cat#565787; RRID:AB_2869711
Anti-mouse SiglecF (E50-2440)	BD Biosciences	Cat#740956; RRID:AB_2740581
Anti-rat AF647	Life technologies	Cat#A21247; RRID:AB_141778
Anti-IFNg Pe-Cy7 (XMG1.2)	BD Biosciences	Cat#557649; RRID:AB_396777
Chemicals, peptides, and recombinant proteins		
Dnase I	Roche	Cat#10104159001
Dispase II	Gibco	Cat#17105041
Collagenase IV	Gibco	Cat#17104019
Tissue freezing medium	Microm-Microtech	Cat#F/TFM-C
Formaldehyde	Sigma	Cat#47608
Polybeads carboxylate microsphere	Polysciences	Cat#07759-15
Sucrose	Biosolve	Cat#192223
Bovine serum albumin	Sigma	Cat#A3294
Vectashield with DAPI	VectorLabs	Cat#H-1200
Fetal bovine serum	Pan biotech	Cat#P30-3306
Trypsin-EDTA	Gibco	Cat#25200056
RPMI 1640 with glutamax	Gibco	Cat#61870010
CFSE	Thermofisher scientific	Cat#C1157
Brefeldin A	Thermofisher scientific	Cat#00-4506-51
Deposited data		
scRNAseq data	This paper	GEO: GSE184096
Experimental models: Cell lines		
PyMT cell line	(You et al.)	Developed by MF. Krummel
Experimental models: Organisms/strains		
Mouse : Cx3cr1$^{EGFP/Kin}$	The Jackson laboratory	JAX : 005582
Mouse: Csf1rmApple	(Hawley et al., 2018)	Developed by D. Hume
Mouse: ΔCsf1rECFP	(Ovchinnikov et al., 2008)	Developed by D. Hume
Mouse: MMTV-PyMT-mCherry-OVA	(Engelhardt et al., 2012)	Developed by MF. Krummel
Software and algorithms		
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij; RRID:SCR_003070
FlowJo v10	FlowJo, Treestar Inc.	https://www.flowjo.com; RRID:SCR_008520
Prism v7	GraphPad Software	https://www.graphpad.com/scientific-software/prism/; RRID:SCR_002798
Imaris v8.0.2	Bitplane	http://www.bitplane.com/; RRID:SCR_007370

Zeiss Zen	Carl Zeiss	http://www.zeiss.com
Diva	BD Biosciences	http://www.bdbiosciences.com/us/instruments/clinical/software/flow-cytometry-acquisition/bd-facsdiva-software/m/333333/overview
OMIQ	OMIQ inc.	https://www.omiq.ai/
Rstudio		https://rstudio.com

