

Spatially Segregated Macrophage Populations Predict Distinct Outcomes In Colon Cancer

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Declaration of Interests

All authors declare no potential conflicts of interest.

Running title

The Spatial Map Of Human Macrophage Niches

Summary

Tumor-associated macrophages are transcriptionally heterogeneous, but the spatial distribution and cell interactions that shape macrophage tissue roles remain poorly characterized. Here, we spatially resolve five distinct human macrophage populations in normal and malignant human breast and colon tissue and reveal their cellular associations. This spatial map reveals that distinct macrophage populations reside in spatially segregated micro-environmental niches with conserved cellular compositions that are repeated across healthy and diseased tissue. We show that IL411⁺ macrophages phagocytose dying cells in areas with high cell turnover and predict good outcome in colon cancer. In contrast, SPP1⁺ macrophages are enriched in hypoxic and necrotic tumor regions and portend worse outcome in colon cancer. A subset of FOLR2⁺ macrophages is embedded in plasma cell niches. NLRP3⁺ macrophages co-localize with neutrophils and activate an inflammasome in tumors. Our findings indicate that a limited number of unique human macrophage niches function as fundamental building blocks in tissue.

Statement of Significance

This work broadens our understanding of the distinct roles different macrophage populations may exert on cancer growth and reveals potential predictive markers and macrophage population-specific therapy targets.

Introduction

As sentinel immune cells within tissues, macrophages execute diverse roles including pathogen defense, antigen presentation, phagocytosis of dying cells, and secretion of signals that drive tissue repair (1–3). Macrophages also play important roles in tumors, as evidenced by the fact that their infiltration within tumors predict poor patient outcomes for most tumor types (4). As a result, tumor associated macrophages (TAMs) were surmised to be a promising cancer therapy target. However, single agents targeting TAMs (e.g., CSF1 pathway inhibitors) have thus far shown minimal efficacy against solid tumors (5,6). This may be in part because such therapies monolithically repress macrophages without consideration for their heterogeneity. Clearly, a better understanding of the molecular and functional diversity of macrophages is needed to facilitate rational macrophage targeting in cancer and to predict clinical outcomes.

Defining molecular diversity of macrophage subsets and their spatial organization relative to one another, and other cells within tissues, is a preeminent goal of immunology. It has long been appreciated that macrophages residing in different tissues are different from one another, reflecting tissue specializations (7,8). More recently, attention has turned to whether even macrophages within a single tissue exhibit diversity (9). For instance, two subsets of Lyve1^{lo} MHC-II^{hi} vs. Lyve1^{hi} MHC-II^{lo} macrophages have been identified in several mouse tissues (10). Another study identified two macrophage subsets in human colon: one ACP5⁺ C1Q⁺ macrophages in lamina propria and second, LYVE1⁺ COLEC12⁺, in colon submucosa (11). Transcriptional macrophage heterogeneity is also apparent in human cancer and murine models of cancer (12–15). Yet, a comprehensive spatial organization of the putative tumor associated macrophage subsets remains to be profiled.

As a prelude to understanding spatial interactions of macrophage subtypes with other cells and their impact on cancer outcomes, we and others used immunostaining to show differential spatial enrichment of specific macrophage populations with distinct T cell or fibroblast subtypes (16–19). However, those studies were limited to examining at most one or two distinct macrophage populations and one other cell type at a time in a single organ. Unbiased and highly multiplexed profiling across all cell-types within a given organ, and across multiple organs, is needed to dissect the spatial organization of macrophages within a tissue, as well as cell-cell interactions that shape macrophage roles in the TME.

Here we employ multiplexed protein analyses to define five human macrophage populations (LYVE1⁺, FOLR2⁺, IL4I1⁺, NLRP3⁺, and SPP1⁺ macrophages) and to spatially map their distributions within human breast and intestinal tissues, as well as their malignant counterparts (breast cancer [BC] and colorectal cancer [CRC]). An important aspect of our approach is that we also simultaneously stain other cell-types in the tissue, providing a comprehensive insight into the multicellular environments that these macrophage populations inhabit. To our surprise, these five macrophage populations were not randomly distributed throughout tissues. Rather, they occupied spatially-distinct niches characterized by unique cellular compositions, discrete histological properties and conferred distinct effects on human patients' outcomes. The LYVE1⁺ and FOLR2⁺ macrophage populations were conserved across both human breast and intestine

