

Spatial Mapping of Cytotoxic T Cell Exclusion Associated with Hypoxic PLAUR+ Macrophages in Triple-Negative Breast Cancer

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Abstract

Cytotoxic T-lymphocyte (CTL) infiltration shapes outcomes in triple-negative breast cancer (TNBC), yet the microenvironmental drivers of CTL exclusion remain poorly defined. Using Xenium 5K spatial transcriptomics on 76 tumor-containing cores from 28 treatment-naïve, early-stage TNBC patients, we identified a subset of hypoxia-associated macrophages, MC2(LDHA_PLAUR), enriched in CTL-low tumors and inversely correlated with CTL abundance. *PLAUR*, a gene induced broadly across macrophage subsets under hypoxia, marked macrophages depleted of neighboring CTLs. PLAUR+ macrophages preferentially co-localized with FB0(MMP11_COL11A1) fibroblasts and engaged in *PLAU*–*PLAUR* ligand–receptor signaling that correlated with TGF- β pathway activation in adjacent stroma. Multiplex immunofluorescence (mIF) validation in an expanded cohort of 134 patients confirmed reduced CD8⁺ T-cell proximity and elevated cancer-associated pSMAD2 activation in fibroblasts around uPAR⁺ macrophages. These findings support a uPAR–TGF- β axis in hypoxic TNBC microenvironment, coupling macrophage–fibroblast crosstalk to spatial CTL exclusion.

INTRODUCTION

TNBC, defined by the absence of estrogen receptor, progesterone receptor, and HER2 expressions, has limited options for targeted therapy compared with other breast cancer subtypes. To overcome these limitations, immune checkpoint inhibitors (ICIs) combined with chemotherapy has since been incorporated into standard care for early-stage and advanced TNBC, establishing anti-tumor immunity as a therapeutically actionable axis in this subtype^{1–3}. However, clinical benefit has remained heterogeneous, and a substantial subset of patients has shown residual disease and subsequently relapsed⁴. In addition, biomarkers that could reliably identify potentially immunotherapy-responsive patients remain limited. PD-L1 expression does not select for benefit in the early-stage setting, and stromal tumor-infiltrating lymphocytes (TILs), although prognostic, have not been established as a selection criterion⁵. Another determinant of this heterogeneity is whether CD8⁺ cytotoxic T-lymphocytes (CTLs) physically reach and infiltrate the tumor^{6,7}.

CTL density carries independent prognostic value in treatment-naïve TNBC, with low CD8⁺ infiltration independently predicting worse breast cancer-specific survival^{8–12}. CTLs serve as primary effectors against tumor cells and its presence act as a critical determinant of therapeutic efficacy in both radiotherapy and chemotherapy¹³. Despite this prognostic significance, the tumor microenvironmental (TME) factors that limit CTL infiltration in TNBC require further characterization. Emerging evidences suggest a spatially restricted immune barrier, yet the cellular and molecular drivers establishing this barrier are poorly defined^{14–16}.

Among the cellular drivers of this spatial barrier, tumor-associated macrophages (TAMs) play a central role in shaping the TNBC immune microenvironment. Under hypoxic conditions, TAMs frequently adopt an SPP1⁺/M2-like phenotype that colocalizes with hypoxic tumor regions and suppresses CTL cytotoxic

function^{17,18}. This hypoxia-driven macrophage reprogramming is often accompanied by the expansion of cancer-associated fibroblasts (CAFs), which remodel the extracellular matrix (ECM) and reinforce a fibrotic, immune-excluding stroma.

PLAUR is expressed across tumor cells, macrophages, and fibroblasts, and it encodes the receptor for urokinase plasminogen activator (uPAR), a mediator of pericellular ECM proteolysis. Its transcription is directly induced by HIF-1 α under hypoxia¹⁹, and uPA–uPAR engagement has been linked to fibroblast activation and TGF- β signaling^{20–22}, making it a candidate convergence point to link these compartments in immune exclusion. Prior scRNA-seq and spatial transcriptomics studies have descriptively mapped TNBC microenvironment cell types and their spatial organization, but have yet linked a defined hypoxic macrophage state to a continuous gradient of CTL exclusion^{6,13,23}.

Because both cytotoxic chemotherapy and checkpoint blockade extensively remodel the TME, the cellular architecture that governs CTL accessibility to tumor cells in the first place is observable only before systemic therapy. Here, we applied Xenium 5K spatial transcriptomics to treatment-naïve, early-stage TNBC and stratified tumors by CTL abundance. *PLAUR*⁺ macrophages were enriched in CTL-low tumors, exhibited a robust hypoxia-associated transcriptional profile, and co-localized with MMP11 + CAFs with elevated TGF- β pathway activity. In turn, local CTL density was reduced in the immediate vicinity of *PLAUR*⁺ macrophages. These associations were confirmed at the protein level by mIF.

RESULTS

Spatially resolved single-cell landscape of the TNBC tumor microenvironment.

Surgical specimens of tumor center and adjacent normal regions were collected from treatment-naïve patients with stage I or II TNBC (Fig. 1a). To characterize the tumor microenvironment of TNBC, we performed in-situ spatial transcriptomic profiling using the Xenium Prime 5K platform (Methods).

Starting with an initial dataset of 612,848 cells, we filtered out low-quality cells displaying fewer than 10 transcript counts. Additionally, using Xenium Explorer, we restricted the analysis to cells with valid centroid coordinates located within tissue microarray cores. The resulting dataset comprised 499,655 cells across 82 cores from 28 patients, representing tumor, and adjacent-normal tissues (Fig. 1b).

Cell type annotation was initially performed across the entire dataset (Fig. 1b, c). Subsequent analyses, unless otherwise indicated (Fig. 2a, b), were restricted to tumor-containing TNBC cores from 28 patients (76 cores in total, including 489,129 cells). The clinicopathological characteristics of 28 patients are summarized in Table 1.

Consistent with the known inter-patient heterogeneity of TNBC, cancer cells formed heterogeneous and patient-specific clusters. Immune and stromal cell populations, including B cells, plasma cells, myeloid cells, T cells, and fibroblasts, were annotated by using lineage-specific markers (Fig. 1c). Cancer cells were annotated based on patient-specific clustering, expression of canonical TNBC-associated genes,

and absence of lineage-specific markers (Methods)²³. A small cluster designated “Low_feature_cell” exhibited a markedly low transcript count per cell and was retained as a separate population (Fig. 1b, c).

To elucidate detailed cell subtype distribution in TNBC, each major lineage was re-clustered and annotated into finer subpopulations within each cell type (Supplementary Fig. 1a-e and Supplementary Data S1, Supplementary Table S5). Cell-type representation across individual patients demonstrated that most non-cancer populations were broadly distributed throughout the cohort, whereas cancer cell clusters remained largely patient-specific (Fig. 1b). These annotated cell populations provided a comprehensive landscape of the cellular composition of the TNBC tumor microenvironment.

Table 1
Clinicopathologic characteristics of the treatment-naïve TNBC patients profiled by
imaging-based spatial transcriptomics.

Characteristic	Xenium cohort, N = 28¹
Age at diagnosis, years	48 (45, 52)
Tumor size, cm	2.0 (1.6, 2.5)
Histologic type	
Invasive carcinoma of no special type	26 (93%)
Metaplastic	2 (7%)
Histologic grade	
Grade 2	5 (18%)
Grade 3	23 (82%)
Nuclear grade	
Grade 2	5 (18%)
Grade 3	23 (82%)
Ki-67 index, %	65 (50, 80)
pT category	
T1	15 (54%)
T2	13 (46%)
pN category	
N0	20 (71%)
N1	8 (29%)
AJCC stage	
I	14 (50%)
IIA	7 (25%)
IIB	7 (25%)
Lymphovascular invasion	
Absent	26 (93%)
Present	2 (7%)
² Follow-up is reported as median (range) of time from surgery to death or last contact.	

Characteristic	Xenium cohort, N = 28 ¹
Stromal TILs, %	35 (10, 70)
sTIL group	
Low ($\leq 10\%$)	10 (36%)
Intermediate (11–59%)	7 (25%)
High ($\geq 60\%$)	11 (39%)
Adjuvant chemotherapy	28 (100%)
Regimen	
AC	20 (71%)
AC+paclitaxel	2 (7%)
AC+docetaxel	6 (21%)
Adjuvant radiotherapy	25 (89%)
Follow-up, months ²	187.7 (88.3–189.0)
Any recurrence	3 (11%)
Distant recurrence	1 (4%)
Death from any cause	1 (4%)
¹ Median (Q1, Q3) or n (%) unless otherwise stated.	
² Follow-up is reported as median (range) of time from surgery to death or last contact.	

Spatial niche identification and CTL group classification

Utilizing spatial coordinates and cell type annotations, we identified spatial neighborhoods with Scimap²⁴ and grouped cells with similar neighbor cell compositions into spatial niches. We identified 12 spatial niches well-aligned with tissue morphology (Fig. 2a) and annotated them based on predominant cell type composition (Fig. 2b). To investigate microenvironmental features associated with CTL infiltration, we stratified tumor-containing cores into CTL-high and CTL-low groups based on their CTL proportion, dichotomized at the cohort median (Methods, Supplementary Fig. 3a-d, Supplementary Table S6). Comparison of spatial niche composition revealed distinct differences between CTL groups. The CTL-low group was enriched for tumoral and CAF niches (Fig. 2c), while the CTL-high group showed enrichment of myoepithelial cells and tertiary lymphoid structure (TLS)-like niches (Fig. 2c).

Hypoxia-associated LDHA⁺ PLAUR⁺ macrophages are enriched in CTL-low tumor microenvironments.

We evaluated the differential abundance of cellular subtypes across the CTL groups and analyzed their correlation with CTL abundance. Notably, MC2(LDHA_PLAUR), a distinct macrophage subpopulation, was significantly enriched in the CTL-low group (Fig. 3a) and exhibited a significant negative correlation with CTL proportion in both whole and stromal niches (Fig. 3b).