Figure 1

Click here to access/download;Figure;Fig (1).TIF

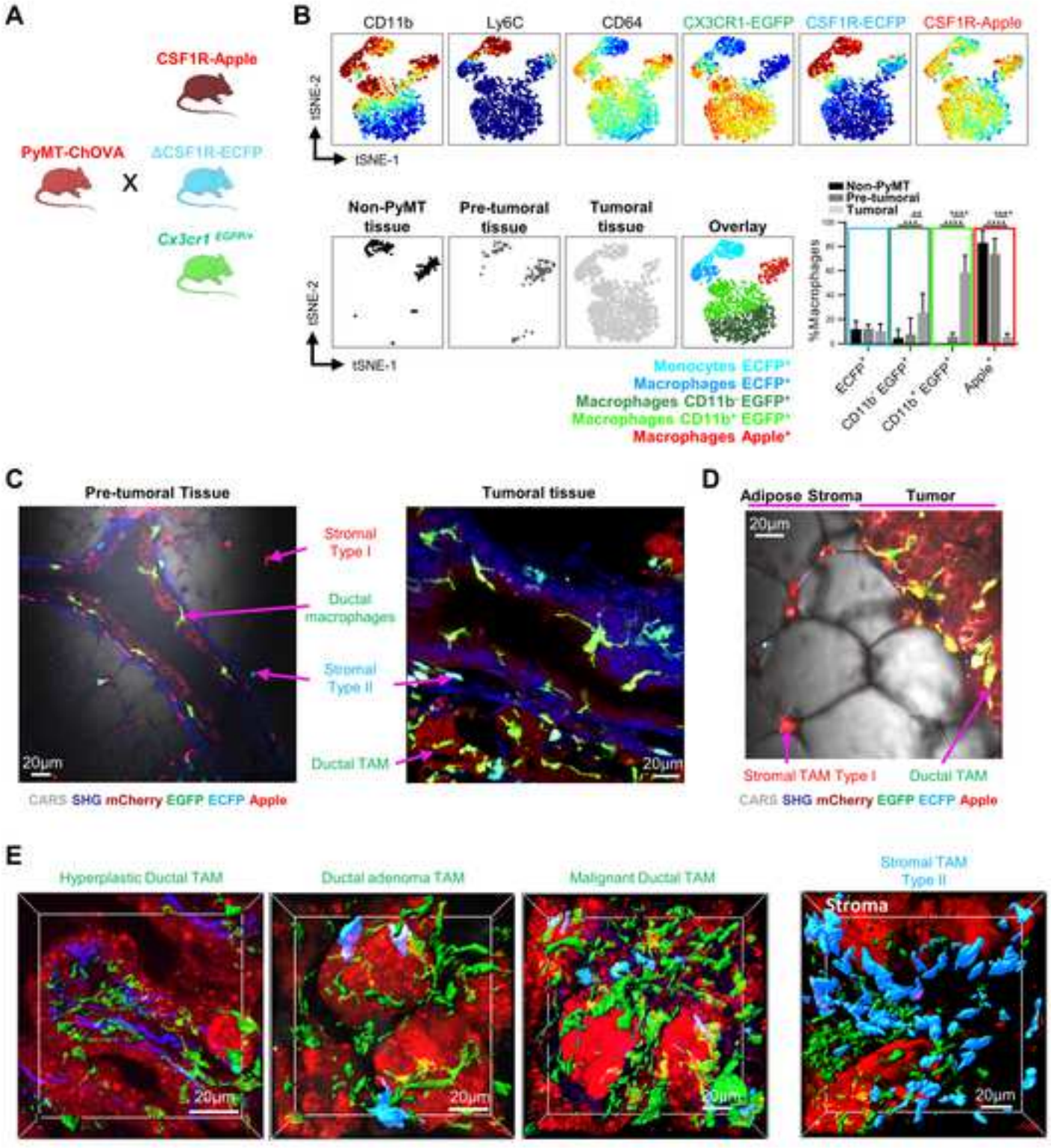

Figure 2

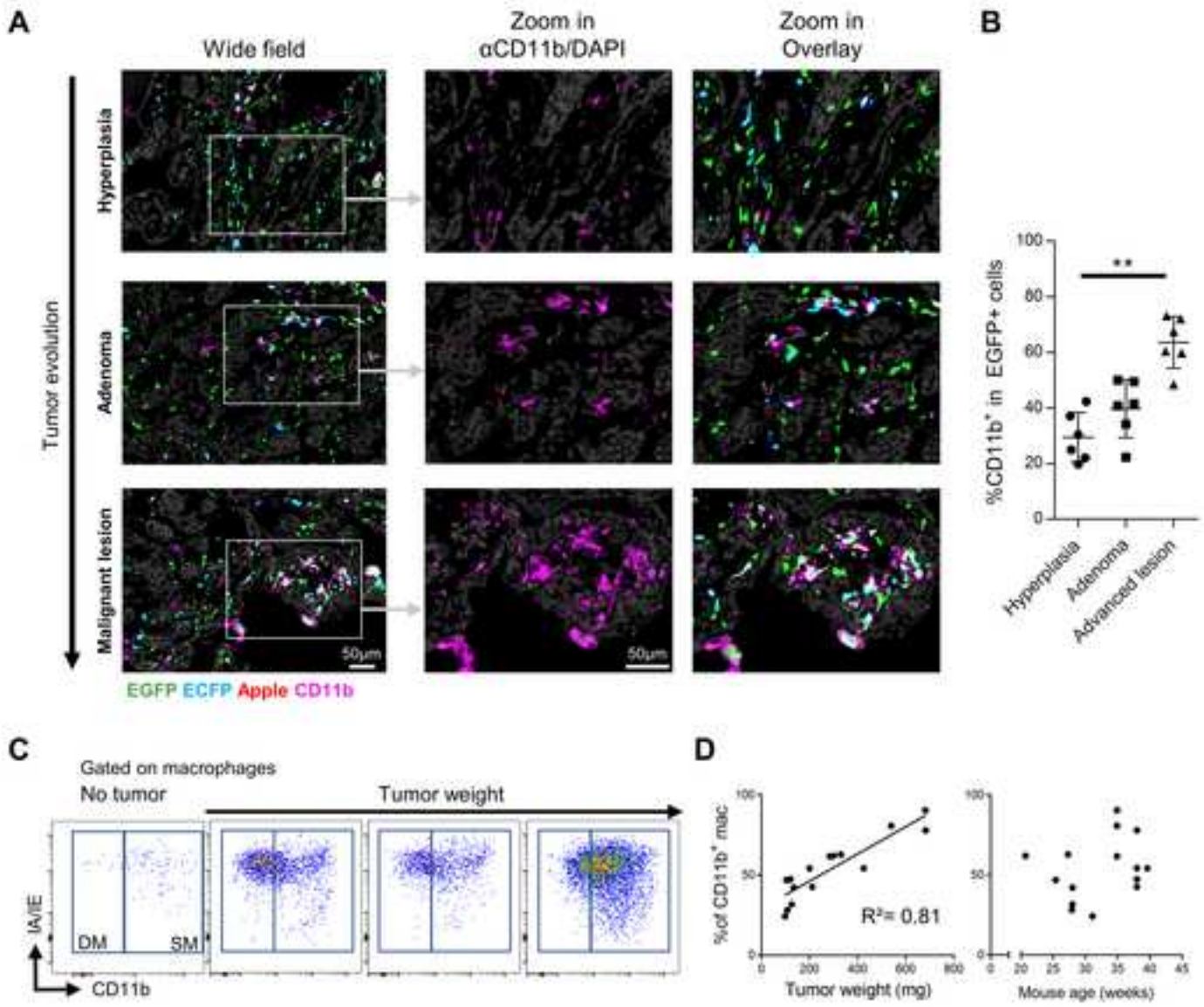

Figure 3

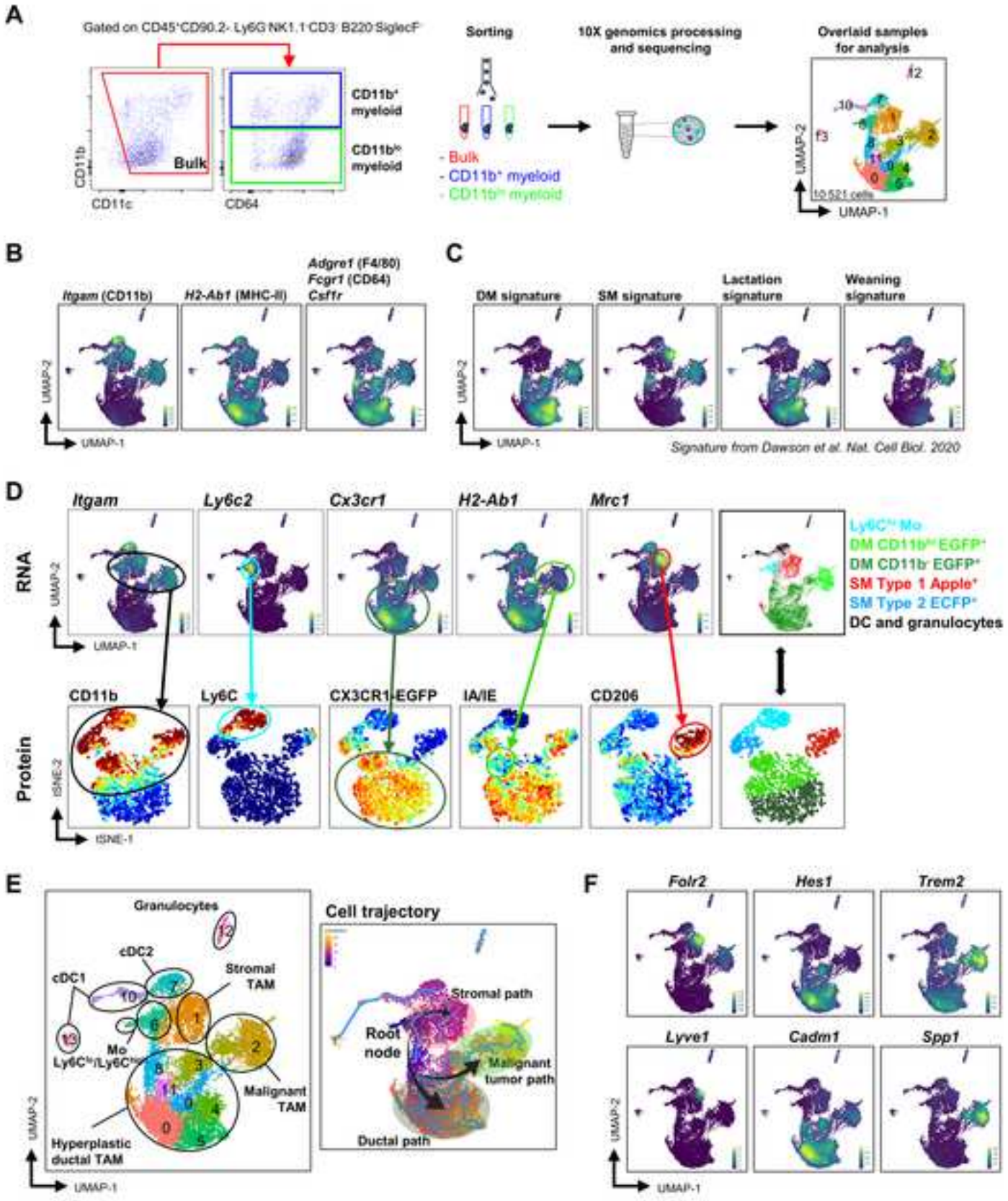

Figure 4

Click here to access/download;Figure;Fig (4).TIF

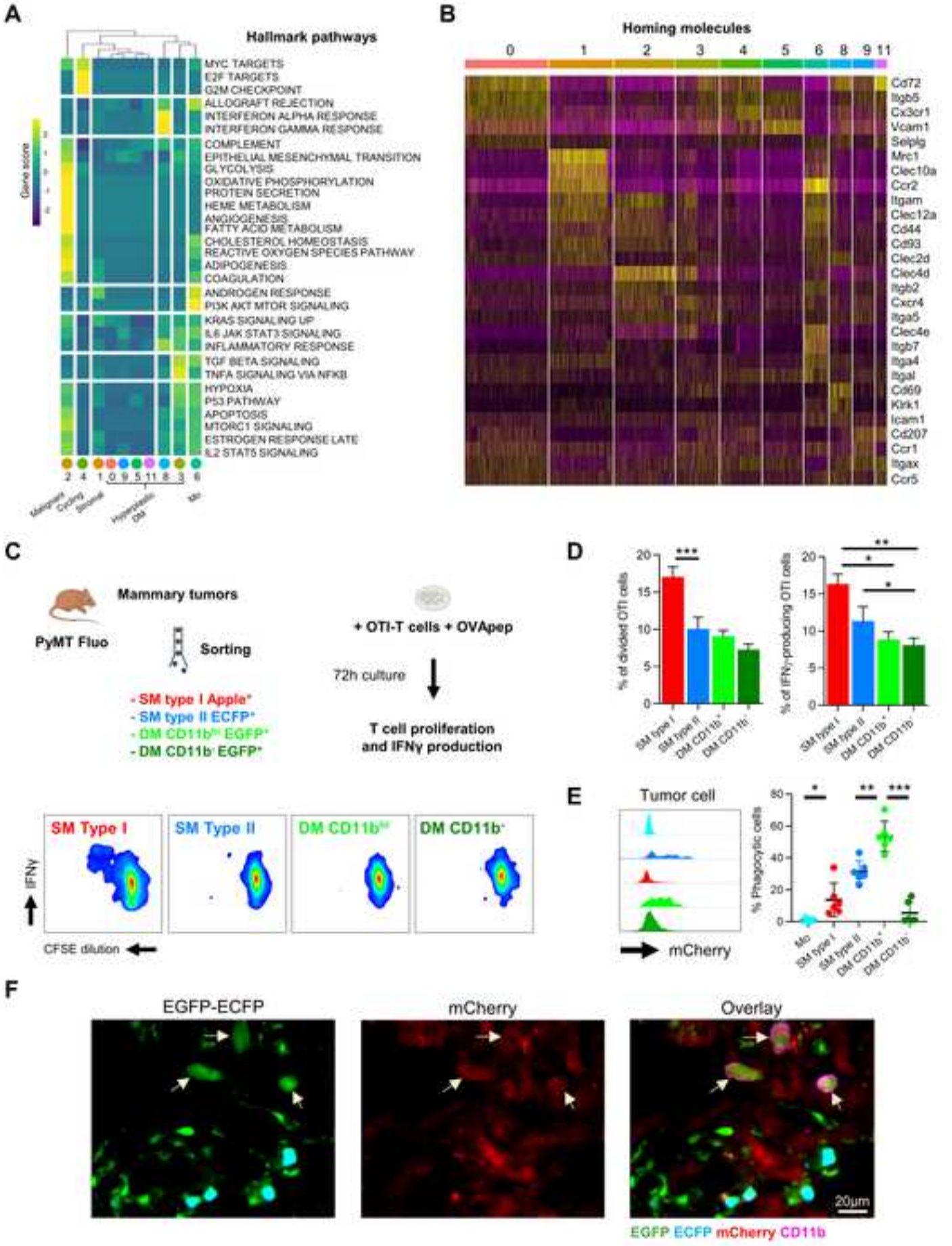