To further characterize this population, myeloid cells were subclustered into ten subtypes: tissue-resident-like (MC0), inflammatory (MC1), LDHA-high/PLAUR-high (MC2), lipid-associated (MC3), MMP9-high (MC4), low-feature (MC5) macrophages, LAMP3 + DC(DC0), pDC(DC1), cDC1(DC2) and mast cells (Fig. 3c). MC1 and MC4 corresponded to previously described IFN γ -responsive (CXCL9/CXCL10) and MMP9 + macrophage populations, respectively^{17,18,23}. MC2(LDHA_PLAUR) was distinguished by elevated expression of *LDHA*, *PLAUR* and glycolysis- and hypoxia-associated genes (Fig. 3d). To determine whether this macrophage state was reproducible across datasets, we examined an independent TNBC single-cell RNA sequencing dataset from Yan et al.²⁵ and identified a highly concordant macrophage subset, “mac-angio”, characterized by elevated expression of hypoxia and glycolysis-related genes including *LDHA*, *PLAUR*, and *SLC2A1* (Supplementary Fig. 5a, b). In our cohort, MC2(LDHA_PLAUR) cells were present in both tumoral and stromal niches (Fig. 2b), prompting us to examine hypoxia directly across niches (Fig. 2b). To evaluate hypoxia levels across the cores, we calculated a hypoxia signature score for each core using the 91 genes shared between the hypoxia hallmark gene set²⁶ and the Xenium prime 5K panel. Hypoxia signature scores were positively correlated with the abundance of MC2(LDHA_PLAUR) but inversely correlated with the proportion of TC3 (CTL) (Fig. 3e, $r = 0.396$).

Together, these findings identify MC2 (LDHA_PLAUR) as a hypoxia-associated macrophage population that is enriched in CTL-low tumors and closely associated with reduced CTL infiltration.

PLAUR expression identifies a macrophage state associated with low CTL infiltration.

To delineate the primary candidate genes associated with underlying CTL exclusion, we screened for candidates whose expression levels inversely correlated with CTL proportions across all spatial niches. Among the top-nine candidates, *LDHA*, *ADM*, *GBE1*, *P4HA1*, and *PLAUR* were closely linked to cellular adaptation to hypoxia (Supplementary Fig. 6a, b). Under hypoxic stress, these genes synergistically orchestrate metabolic reprogramming (*LDHA*, *GBE1*), extracellular matrix (ECM) remodeling (*P4HA1*, *PLAUR*), and angiogenesis (*ADM*) to sustain cell survival and motility within the TME. Among these, we focused on *PLAUR* due to its established roles in fibroblast activation via the canonical TGF- β signaling axis^{27–29}. *PLAUR* expression in MC2 macrophages correlates negatively with CTL infiltration across samples (Fig. 4a). *PLAUR* expression was significantly elevated in the CTL-low group than CTL-high group (Fig. 4b). Although initially identified within the MC2 macrophage subset, elevated *PLAUR* expression was also observed across multiple macrophage subpopulations (Fig. 3d), suggesting that *PLAUR* represents a broader macrophage activation state. Consistent with its association with hypoxia, *PLAUR*+ macrophages exhibited significant higher hypoxia signature scores (calculated excluding *PLAUR*) than *PLAUR*- macrophages (Fig. 4c). In addition, overall macrophage-derived *PLAUR* expression

was similarly upregulated in CTL-low group (Fig. 4d). To assess the topographical relationship between these cells, we evaluated the spatial distribution of CTLs relative to PLAUR+ macrophages. CTLs were significantly less enriched around PLAUR+ macrophages than around PLAUR- macrophages (Fig. 4e), indicating that *PLAUR* expressing macrophages are preferentially localized within CTL-low regions of the TME. Collectively, these findings suggest that hypoxia-associated *PLAUR* expression, broadly distributed across macrophage subsets rather than restricted to MC2, is spatially associated with local CTL exclusion, positioning PLAUR+ macrophages as key contributors to the immunosuppressive niche characterizing the CTL-low tumor microenvironment.

PLAU – PLAUR signaling between specific fibroblasts and PLAUR+ macrophages in CTL-low TNBC

To characterize the niches in which PLAUR+ macrophages accumulate, we quantified the proportion of PLAUR+ macrophages among total macrophages within each spatial niche at the core level. PLAUR+ macrophages were enriched in tumor-associated niches (Fig. 5a, left), and among MC2(LDHA_PLAUR), the highest PLAUR+ fraction was observed in the CAF(MMP11)_Immune niche (Fig. 5a, right). Neighborhood enrichment analysis further demonstrated that these niches are located adjacent to tumoral niches, suggesting that PLAUR+ macrophages preferentially localize within fibroblast-rich stromal regions bordering the tumor (Supplementary Fig. 7 and Methods). Cell type-level neighborhood enrichment further showed that PLAUR+ macrophages preferentially colocalized with cancer-associated fibroblasts (CAFs), specifically FB5(EGR3_MMP13), FB3(VEGFA_NDRG1), and FB0(MMP11_COL11A1) compared with PLAUR- macrophages (Fig. 5b).

Given this spatial proximity, we next examined which cell types express *PLAU*, the cognate ligand of *PLAUR*, and could therefore directly signal to PLAUR+ macrophages. *PLAU* was predominantly expressed in fibroblasts, with the highest expression in FB0(MMP11_COL11A1), FB4(SFRP4_THBS1) and FB5(EGR3_MMP13), while *PLAUR* expression was highest in MC2(LDHA_PLAUR) among macrophages (Fig. 5c). As FB5(EGR3_MMP13) comprised only 517 cells and was heavily dominated by a single patient (#Pt476) (Supplementary Fig. 2), and FB4(SFRP4_THBS1) showed no preferential co-localization with PLAUR+ macrophages (Fig. 5b), we focused subsequent analyses on FB0(MMP11_COL11A1), which combined high *PLAU* expression with consistent spatial proximity to PLAUR+ macrophages.

To determine whether this spatial co-localization reflects an active *PLAU*–*PLAUR* signaling axis, we first performed ligand-receptor analysis between all FB(FB0–FB5) and MC(MC0–MC5) subclusters using LIANA³⁰, separately for each CTL group. Among interactions enriched in the CTL-low group, *PLAU*(FB0/FB1) – *PLAUR*(MC3/MC2) ranked within the top 25 (Supplementary Fig. 8a). Specifically, *PLAU*–*PLAUR* ranked third among the 25 FB0-MC2 ligand-receptor pairs enriched in the CTL-low group (Supplementary Fig. 8b). To assess this signaling at the individual core level, we computed *PLAU*–*PLAUR* interaction scores for all 36 FB-MC combinations and compared them between CTL groups. Scores from FB0(MMP11_COL11A1) and FB1(POSTN_COL5A1) to MC0, MC1 and MC2 were significantly higher in CTL-low cores, including MC2(LDHA_PLAUR) (Fig. 5d). Consistent with this, representative Xenium images illustrated that *PLAU* transcripts within FB0(MMP11_COL11A1) fibroblasts were frequently found

in close spatial proximity to *PLAUR* transcripts within MC2(LDHA_PLAUR) macrophages, providing spatial support for local ligand–receptor engagement (Fig. 5e).

Collectively, these results identify FB0(MMP11_COL11A1) as a spatially proximal and a candidate source of *PLAU* for PLAUR+ macrophages and suggest that the *PLAU-PLAUR* signaling axis is preferentially associated with CTL-low tumors.

PLAUR+ macrophages are associated with TGF-β pathway activation

Having identified enhanced *PLAU*(FB0)–*PLAUR*(MC) signaling in CTL-low tumors, we next investigated whether spatial niches and cell types exhibited evidence of downstream pathway activation. PLAUR+ macrophage-enriched niches, including CAF(MMP11)_Immune, Tumor_Center, Inner_Interface, and Outer_Interface, displayed significantly higher hypoxia (median 0.014 vs. 0.011; Mann–Whitney U test, $p = 7.01 \times 10^{-4}$) and TGF-β signaling (median 0.016 vs. 0.013; $p = 1.27 \times 10^{-4}$) scores than other spatial niches. (Fig. 6a). Similarly, the fibroblast subsets that preferentially co-localized with PLAUR+ macrophages, FB0, FB3, and FB5, also exhibited elevated hypoxia (median 0.023 vs. 0.010; Mann–Whitney U test, $p = 3.00 \times 10^{-30}$) and TGF-β signaling (median 0.032 vs. 0.009; $p = 5.70 \times 10^{-32}$) scores relative to other cell types. (Fig. 6b), consistent with *PLAU-PLAUR* engagement occurring within a hypoxic and TGF-β-activated microenvironment^{19,21,31}.

Given that TGF-β downstream signaling is a principal driver of myofibroblast differentiation and CAF activation^{27,29,32}, we examined whether *PLAUR* expression in macrophages was associated with TGF-β pathway activity in the neighboring stromal cells. Indeed, per-core *PLAUR* expression in macrophages was positively correlated with the TGF-β signaling score of neighboring cells (Spearman $r = 0.388$, $p = 5.271 \times 10^{-4}$, permutation $p = 3.992 \times 10^{-3}$, $n = 76$ cores) (Fig. 6c), supporting a model in which *PLAU*-driven activation of PLAUR+ macrophages contribute to local TGF-β-driven fibroblast activation.