Figure 5

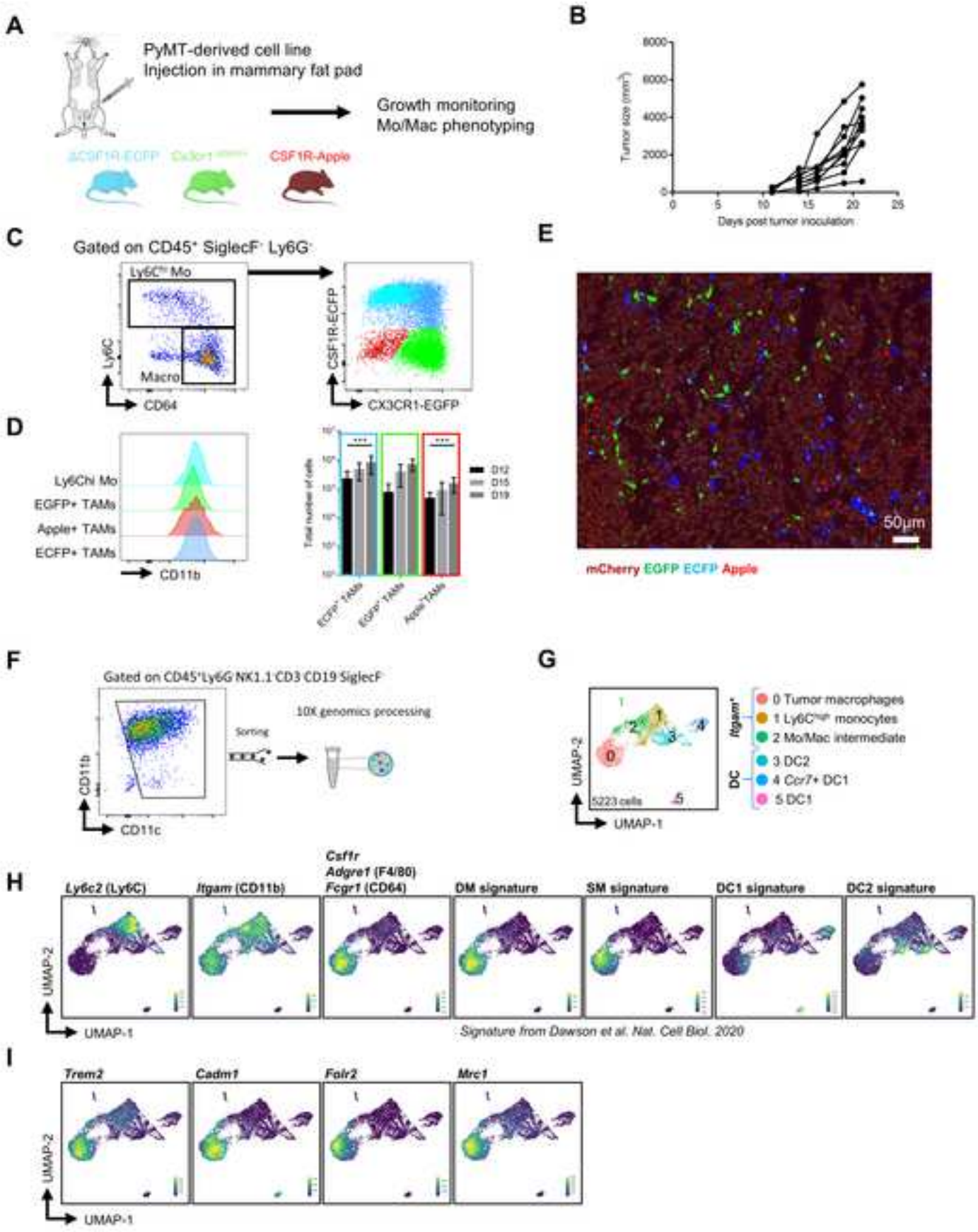

Figure 6

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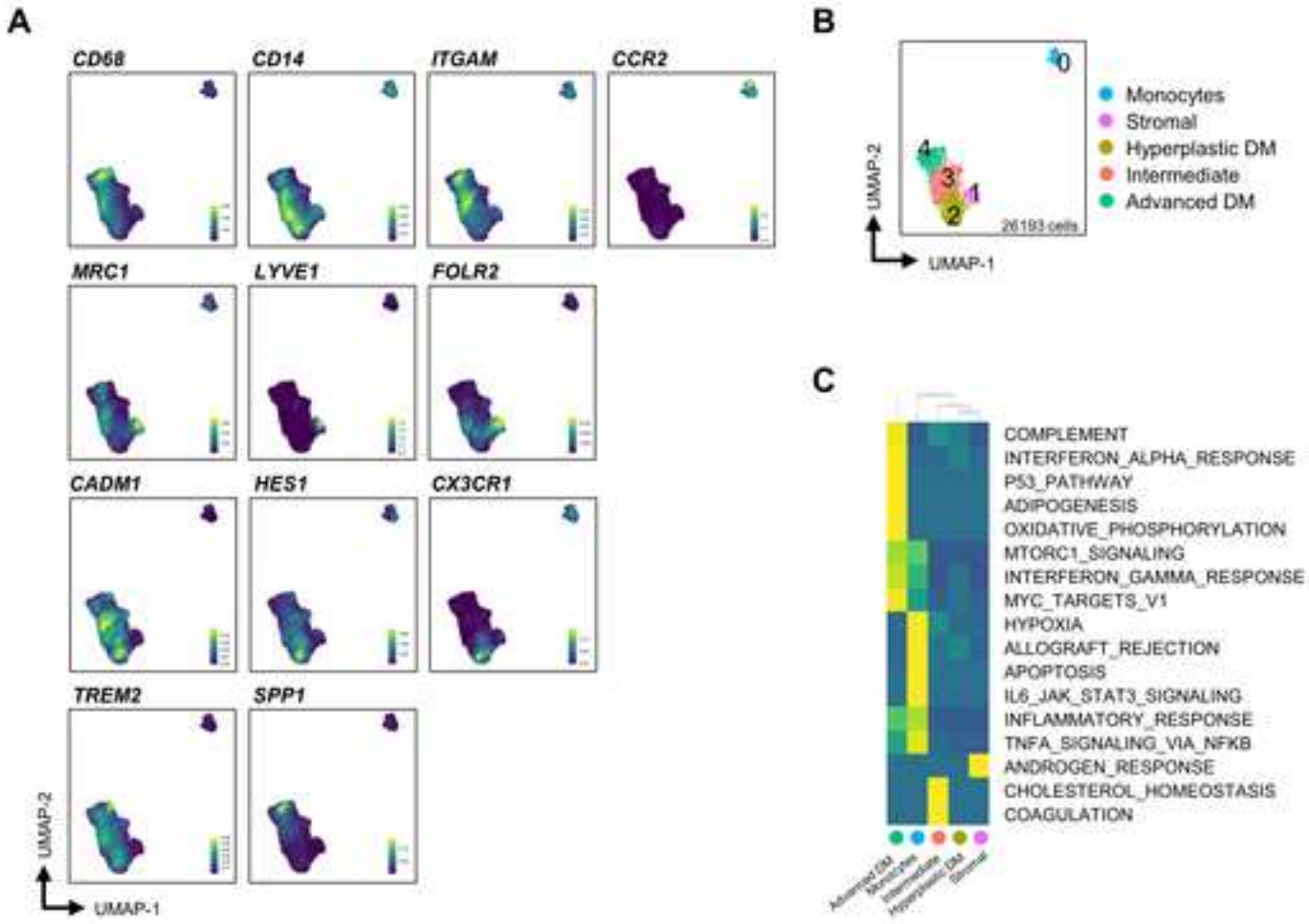


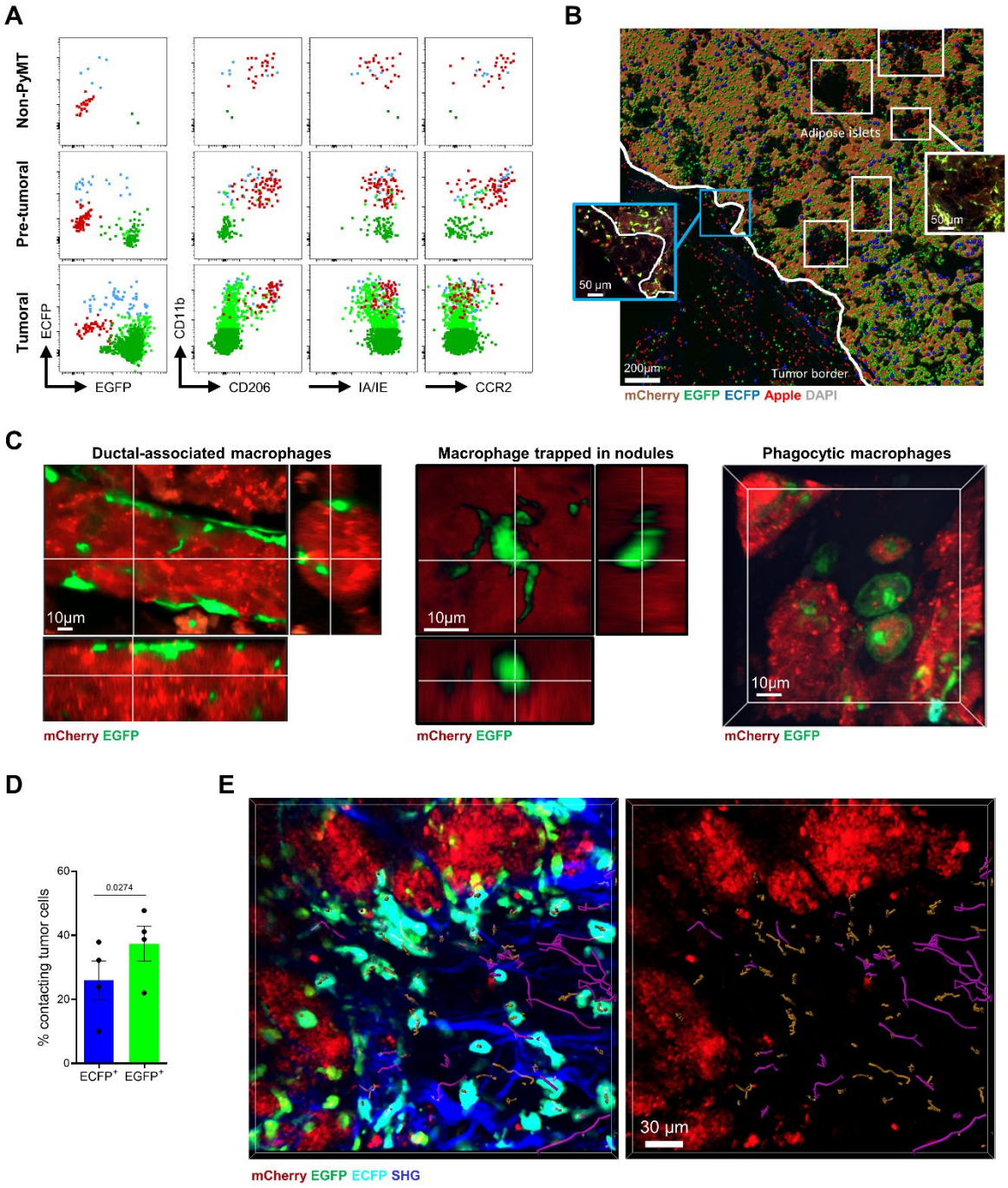


Figure S1. Breast macrophage niches evolve during tumor development. Related to Figure 1.

A. Overlaid dot plots of flow cytometry analysis of the phenotype of the different color-coded macrophage subsets. Scale bar 200µ and 50 µ for the zoom. **B**. Representative cryo-section of tumor (orange surface) shows the distribution of the different TAM subsets Apple$^+$(red spots), EGFP$^+$ (green spots) and ECFP$^+$ (blue spots) with zoom on tumor border and adipose islets. **C**. TPLSM 3D orthogonal projections illustrate the different morphologies of ductal EGFP$^+$ cells. Scale bar 10µ. **D**. Quantification of ECFP$^+$ and EGFP$^+$ cell proportion among DAPI$^+$ cells and their relative distance to tumor nodules (Bar graphs represent mean ± SEM of n=4 mice, Paired t-test was performed). **E**. Representative image of intravital tracking of ECFP$^+$ SM type II. Scale bar 30µ.