To validate this relationship at the protein level, we performed mIF on the discovery cohort (Xenium 5K) and an expanded cohort of 134 treatment-naïve TNBC patients, which included 27 of the patients profiled by Xenium. We assessed the spatial localization of CD8⁺ T cells and α-SMA⁺ CAFs relative to CD68⁺ uPAR⁺ macrophages, and quantified nuclear pSMAD2 as a readout of TGF-β pathway activation (Fig. 6d). CD8⁺ T cell enrichment was reduced around uPAR⁺ macrophages compared to uPAR[−] macrophages ($n = 107$ evaluable patients; Fig. 6e), while CAFs surrounding uPAR⁺ macrophages exhibited higher nuclear pSMAD2 positivity ($n = 107$; Fig. 6f). To confirm that the findings were not driven by the discovery samples, the analyses were repeated in the independent subset of patients not profiled by Xenium ($n = 83$ and $n = 87$, respectively; Supplementary Fig. 10a, b), with concordant results. These findings indicate that the uPAR⁺ macrophage niche is characterized by concurrent CD8⁺ T cell exclusion and enhanced CAF activation.

We next examined how the CAF–CD8 T cell relationship around uPAR⁺ macrophages relate to conventional sTIL and prognosis. Across patients, a higher fraction of uPAR⁺ macrophages that were CAF-adjacent and CD8 T cell-deficient correlated with lower sTIL (%) (Supplementary Fig. 11a). This allowed us to define a patient subgroup enriched for such CAF-adjacent, CD8-deficient uPAR⁺ macrophages, which showed a significantly higher fraction of CAF-only-adjacent uPAR⁺ macrophages, lower sTILs (median 20% vs 40%, $p = 0.044$), larger tumors (higher pT/stage), and a markedly higher frequency of lymphovascular invasion (44% vs 9%, $p < 0.0001$) (Supplementary Fig. 11b,c, and Supplementary Table S2). Kaplan–Meier analysis showed shorter overall survival in C2 than in the combined C1/C3 subgroups (log rank $p = 0.032$; Supplementary Fig. 11d), with 11 deaths among the 118 patients analyzed (4 of 25 in C2 and 7 of 93 in C1/C3). Recurrence-free survival did not differ significantly (14 events; log-rank $p = 0.11$; Supplementary Fig. 11e). Unadjusted hazard ratios were 3.39 (95% CI 1.03–11.10) for overall survival and 2.41 (95% CI 0.80–7.29) for recurrence-free survival (Supplementary Table S3). Because only 11 deaths occurred, a model containing all clinicopathologic covariates simultaneously was not estimable; covariates were therefore added one at a time. Adjustment for pT category attenuated the association only partially (HR 2.56, 95% CI 0.76–8.62; Supplementary Table S4), whereas adjustment for lymphovascular invasion abolished it (HR 1.23, 95% CI 0.33–4.60; Supplementary Table S4). Lymphovascular invasion itself remained strongly prognostic after adjustment for C2 (HR 10.20, 95% CI 2.59–40.13, $p < 0.001$; Supplementary Table S4).

Additionally, we assessed the relationship between neoadjuvant therapy response and *PLAUR* in myeloid cell types from an independent dataset of Yan et al.²⁵. Myeloid cells overall expressed significantly higher *PLAUR* in the residual disease (RD) group compared to the pathological complete response (pCR) group ($p = 1.34 \times 10^{-31}$). Similarly, more modest trend was observed within the mac_angio macrophage subtype which resembles MC2 in our in-house data specifically ($p = 0.0213$) (Supplementary Fig. 5c, d).

Additionally, *PLAUR* expression in myeloid cells was positively correlated with TGF- β downstream signaling score in CAFs ($p = 0.440$, $p = 0.031$; Supplementary Fig. 5e), whereas it was weakly negatively correlated with T cell proportion ($\rho = -0.249$, $p = 0.013$; Supplementary Fig. 5f) in the Yan et al.²⁵ cohort.

Taken together, these results suggest *PLAU*(FB0)–*PLAUR*(MC) signaling as a candidate axis linking hypoxic niches and TGF- β -driven CAF activation and immune remodeling in the tumor microenvironment.

DISCUSSION

The importance of immune cell abundance in TNBC has long been emphasized. Among these cells, CD8⁺ T cells are known to be associated with post-chemotherapy responses and postoperative prognosis. However, many aspects of how the TME regulates the infiltration and function of anti-tumor immune cells remain to be explored. Utilizing in situ spatial transcriptomics, we characterized cellular heterogeneity within the TNBC TME at single-cell resolution and identified distinct cellular and spatial

features associated with CTL abundance. The CTL-low group was enriched for hypoxia-associated cell types, specifically FB3(VEGFA_NDRG1) and MC2(LDHA_PLAUR).

In addition, we elucidated 12 spatial niches that closely corresponded to tissue morphology. Notably, reflecting its association with hypoxia, MC2 was enriched in the Tumor Center niche compared to other macrophages. The CTL-low group contained a higher abundance of Immune niches enriched for FB0(MMP11_COL11A1). FB0 fibroblasts have been associated with high levels of MMP11 to facilitate tumor invasion, suggesting that the enrichment of this fibroblast population may contribute to the expansion of tumoral niches in the CTL-low group.

Among the genes expressed by MC2 and associated with low CTL infiltration, we focused on PLAUR, which encodes the urokinase-type plasminogen activator receptor (uPAR). PLAUR/uPAR expression can be induced by hypoxia through HIF-1-dependent transcriptional activation and by TGF- β through TGF- β receptor–JNK/AP-1 signaling^{19,33–36}. In addition to being a transcriptional target of TGF- β in some cellular contexts, uPAR binds *PLAU*-encoded urokinase-type plasminogen activator (uPA), thereby localizing plasmin generation at the cell surface^{21,22,31,37}. The resulting plasmin can activate latent TGF- β . TGF- β signaling has previously been associated with low stromal TIL environment, and our study further demonstrates its association with the CTL-low TME.

We also observed increased interaction between *PLAU* expressed by the FB0 subtype and *PLAUR* expressed by macrophages. These findings suggest a possible model in which PLAUR+ macrophages bind to *PLAU* expressed by surrounding fibroblasts and contributes to local TGF- β activation. Increased TGF- β signaling may promote adjacent fibroblasts toward an activated myofibroblast phenotype, which may create a physical barrier through extracellular matrix remodeling and create a desmoplastic TME that impairs CTL infiltration^{28,38–40}. Furthermore, enhanced TGF- β signaling suggests that the cytotoxic function in surrounding CD8 + T cells may also be suppressed^{41,42}.

In an external single-cell cohort, *PLAUR* expression in myeloid cells was higher in tumors with residual disease than in those achieving pathological complete response after neoadjuvant chemotherapy, linking this macrophage program to treatment resistance (Supplementary Fig. 5c,d). Consistent with this finding, the clinical relevance of the PLAUR+ macrophage program was also reflected in the tissue architecture of our cohort. Because immune exclusion was spatially heterogeneous, with CTL-high and CTL-low regions frequently co-occurring within the same tumor, immune exclusion is best understood as a locally organized process rather than a uniform tumor-wide phenotype. We therefore characterized patients by the CSR-normalized spatial enrichment of nuclear-pSMAD2+ CD8+ T cells and of α SMA+ myCAFs around uPAR+ macrophages, and unsupervised clustering delineated an immune-excluded subgroup (C2) defined by low CD8 and high myCAF enrichment. A greater burden of this uPAR+ macrophage–associated exclusion program was associated with more aggressive features, most notably lymphovascular invasion and higher T category.

This clinical association was concentrated in lymphovascular invasion. Adjustment for lymphovascular invasion returned the hazard ratio for C2 essentially to unity, whereas adjustment for pT category did not. This may reflect simple confounding, since C2 tumors were also larger and of higher T category. Alternatively, lymphovascular invasion may lie downstream of immune exclusion. uPA and uPAR constitute a pericellular proteolytic system whose canonical role is matrix degradation during invasion and dissemination⁴³, and uPA together with PAI-1 is among the best-validated prognostic biomarkers in node-negative breast cancer, having reached level-1 evidence^{44,45}. A niche that concurrently remodels the matrix and restricts cytotoxic T cell access would therefore be expected to favor vascular invasion, in which case adjustment for lymphovascular invasion could remove part of the biological association rather than simply account for confounding. These possibilities cannot be separated with only 11 deaths, and a larger cohort with sufficient events for formal mediation analysis will be required.

These findings align with emerging uPAR-targeted therapeutic approaches in TNBC. Previous work showed that uPAR CAR T cells can eliminate both tumor cells and fibrotic stroma, clearing metastases in preclinical models⁴⁶, highlighting the therapeutic potential of targeting the broad uPAR-associated TME. Our findings extend this concept by demonstrating that PLAUR⁺ macrophages are spatially associated with FB0, FB3, and FB5 fibroblasts in a TGF- β -activated and CTL-excluding state. This spatial relationship suggests that targeting both uPAR⁺ macrophages and the associated fibrotic compartments may provide a potential strategy to overcome CTL exclusion in TNBC.

A key limitation of this study is the lack of functional validation regarding the *PLAU-PLAUR* interaction in latent TGF- β activation. Additionally, the use 0.6-mm tissue cores may not fully capture intratumoral spatial heterogeneity. We sought to reduce this limitation by analyzing three cores per patient, and core-level analysis revealed a consistent negative correlation between CTLs and PLAUR⁺ macrophages. Lastly, the targeted probe set of the Xenium 5K panel limited transcriptome-wide characterization and contributed to a population of cells with insufficient features for detailed annotation.

Nevertheless, our study provides a detailed spatial characterization of the TNBC microenvironment and identifies hypoxia-associated PLAUR⁺ macrophages as a potential contributor to fibroblast remodeling and CTL exclusion.

METHODS

Study design and patients

Breast cancer specimens from treatment-naïve patients with early-stage (stage I or II) TNBC who underwent surgery at Asan Medical Center between 2008 and 2010 were collected. No patient received neoadjuvant chemotherapy. Clinical and pathological data, including age, tumor size, histologic and nuclear grade, T and N category, clinical stage, lymphovascular invasion, stromal tumor-infiltrating lymphocytes, adjuvant treatment, recurrence and survival at diagnosis, were retrieved from electronic medical records.