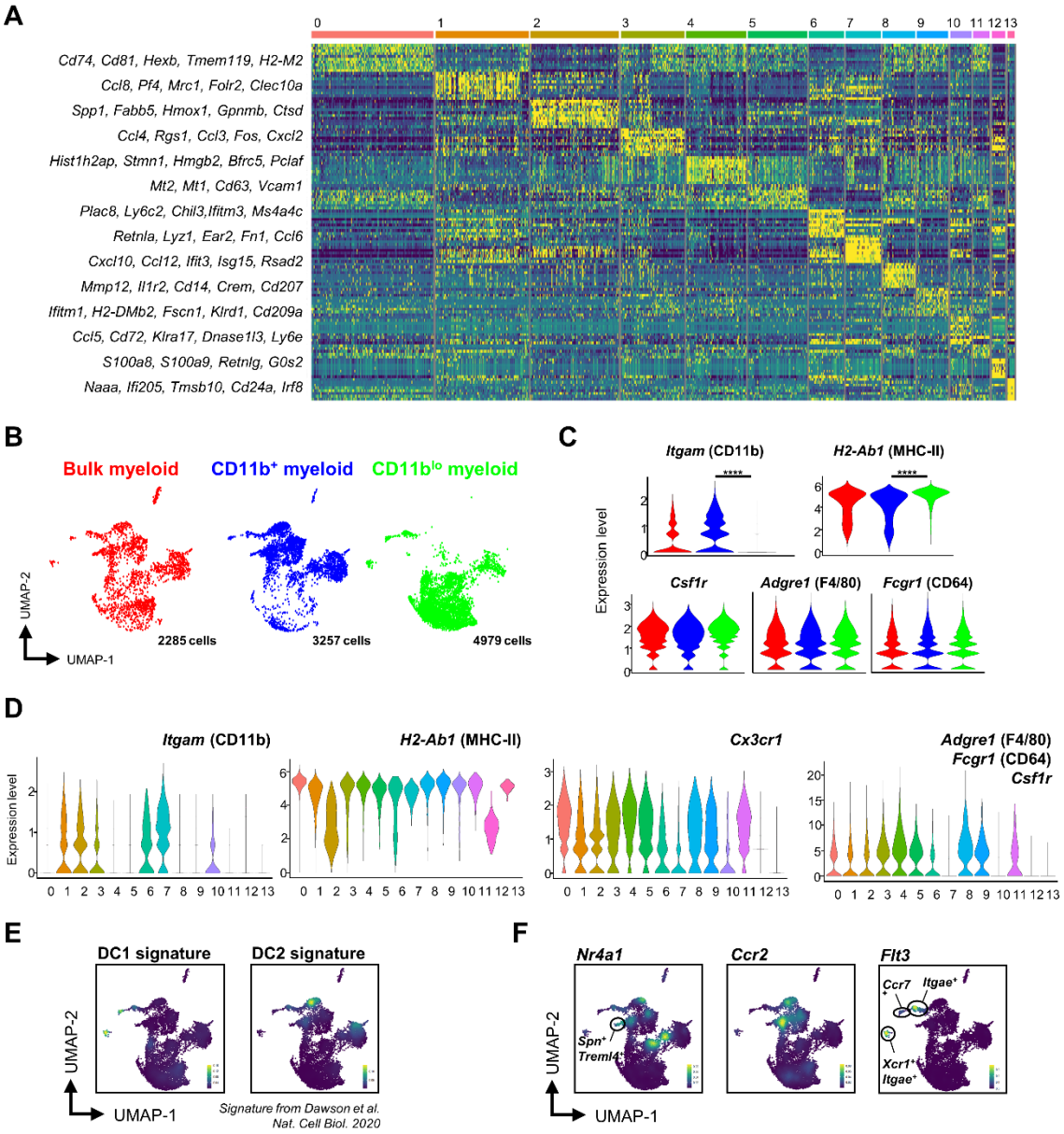
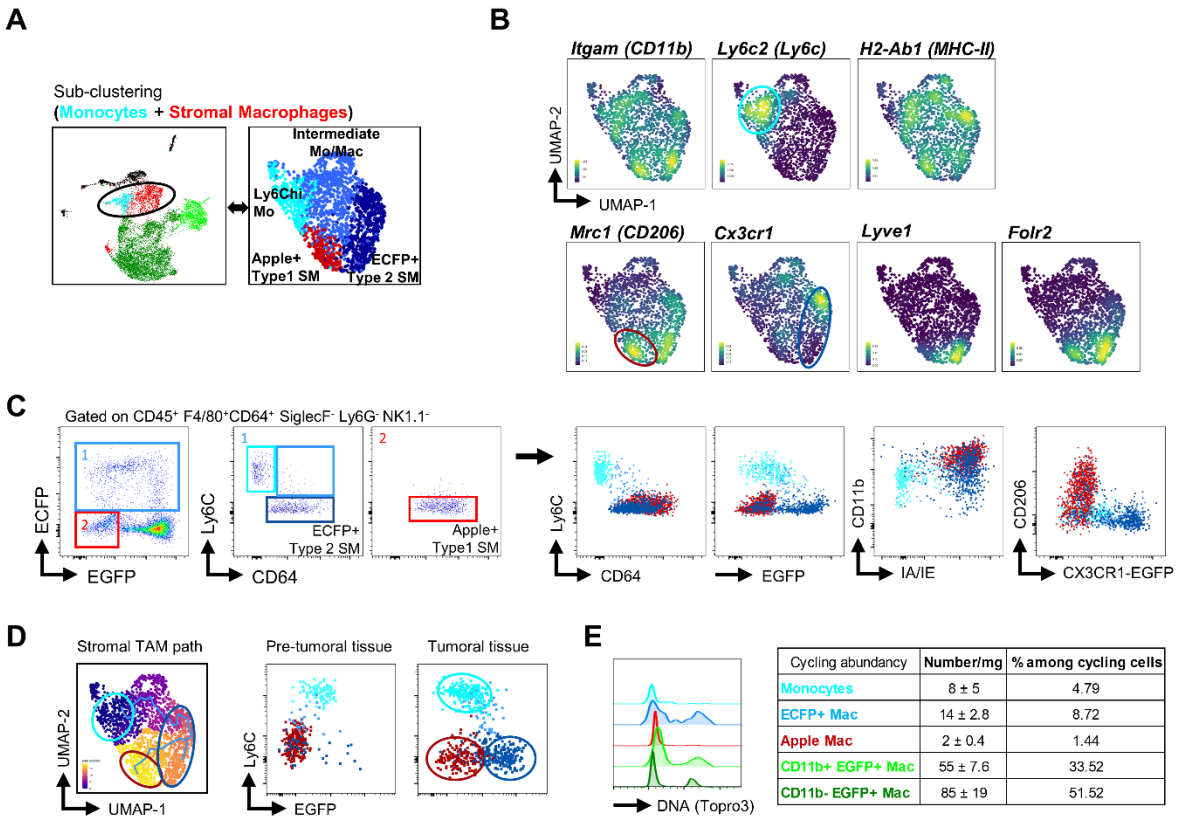