The two profiling platforms were applied sequentially and with distinct purposes. Imaging-based spatial transcriptomics was performed first and was used to generate the hypothesis that a PLAU-high hypoxic macrophage state contributes to immune exclusion which was then tested by multiple IF on deeper sections of the same tissue microarray series. Analyses of the two platforms are therefore reported as discovery and validation (Supplementary Fig. 9a), respectively.

Tissue micro array construction

Tissue microarrays (TMAs) had been constructed from formalin-fixed, paraffin-embedded tumor blocks of this cohort. For each case, a representative area of invasive carcinoma was marked on a hematoxylin and eosin (H&E)-stained slide by three breast pathologists (B.K.J., H.J.L. and G.G.), and three 0.6-mm-diameter cores were punched from the corresponding donor block and transferred into a recipient paraffin block using an automated tissue arrayer. The three cores of a given case were placed at consecutive positions within a single row of the array, and all cores from a given case were placed within a single recipient block, so that every core could be assigned unambiguously to its case of origin and no patient was represented on more than one block. Five blocks (designated TNBC 22 to TNBC 26) were prepared, each comprising 10 or 11 rows of 12 core positions and accommodating 40 to 44 cases, for a total of 194 arrayed cases. Serial 4- μ m sections were cut from each block; the first section of each set was stained with H&E for quality control and subsequent sections were used for spatial transcriptomic and multiplex immunofluorescence (mIF) analysis.

Histologic analysis and case eligibility

Because the arrays had been assembled as part of an institutional breast cancer TMA series, eligibility for the present study was verified by central pathologic review after array construction. Review was performed by three breast pathologists (B.K.J., H.J.L., and G.G.) on H&E-stained sections together with the original receptor immunohistochemistry. TNBC was defined as a tumor negative for estrogen receptor and progesterone receptor with a HER2 immunohistochemical score of 0 or 1+, or a score of 2+ without gene amplification on in situ hybridization, in accordance with the ASCO/CAP guidelines⁴⁷. Histologic type and grade were assigned according to the fifth edition of the WHO Classification of Breast Tumours⁴⁸; all eligible tumors were invasive breast carcinoma of no special type.

Stromal tumor-infiltrating lymphocyte (sTIL) percentage was quantified as the area of TILs relative to the stromal area on whole-slide H&E images by pathological review. The assessment of sTIL was measured by pathologists using the recommendations by an international TILs working group 2014⁴⁹.

Of the 194 arrayed cases, 47 (118 cores) were excluded because central review showed that the receptor profile did not fulfil the definition of TNBC, or that the arrayed cores contained no evaluable tumor tissue. As these cases fell outside the predefined cohort, clinical data was not retrieved for them under the minimal-data principle of the institutional review board approval, and they are therefore not characterized further. Review and exclusion were based solely on morphology and receptor status and were performed

before any staining or image quantification and without knowledge of clinical outcome. The remaining 147 patients contributed 422 cores.

Each core was annotated for tissue content as invasive carcinoma, carcinoma in situ or non-neoplastic breast tissue. Quantitative analyses were restricted to cores containing invasive carcinoma; cores composed exclusively of carcinoma in situ or non-neoplastic tissue were not analyzed, leaving 358 cores from 141 patients.

Xenium in situ RNA experiments

TMA sections were sectioned at 4 μ m and were placed within the Sample Area of the Xenium slides (10X Genomics, PN3000941) by the pathology laboratory (Superbiochips, South Korea). Deparaffinization and decrosslinking were conducted following the manufacturers' guides (CG000580, Rev E). Subsequently, the TME slides were continued according to the Xenium Prime In Situ Gene Expression with Cell Segmentation Staining guide (CG000760, Rev C). Post-Xenium H&E staining was performed by conventional methods in pathology laboratory (Core Research Facility of Seoul National University, South Korea).

Data processing and Batch Integration

For quality control and pre-processing of individual Xenium slides, we utilized the Scanpy (v1.9.8) framework. Cells with fewer than 10 total counts and genes detected in fewer than five cells were excluded, yielding 499,655 cells across 82 cores from 28 patients after QC. The raw expression values were preserved in the counts layer before normalization. Individual cellular expression values were normalized to total counts, log-transformed, and scaled with a maximum value threshold of 10 to establish a stable reference in `adata.raw`. Processed datasets from individual Xenium slides were concatenated using an inner join and integrated using single-cell Variational Inference (scVI). The scVI model was trained on the raw counts layer with the slide identifier as the batch covariate, configured with two layers, a 30-dimensional latent space, and a negative binomial gene likelihood (`gene_likelihood="nb"`). The resulting latent representation (`X_scVI`) was used to construct a nearest-neighbor graph, followed by UMAP visualization and Leiden clustering (resolution = 0.4). Except for cell type annotation and niche identification, downstream analyses were restricted to tumor-containing cores ($n = 76$) from 28 patients.

Cell type annotation

Cells were first classified into cancer and non-cancer populations. Cancer cells were identified based on the following criteria: (1) clusters exhibited high inter-patient heterogeneity, consistent with the well-documented patient-specific heterogeneity of cancer cells, such that clusters predicted to be cancer cells were largely unique to individual patients; (2) clusters expressed conventional TNBC cancer-associated genes, such as *EPCAM*, *CCT3*, *TP53*, *MKI67*, and *MYC*; and (3) clusters lacked expression of marker genes specific to other cell types, including *CD2* (T cells), *CD68* (macrophages), *MS4A1* (B cells),

MS4A2 (mast cells), *CD38* (plasma cells), *LAMP3* (*LAMP3*⁺ DCs), *ADIPOQ* (adipocytes), *DST* (myoepithelial cells), *POSTN* (fibroblasts), and *PECAM1* (endothelial cells). The remaining non-cancer cells were re-clustered and annotated into major cell types, and broad lineages, including myeloid cells, T cells, fibroblasts and vascular cells, were further sub-clustered using the Leiden algorithm on the scVI latent space. At each level, cluster-specific marker genes were identified by differential expression analysis using *sc.tl.rank_genes_groups* (Scanpy; method = "wilcoxon", one-versus-rest), and clusters were manually annotated by comparing the top-ranked markers with canonical cell-type markers and previously reported TNBC single-cell signatures^{23,25}. This yielded two annotation levels: major cell types (e.g., fibroblasts, macrophages) and subclusters, which were named by lineage and their most distinctive marker genes (e.g., MC2(LDHA_PLAUR)) (Supplementary Fig. 1a–e). Unless otherwise stated, all analyses used subcluster-level annotations.

PLAUR + macrophage definition

Within the macrophage subtype (MC) population, cells with at least one detected PLAUR transcript in the raw count matrix were classified as PLAUR⁺, and the remaining MC cells as PLAUR[−].

Spatial niche identification

Spatial niches were identified using the *spatial_count* function in the Scimap package (v2.3.5), which defines the cellular neighborhood of each cell based on the composition of neighboring cells within a 50 μ m radius. Cells with similar neighborhood compositions were subsequently grouped using the *spatial_cluster* function (k-means clustering, *k* = 12), with each resulting cluster representing a distinct spatial niche. The number of clusters (*k* = 12) was chosen empirically, as this value best aligned with tissue morphology and provided clear discrimination of tumoral and stromal niches.

CTL group classification

For each tumor-containing core, the proportion of cytotoxic T lymphocytes (TC3(CTL), subclusters) among all cells (whole-niche, no compartment restriction) was calculated. Cores were then dichotomized into CTL-high and CTL-low groups using the cohort median proportion (0.0045) as the classification threshold: cores with a CTL proportion $\geq$ median were assigned to the CTL-high group, and those below the median to the CTL-low group. This yielded 38 CTL-high and 38 CTL-low cores (*n* = 76 tumor-containing cores). Within-patient consistency of core-level TC3(CTL) proportion was quantified using the intraclass correlation coefficient (ICC(1)) from a random-intercept mixed model, restricted to patients with ≥ 2 evaluable cores. 95% confidence intervals were estimated by patient-level bootstrap resampling (*n* = 1,000), and a one-way ANOVA (patient as factor) was performed as a complementary test (Supplementary Fig. 4a). Within-patient variability was further summarized as the standard deviation of core-level CTL proportion per patient (Supplementary Fig. 4b).

Milo differential abundance test

To examine differential abundance of cell types between CTL-low and CTL-high groups, we implemented Milo (Dann et al., 2022) using the *milo* module in *pertpy* package (v0.10.0). First, a k-nearest neighbor

graph was constructed using the `sc.pp.neighbors` function with `n_neighbors = 30`. Second, neighborhoods were defined using the `milo.make_nhoods` function with `prop = 0.1`. Differential abundance testing was performed using the `milo.da_nhoods` function with `edgeR` as the statistical testing method. Neighborhoods were visualized based on the following criteria: spatial FDR < 0.05, fraction of significant neighborhoods > 0.3, minimum number of patients ≥ 5 , and minimum cell count per patient ≥ 50 .

Neighborhood enrichment analysis

Spatial neighbor graphs were constructed independently for each core (`coord_type = "generic"`, `radius = 50` μm) using `sq.gr.spatial_neighbors`, and pairwise niche-level or cell-level neighborhood enrichment z-scores were computed via permutation testing (1,000 permutations) using `sq.gr.nhood_enrichment` in `Squidpy` (v1.2.2).

Gene set scoring

Hypoxia and TGF- β downstream signaling activity were quantified at the single-cell level using `sc.tl.score_genes` (Scanpy) with gene signatures derived from the MSigDB Hallmark collection (HALLMARK_HYPOXIA and HALLMARK_TGF_BETA_SIGNALING, respectively), restricted to genes present in the Xenium panel. Of the 200 genes comprising HALLMARK_HYPOXIA, 91 were present in the panel and used for scoring; of the 54 genes comprising HALLMARK_TGF_BETA_SIGNALING, 36 were present and used.