Figure S2. Stromal and ductal TAMs harbor localization-dependent transcriptomic profiles. Related to Figure 3.

A. Heatmap of the 5 most expressed genes per cluster. **B**. UMAP representation of the three sorted compartments (Bulk, CD11b$^+$-enriched, CD11b$^-$-enriched). **C**. Violin plot of *Itgam*, *H2-Ab1, Csf1r, Adgre1* and *Fcgr1* expression in the three compartments. **D**. Violin plot visualization of indicated transcript expression, and the macrophage signature (combined *Adgre1*, *Fcgr1* and *Csf1r* score) in each cluster. **E**. UMAP visualization of mammary tissue-associated signatures of DC1 and DC2 obtained from Dawson *et al*. **F**. UMAP visualization of the expression of indicated transcripts.



Cycling abundancy	Number/mg	% among cycling cells
Monocytes	8 ± 5	4.79
ECFP+ Mac	14 ± 2.8	8.72
Apple Mac	2 ± 0.4	1.44
CD11b+ EGFP+ Mac	55 ± 7.6	33.52
CD11b- EGFP+ Mac	85 ± 19	51.52

Figure S3. Stromal and ductal TAMs harbor distinct differentiation paths. Related to Figure 3.

A. scRNAseq sub-clustering of monocytes and stromal macrophages (Cyan + red clusters corresponding to cluster 6 and 1 respectively of the former analysis). UMAP visualization of the 1 and 6 sub-clustering after Louvain graph-based clustering shows 4 new clusters. **B**. UMAP visualization of the indicated transcript expression. **C**. Representative flow cytometry dot plots gated on ECFP^{+} monocytes/TAM and Apple^{+} TAM. **D**. Trajectory analysis on the scRNAseq sub-clustering shows lineage relationships between the 4 new identified clusters and correspondence with flow cytometry analysis. Pink arrows indicate the path orientation considering monocyte cluster as starting point. **E**. Overlaid histogram of DNA content, measured by topro-3 staining gated on all subsets (color code is indicated) Table indicate the absolute number /mg of tumor of cycling cells and the proportion of each subset among total cycling cells mean (mean ± SD are presented n= 3 mice).