Ligand-Receptor Interaction Analysis

Cell-cell communication was inferred using LIANA+ (v1.6.1) in Python. Within tumor-containing cores, cells were grouped by cell type annotation (subclusters), and the consensus rank-aggregation method (`li.mt.rank_aggregate`; `resource_name = "consensus"`, `expr_prop = 0.1`) was applied separately to CTL-high and CTL-low cores to obtain a magnitude rank for each ligand-receptor pair across all source-target cell type combinations. The magnitude rank is a consensus score aggregated across multiple scoring methods, ranging from 0 to 1, with lower values indicating stronger predicted interactions. Interactions were defined as enriched in the CTL-low group when they had a lower magnitude rank in CTL-low than in CTL-high cores. For all fibroblast (FB0-FB5) $\rightarrow$ macrophage (MC0-MC5) source-target pairs combined, CTL-low-enriched interactions were ranked by the difference in magnitude rank between groups (CTL-high – CTL-low), and the top 25 were visualized (Supplementary Fig. 8a). For FB0(MMP11_COL11A1) $\rightarrow$ MC2(LDHA_PLAUR), all 25 CTL-low-enriched interactions were visualized, ranked by the same rank difference (Supplementary Fig. 8b). To quantify *PLAU-PLAUR* signaling strength at the individual core level across all 36 fibroblast-macrophage subtype combinations (FB0-FB5 $\times$ MC0-MC5), we computed, independently of the LIANA statistical framework, a per-core ligand-receptor interaction score directly from raw expression values, defined as the product of mean *PLAU* expression in the source FB subtype and mean *PLAUR* expression in the target MC subtype, restricted to cores containing both subtypes. Core-level scores were compared between CTL-high and CTL-low cores using two-sided Mann-Whitney U tests, and p-values across all 36 combinations were adjusted for multiple comparisons using the

Benjamini-Hochberg (BH) procedure (q values < 0.05 considered statistically significant). Only the six FB-MC combinations reaching this significance threshold are shown (Fig. 5d).

Spatial CTL enrichment around PLAUR+ vs PLAUR-macrophages

Macrophages were classified as PLAUR+/PLAUR- based on *PLAUR* transcript count (> 0 vs. 0), and a BallTree was used to count TC3(CTL) cells and total cells within a 50 μm radius of each macrophage. Within each core, CTL and total neighbor counts were pooled across macrophages in each PLAUR group (rather than averaging per-macrophage proportions) to compute a local CTL proportion. This pooled proportion, which gives proportionally more weight to macrophages with larger neighbor pools, was then compared to the core's overall CTL proportion (expected baseline) via log 2 ratio ($\log_2[(\text{pooled proportion} + \text{pseudocount}) / (\text{baseline proportion} + \text{pseudocount})]$, pseudocount = 1×10^{-3}), yielding one enrichment value per core per group. Cores served as the statistical unit, and PLAUR+ vs. PLAUR-enrichment was compared using a paired Wilcoxon signed-rank test (within-core pairing).

scRNA-seq data analysis

scRNA-seq data from Yan *et al.*²⁵ were retrieved in AnnData format from the CELLxGENE collection (<https://cellxgene.cziscience.com/collections/0a117356-ecaa-4b82-a454-c15a4c9ec507>). The name of dataset is "Single-cell RNA-seq data from untreated tissue samples of treatment-naive triple-negative breast cancer patients". The pre-processed and pre-annotated AnnData object, including quality control, normalization, dimensionality reduction, clustering, and cell type annotations as provided by the original authors, was used without further re-clustering or re-annotation. Donor-level mean *PLAUR* expression was calculated across myeloid ("Mye", *author_cell_type*) cells, excluding donors with < 5 myeloid cells; T cell proportion was calculated as the fraction of T cells ("T", *author_cell_type*) among all cells per donor. TGF- β signaling activity was scored per cell using scanpy.tl.score_genes with the 54-genes comprising HALLMARK_TGF_BETA_SIGNALING set (*tgfb_score*), and donor-level CAF TGF- β score was calculated as the mean *tgfb_score* across CAF (*cell_state*) cells.

Opal multiplex immunofluorescence

Staining, scanning and image analysis were performed by prismCDX (Gyeonggi-do, Korea). Sections 4 μm thick were cut from the recipient blocks, baked at 60°C for at least one hour, and stained on a Leica Bond Rx automated stainer (Leica Biosystems). After dewaxing, epitope retrieval was performed with Bond Epitope Retrieval 2 for 30 minutes. Six markers were then detected in sequential rounds, each comprising protein block, primary antibody, HRP-conjugated secondary antibody (TheraNovis) and tyramide-based signal amplification with an Astra-TSA fluorophore (TheraNovis), followed by a 20-minute incubation in Bond Epitope Retrieval 1 to strip bound antibodies before the next round. Markers were applied in the order CD8, α -smooth muscle actin (αSMA), phosphorylated Smad2 (pSmad2), urokinase plasminogen activator receptor (uPAR), CD68 and pan-cytokeratin. Nuclei were counterstained with DAPI after the final retrieval step and slides were mounted with ProLong Gold antifade reagent

(Invitrogen). Antibody sources, dilutions and fluorophore assignments are listed in Supplementary Table S1.

Slides were scanned at 20× magnification on a Phenolmager HT (Akoya Biosciences). Representative fields selected in Phenochart (v2.2.0) were used to train a single inForm algorithm (v3.1, Akoya Biosciences), which was then applied to all cores in batch. Multispectral images were unmixed against a spectral library, tissue was segmented into cytokeratin-positive (CK⁺) and cytokeratin-negative (CK⁻) compartments, and individual cells were segmented on the DAPI channel and phenotyped according to the subcellular compartment and intensity of each marker, allowing nuclear and cytoplasmic pSmad2 to be scored as separate phenotypes. Per-core cell counts, densities, tissue areas and proximity statistics were consolidated using the phenoptr and phenoptrReports packages in R (v4.2.1) and exported for downstream analysis.

The following phenotypes were defined: uPAR-positive and uPAR-negative macrophages (CD68⁺ CK⁻, uPAR⁺ or uPAR⁻); active myCAF (αSMA⁺ with nuclear pSmad2) and latent myCAF (αSMA⁺ with cytoplasmic pSmad2); active and latent CD8 + T cells (CD8⁺ with nuclear or cytoplasmic pSmad2, respectively); and uPAR-positive tumor cells (CK⁺ uPAR⁺). Nuclear pSmad2 localization was taken to indicate the TGF-β-activated form.

Core-level quality control after multiplex immunofluorescence

Stained sections were reviewed before quantification and cores were excluded if they showed tissue folding, detachment, or displacement resulting in overlap with an adjacent core, since such cores cannot be assigned unambiguously to a single case. After this quality control step, 299 cores from 134 patients were available for analysis; one core was analyzed in 35 patients, two in 33 and three in 66. The flow of cases from array construction to the final analytic cohort is summarized in Supplementary Fig. S9.

Spatial quantification of multiplex immunofluorescence result

Pairwise cell-to-cell proximity was quantified from the inForm Count Within output, which reports, for an index (From) phenotype and a target (To) phenotype at a given radius, the total number of index cells and the number of index cells having at least one target cell within that radius. A radius of 50 μm was used for all analyses, and the whole-core compartment was used unless otherwise stated.

Proximity was corrected for target-cell abundance by referencing the observed value to that expected under complete spatial randomness. The observed contact fraction was calculated as the number of index cells with at least one target cell within r divided by the total number of index cells. The expected fraction was calculated as $1 - \exp(-\lambda\pi r^2)$, where λ is the density of the target phenotype in the same

compartment. The enrichment score was defined as the ratio of observed to expected fractions, such that values above 1 indicate spatial attraction and values below 1 indicate spatial exclusion.

The TGF- β activation state of cells neighboring a given macrophage population was expressed as the fraction of neighbor-bearing index cells whose target was in the activated (nuclear pSmad2) rather than the latent (cytoplasmic pSmad2) form, calculated as $A/(A + L)$, where A and L are the numbers of index cells bearing an active or latent target within r. Counts were pooled across a patient's cores before the ratio was formed. Patients in whom neither macrophage population had any myCAF within r ($A + L = 0$) have an undefined ratio and were excluded, leaving 107 of 134 patients evaluable for this comparison. Core background values were computed from total cell counts in the same patients using the same expression.

Because a patient may contribute up to three cores, which are not statistically independent, all metrics were aggregated to the patient level before analysis. Aggregation was performed by pooling constituent counts across a patient's qualifying cores rather than by averaging core-level ratios, so that each core contributes in proportion to the number of cells it contains. For proximity enrichment, the observed and expected numbers of index cells bearing a target within r were summed separately across core, and the enrichment score was defined as the ratio of pooled observed to pooled expected counts. Patient identity was defined by pathology accession number.

Statistics

Unless otherwise stated, Xenium analyses were performed at the core level, because spatial niches are defined within individual cores and are not preserved by patient-level averaging. Multiplex immunofluorescence metrics are not niche-resolved and were therefore aggregated to the patient level before analysis. Nonparametric Mann-Whitney U tests were used for two-group comparisons at the core level (e.g., TC3(CTL) proportion between CTL-high and CTL-low groups), and paired Wilcoxon signed-rank tests were used for paired comparisons within individual cores (e.g., local CTL infiltration around PLAUR+ versus PLAUR- macrophages). Spearman's correlation was used to assess associations between continuous variables, including cell type proportions, hypoxia score, and *PLAUR* expression relative to TC3(CTL) proportion; resulting p-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR), with q values < 0.05 considered significant. All statistical analyses were performed in Python (v3.10.19) using Scanpy (v1.9.8), pertpy (v0.10.0), Squidpy (v1.2.2), LIANA+ (v1.6.1), SciPy (v1.15.3) and scikit-learn (v1.7.2), and in R (v4.2.2) using tidyverse (v2.0.0), survival(v3.8.6) and survminer (v0.5.1).

Study approval

This study received approval from the Institutional Review Board of Asan Medical Center (IRB No. 2025 – 1209) and adhered to the principles of the Declaration of Helsinki of 1975, as revised in 1983. This study waived the requirement for informed consent given the retrospective analysis of archival tissue and de-identified clinical data.

DATA AVAILABILITY

Xenium data from TNBC tissues including tumor regions have been uploaded into the Gene Expression Omnibus Repository (GEO) under accession number GSE347131.

Abbreviations

CTL

Cytotoxic T-lymphocyte

TNBC

triple-negative breast cancer

TME

tumor microenvironment

CAF

cancer-associated fibroblast

Stromal tumor-infiltrating lymphocyte

sTIL

Declarations

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COMPETING INTERESTS

All authors declare no financial or non-financial competing interests.

Author Contribution

S.C. and B.K.J. conceptualized the study, designed and performed the experiments, and analyzed the data. S.C., H.C., and B.K.J. wrote the original draft of the manuscript. H.C., B.K.J., S.J., and H.J.K. reviewed and edited the manuscript. H.C. and S.J. provided helpful discussions and suggestions. H.J.L.,

B.K.J., and G.G. provided patient data and resources. G.G. participated in tissue microarray block construction. M.O. assisted in writing and validating the code for analysis. H.J.K. provided computational resources for analysis. B.K.J. and H.J.K. supervised the data analysis and overall project. All authors have read and agreed to the published version of the manuscript.

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Data Availability

Xenium data from TNBC tissues including tumor regions have been uploaded into the Gene Expression Omnibus Repository (GEO) under accession number GSE347131.

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Figures

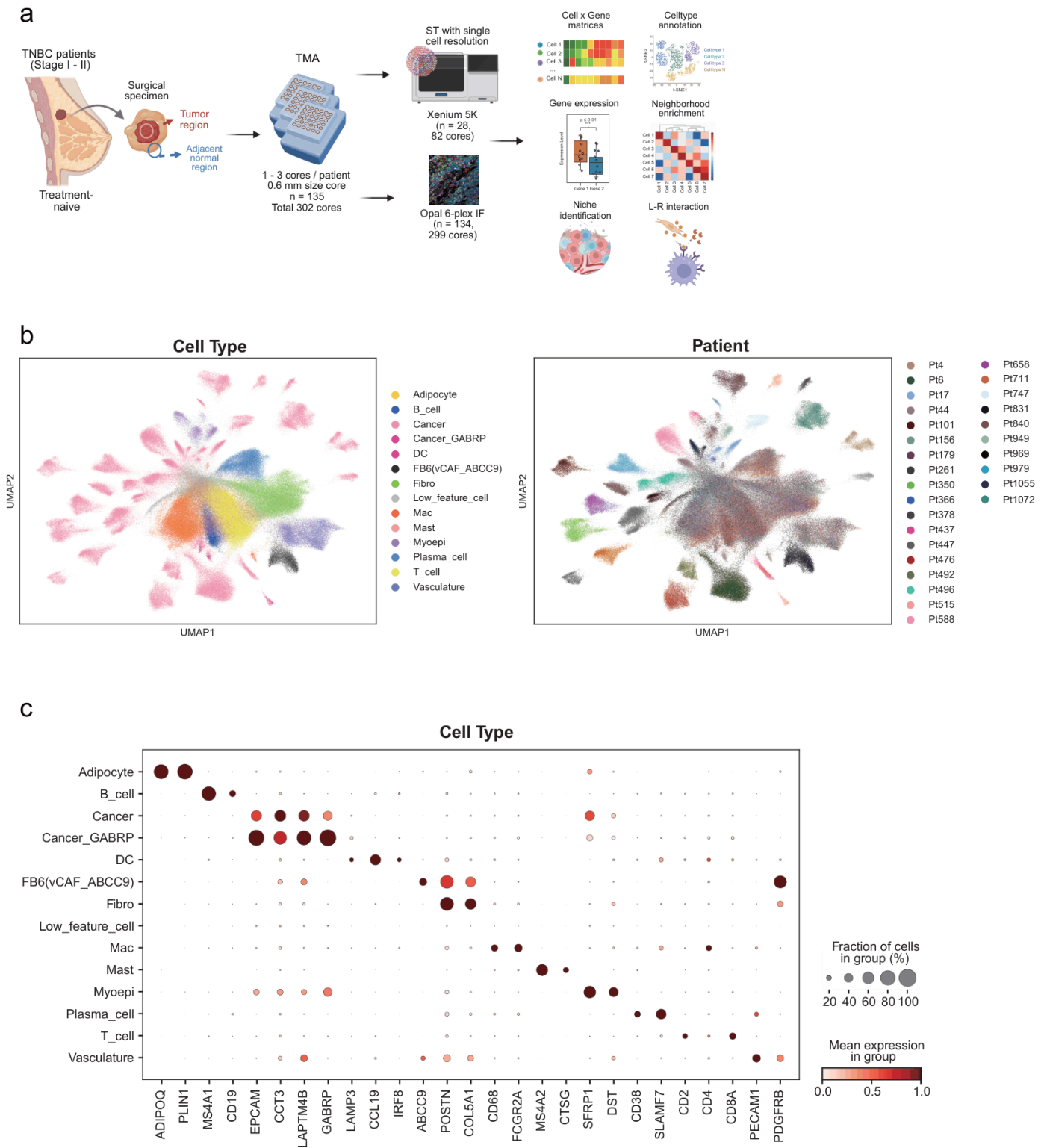


Figure 1

Spatially resolved single-cell landscape of the TNBC tumor microenvironment.

(a) Experimental scheme for spatial transcriptomic profiling of patients with TNBC

(b) Uniform manifold approximation and projection (UMAP) of the integrated Xenium 5K data from 499,655 cells colored by global cell types (left) and patients (right).

(c) Dot plot showing canonical marker gene expression across global cell types.

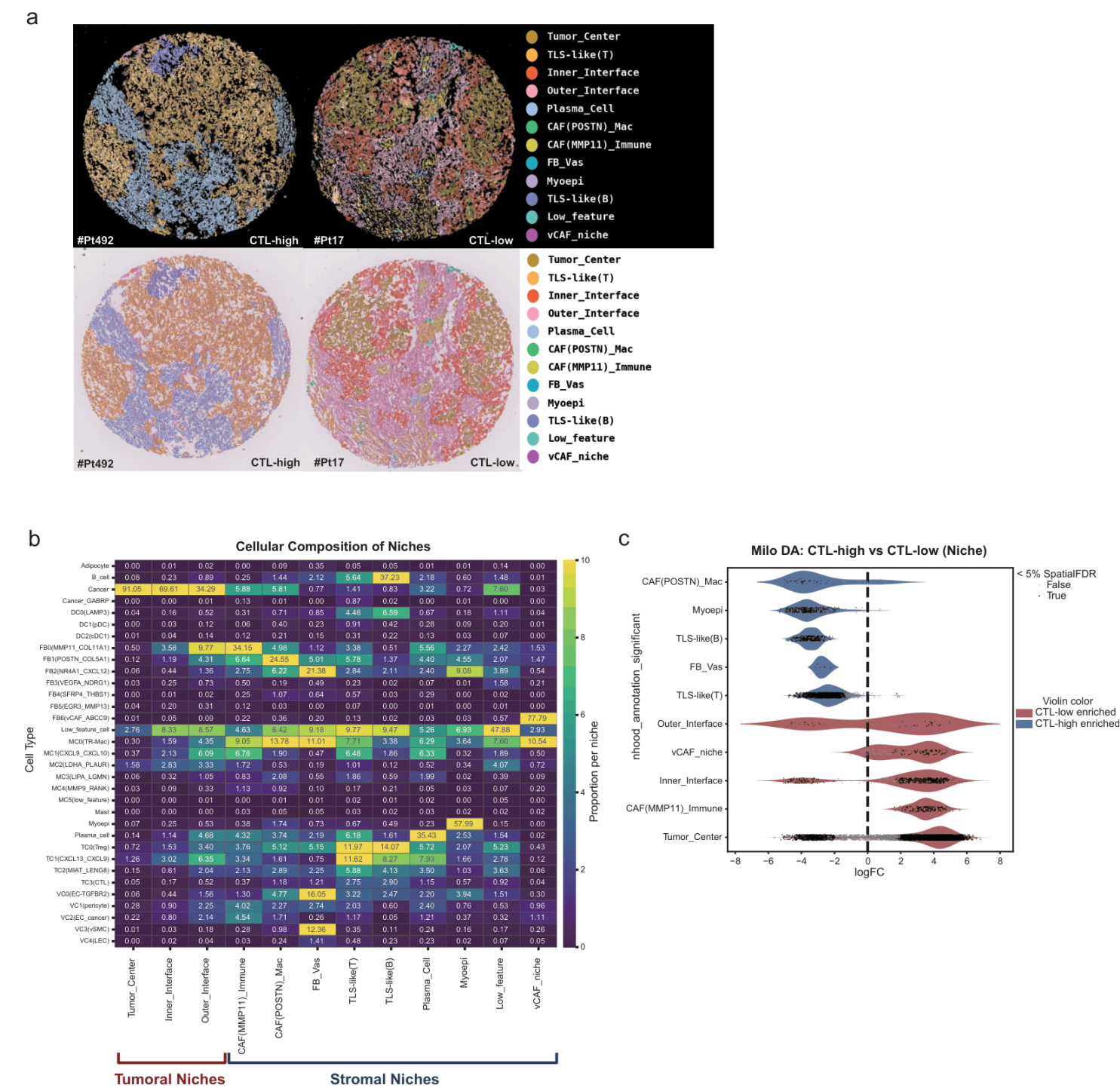


Figure 2

Spatial niche identification and differential niche abundance between CTL groups

(a) Representative spatial niche maps (top) and corresponding H&E images (bottom) from CTL-high (#Pt492, left) and CTL-low (#Pt17, right) cores, demonstrating that niches identified by spatial clustering (Scimap) align with tissue morphology. Niche colors correspond to the legend on the right.