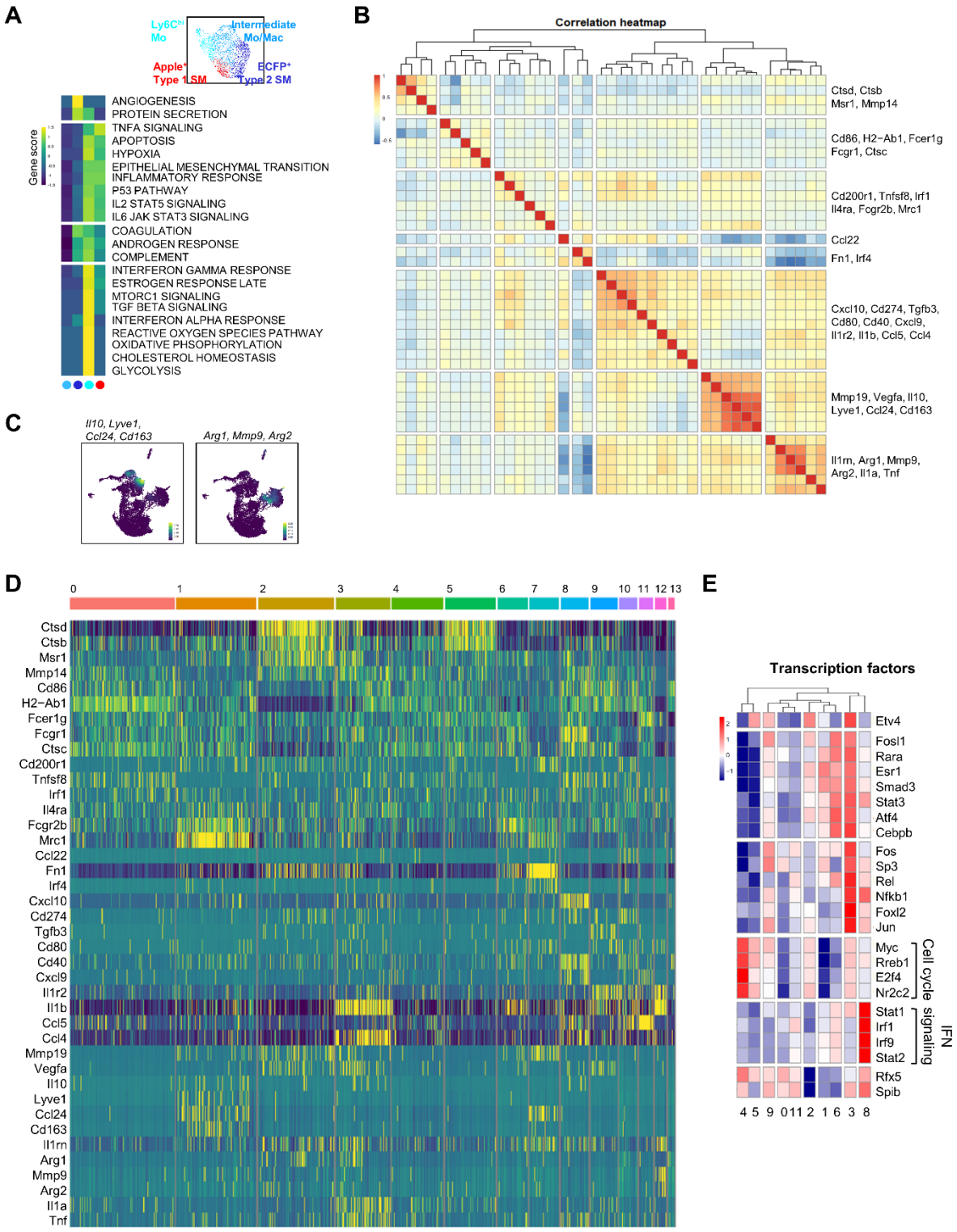


Figure S4. TAM subsets exhibit distinct polarization. Related to Figure 4.

A. Heatmap shows Hierarchical clustering (using pheatmap function in R) of the different biological hallmark k/K scores obtained from GSEA for the monocyte and stromal TAM sub-clustering. **B**. Correlation heatmap of genes associated to M1 or M2 polarization. **C**. UMAP visualization of the score associated to the indicated transcripts. **D**. Heatmap of the expression of M1 and M2-associated transcripts in TAM clusters. **E**. Heatmap shows hierarchical clustering of indicated transcription factors identified through transcriptomic analysis for each TAM clusters.

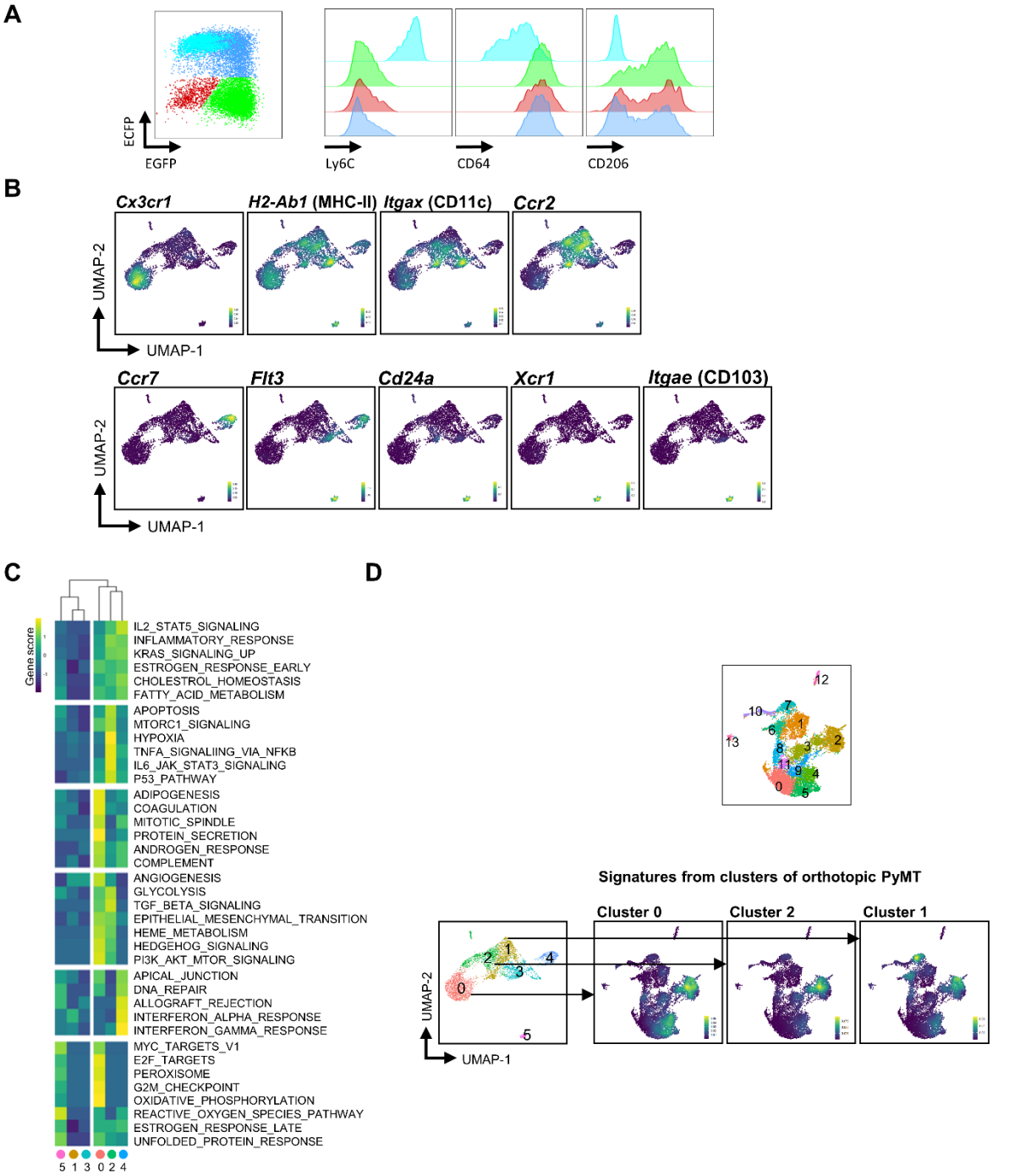


Figure S5. TAM heterogeneity is associated to niche diversity. Related to Figure 5.

A. Representative expression of Ly6C, CD64 and CD206 gated on each fluorescent monocyte and TAM subsets in the orthotopic PyMT tumor model by flow cytometry. **B**. UMAP visualization of the expression of indicated gene transcripts identifying different myeloid subsets by scRNAseq. **C**. Heatmap shows Hierarchical clustering of the different biological hallmark k/K scores obtained from GSEA for each cluster. **D**. Transcriptomic signatures from cluster 0, 1 and 2 of the orthotopic tumor model were projected on the UMAP visualization of the spontaneous PyMT model.