(b) Heatmap of cellular composition across spatial niches, showing the proportion (%) of each cell type within each niche (column sums to 100%). Niches are grouped into tumoral and stromal compartments as indicated by brackets below. Color scale is capped at 10%.

(c) Milo differential abundance analysis (milopy) of spatial niches between CTL-high and CTL-low cores. Only niches passing $\text{SpatialFDR} < 0.05$, $\text{frac_sig} \geq 0.3$, and ≥ 5 patients with ≥ 50 cells/patient are shown; Each dot represents one neighborhood; violins show the distribution of log fold-changes, colored by enrichment direction (red, CTL-low; blue, CTL-high). Black dots, $\text{SpatialFDR} < 5\%$.

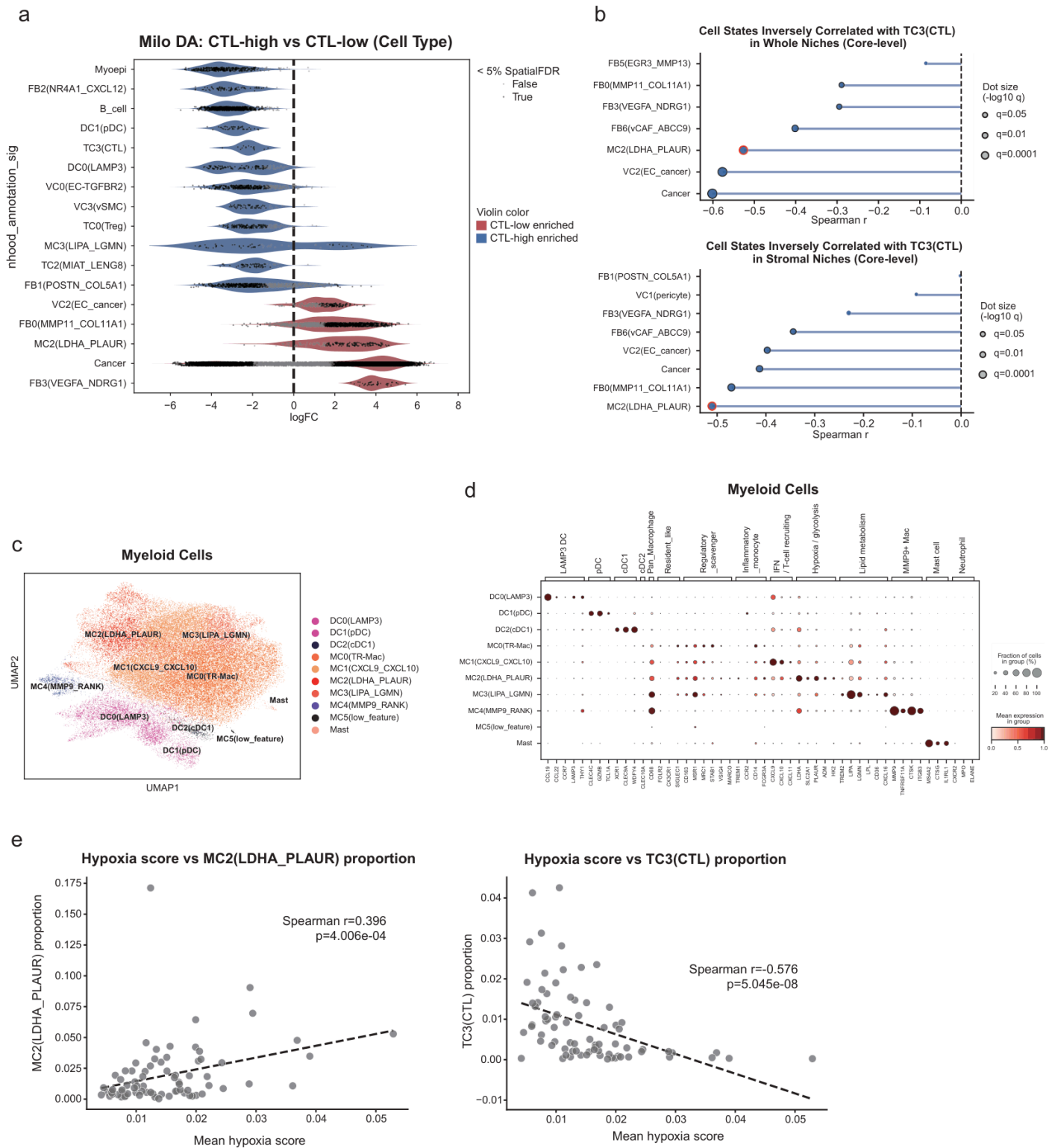


Figure 3

Hypoxia-associated macrophage subtype is enriched in CTL-low group.

(a) Milo differential abundance analysis of cell types between CTL-high and CTL-low cores. Only cell types passing SpatialFDR < 0.05, frac_sig ≥ 0.3, and ≥5 patients with ≥50 cells/patient are shown. Each

dot represents one neighborhood; violins show the distribution of log fold-changes, colored by enrichment direction (red, CTL-low; blue, CTL-high). Black dots, SpatialFDR < 5%.

(b) Spearman correlation between cell type proportion and TC3(CTL) proportion across whole niches (top) and stromal niches (bottom) (core-level). Cell types with negative correlation are shown; dot size indicates $-\log_{10} q$ (BH-FDR). MC2(LDHA_PLAUR) is highlighted (red circle).

(c) UMAP of myeloid subtypes, including macrophage (MC0–MC5), dendritic cell (DC0–DC2), and mast cell populations.

(d) Dot plot of myeloid subtype marker genes grouped by functional modules.

(e) Scatter plots showing Spearman correlations between mean hypoxia score and MC2(LDHA_PLAUR) proportion (left) or TC3(CTL) proportion (right) across whole niches (core-level). Each dot represents one core ($n = 76$). Dashed lines indicate linear fits. Spearman's r and associated p -values are indicated on the plot.

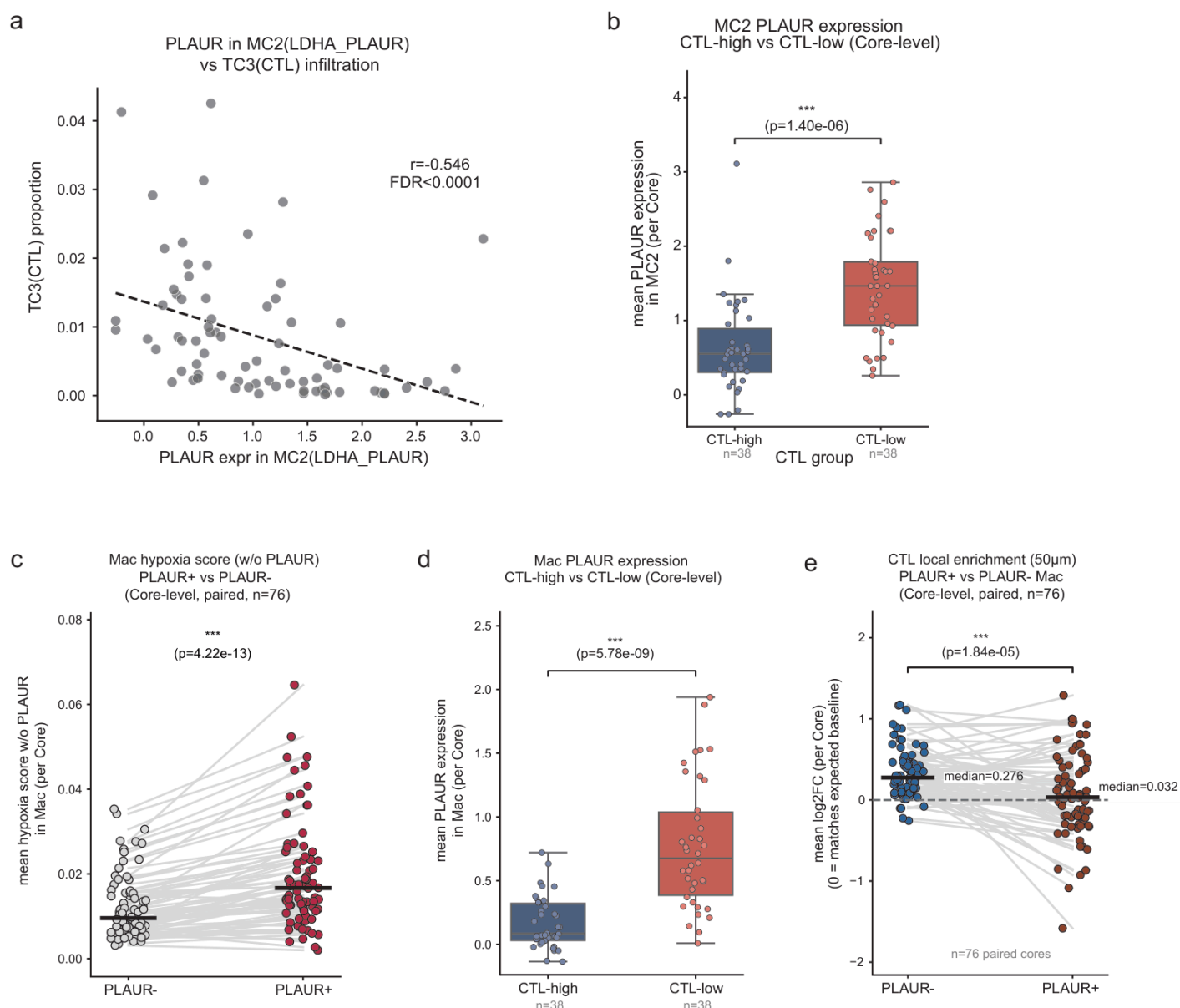


Figure 4

PLAUR+ macrophage states coincide with reduced CTL infiltration.

(a) Scatter plot of *PLAUR* expression in MC2(LDHA_PLAUR) cells versus TC3(CTL) proportion across cores (n = 76). Each dot represents one core. Dashed line indicates the linear fit. Spearman $r = -0.546$, FDR < 0.0001.

(b) Box plot showing the mean *PLAUR* expression in MC2(LDHA_PLAUR). Each dot represents one core, colored by CTL group. Core-level mean, Mann-Whitney U test, $p = 1.40e-06$

(c) Paired comparison of mean hypoxia signature score excluding *PLAUR* between PLAUR+ and PLAUR- macrophages within the same core. Core-level, Wilcoxon signed-rank test (paired, two-sided), n = 76

cores, $p = 3.61e-14$. Each dot represents one core. Each line connects the PLAUR- and PLAUR+ mean values from the same core.

(d) Box plot showing the mean *PLAUR* expression in macrophages. Each dot represents one core, colored by CTL group. Core-level mean, Mann-Whitney U test, $p = 5.78e-09$

(e) Core-level local CTL enrichment ($\log_2FC$ vs. core baseline) within 50 μm of PLAUR+ vs. PLAUR- macrophages (paired, $n = 76$ cores). Gray lines connect paired values within the same core; horizontal bars indicate medians. Wilcoxon signed-rank test (one-sided), $p = 1.84e-05$

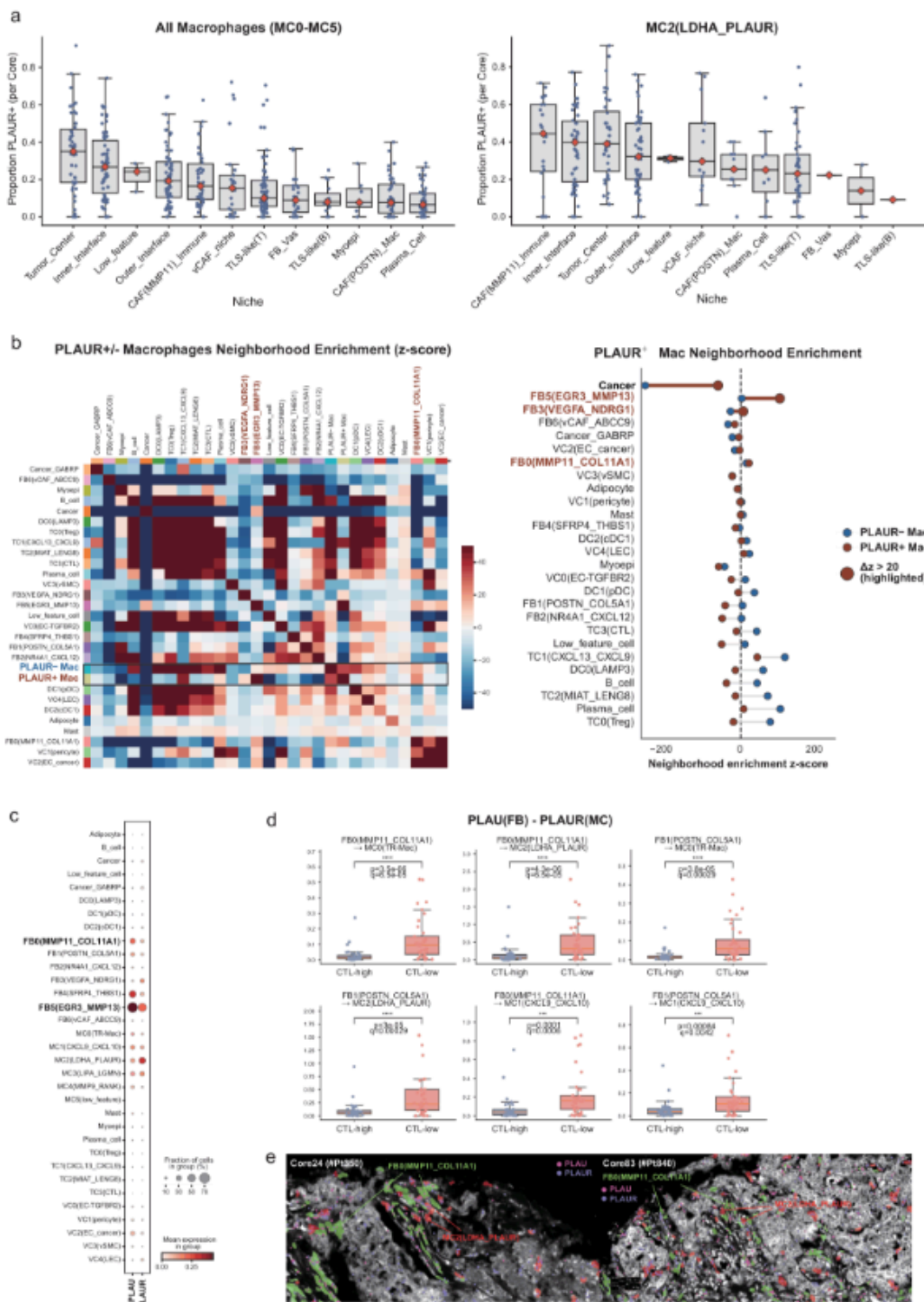


Figure 5

PLAUR+ macrophages engage FB0(MMP11_COL11A1) fibroblasts via *PLAU-PLAUR* signaling in CTL-low TNBC

(a) Box plots showing the proportion of PLAUR+ cells among all macrophages (MC0–MC5; left) and among MC2(LDHA_PLAUR) (right) per core, across spatial niches. Each dot represents one core; red

diamond indicates the mean.

(b) Squidpy neighborhood enrichment z-scores between cell types, with PLAUR+ and PLAUR- macrophages as separate categories (left; boxed rows). Paired z-scores for PLAUR- (blue) and PLAUR+ (red) macrophages per cell type, sorted by Δz (PLAUR+ - PLAUR-) (right); large dots, $\Delta z > 20$. Fibroblast subsets preferentially enriched with PLAUR+ macrophages are highlighted in red. Cancer is shown in bold.

(c) Dot plot shows the mean expression (color) and fraction of expressing cells (dot size) of *PLAU* and *PLAUR* across all annotated cell types in the TNBC cohort.

(d) Core-level *PLAU*(FB) $\times$ *PLAUR*(MC) interaction scores (product of mean *PLAU* in the source fibroblast and mean *PLAUR* in the target macrophage) comparing CTL-high and CTL-low cores. Of 36 FB-MC combinations tested, the six with $q < 0.05$ are shown. Each dot represents one core; boxes show median and IQR. Mann-Whitney U test with BH correction.

(e) Representative Xenium in situ images from two TNBC cores (Core24, #Pt350, left; Core83, #Pt840, right) showing *PLAU* (magenta) and *PLAUR* (purple) transcripts overlaid on segmented FB0(MMP11_COL11A1) fibroblasts (green) and MC2(LDHA_PLAUR) macrophages (red). Scale bar, 100 μm

(CAF(MMP11)_Immune, Tumor_Center, Inner_Interface, Outer_Interface); blue, all other niches.

(b) Box plots of the mean hypoxia signature score (left) and mean TGF- β downstream signaling score (right) per core, across cell types. Red, cell types highly colocalized with PLAUR+ macrophages (FB3(VEGFA_NDRG1), FB5(EGR3_MMP13), FB0(MMP11_COL11A1)); blue, all other cell types.

(c) Scatter plot of the rank-based correlation between mean *PLAUR* expression in macrophages and TGF- β downstream signaling score of neighboring cells within 50 μ m (core-level). Each dot represents one core. Spearman's ρ , associated p-value, and permutation-based p-value are indicated.

(d) Representative multiplex immunofluorescence (mIF) of treatment-naïve TNBC. Top, matched 200x fields from a uPAR+macrophage-high core (left) and -low core (right) (DAPI, blue; CD68, cyan; uPAR, orange; α SMA, green; pSMAD2, red; CD8, yellow). Bottom, the boxed region in the uPAR+Mac-high core shown as single-channel grayscale panels. Red arrowheads, uPAR+/CD68+ macrophages; yellow arrowheads, α SMA+ fibroblasts with nuclear pSMAD2 (activated CAFs); white arrowheads, CD8+T cells. Scale bars, 20 μ m.

(e) Reduced local CD8+ T-cell contact around uPAR+ macrophages. CD8+ T-cell contact enrichment within 50 μ m (observed/expected under spatial randomness) around uPAR+ versus uPAR- macrophages, paired within patient (n = 107). Counts were pooled across cores per patient; The analysis was restricted to cores in which both macrophage populations had ≥ 5 CD8+ target cells within 50 μ m, as the observed/expected ratio is unstable at very low target counts. Values below 1 (dashed line) denote contact below chance expectation. Boxes, median and IQR; grey lines connect paired per-patient values. Paired Wilcoxon signed-rank $P < 0.0001$; matched-pairs rank-biserial $r = -0.66$.

(f) uPAR+ macrophage neighborhoods carry greater TGF- β signaling. Fraction of neighboring α SMA+ CAFs positive for nuclear pSMAD2 within 50 μ m of uPAR+ versus uPAR- macrophages (n=107). Counts were pooled across cores; patients with an undefined ratio (no myCAF contact) were excluded. Paired Wilcoxon signed-rank $P < 0.0001$; $r = 0.47$. Boxes, median and IQR; grey lines connect paired values.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MergedSupplementaryMaterialsFigureTablesDataDescriptions.pdf](#)
- [SupplementaryData1.xlsx](#)