tissues, with a subset of FOLR2⁺ tissue resident macrophages (TRMs) enriched in the plasma cell niche. IL4I1⁺ macrophages were present in regions exhibiting high cell turnover in both healthy and cancerous tissues where they were engaged in efferocytosis. This population correlated with anti-PD1 treatment response in BC and favorable CRC patients' outcome. The SPP1⁺ and NLRP3⁺ macrophages only emerged in diseased tissue. SPP1⁺ macrophages were associated with hypoxia and tumor necrosis and predicted poor outcome in CRC patients. Furthermore, NLRP3⁺ macrophages harboring active inflammasomes were spatially associated with infiltrating neutrophils across BC, CRC, and Crohn's disease (CD), suggesting that inflammasome activation contributes to neutrophil tissue accumulation.

To foster widespread accessibility to the wealth of images acquired in this work, we have developed a cloud-based image viewer. Overcoming barriers of entry for non-experts, it operates effortlessly in web browsers, eliminating the need for extensive computational resources and coding expertise. By accepting standard tiff images and employing efficient pre-processing, the IF Viewer enables swift and intuitive exploration for researchers and non-experts alike, offering an invaluable visual resource for comprehending the scope of our findings.

Taken together, our work reveals an elaborate spatially-organized network of five different macrophage populations embedded in distinct tissue niches. As we show that the existence of these macrophage populations, and their surrounding niches, are conserved across multiple organs and clinical tissue states (healthy, cancerous and inflamed), this work conceptualizes the macrophage niche as a fundamental and conserved tissue building block. Importantly, we establish clinical-grade antibodies to identify these human macrophage populations, enabling laboratories worldwide to define distinct macrophage populations *in situ* in human clinical specimens. We demonstrate strategies to identify new candidate predictive and prognostic markers and macrophage-targeted cancer therapy targets. Finally, we provide an online resource (https://magdalenamat.github.io/Spatial_MAC_Map/) to enable easy exploration and downloading of images acquired during this study.

Results

Experimental approach

This work aimed to reveal the spatial distribution of distinct human macrophage subsets and the cellular composition of their respective niches. We focused on BC and CRC because macrophage abundance (as assessed by CD68 staining) predicts the opposite clinical outcomes in BC and CRC patients (4,20). With high CD68 macrophage infiltration associated with bad outcome in BC, but good outcome in CRC. We thus hypothesized that comparing BC to CRC macrophages might reveal clinical markers associated with different macrophage functional polarization states. We used four public scRNAseq datasets of CRC and BC (21–23) to discover markers of distinct macrophage subtypes (**Fig 1A**) and established a panel of 6 antibodies that are compatible with formalin-fixed, paraffin-embedded (FFPE) tissue. These antibodies identified five discrete macrophage populations *in situ*. We subsequently used whole section IHC, 4-color immunofluorescence (IF), and 36-antibody CODEX assays on Tissue Microarrays (TMAs) to discover distinct spatial macrophage niches and the possible functions these spatially-resolved TAM subsets fulfill in the TME and as TRM in normal tissue (**Fig 1B**, results in **Fig2-6**). In addition, we used CosMx protein profiling to characterize macrophage subset immune polarization in more detail. Figure references with 'v' prefix link to immunofluorescence images of large regions of the entire conventional paraffin sections imaged, and are frequently accompanied by additional images showing the same phenomenon across different patients.

Identification of macrophage subset markers by single-cell RNA-sequencing

To discover markers of different macrophage populations, we integrated, clustered, and compared monocyte and macrophage scRNAseq data spanning 18,698 cells from 128 samples derived from 92 patients across four published studies of BC and CRC (**Fig 1A, C, S1A**). We defined 11 transcriptional clusters marked by differential enrichment of genes and differentiated three monocytes, five TAM, and three TRM subsets (**Fig 1C-D**). We selected a clustering resolution that separated known myeloid subtypes as follows: TRMs (*LYVE1*⁺) form TAMs (*TREM2*⁺*APOE*⁺), and Patrolling (*CDKN1C*⁺*FCGR3A*⁺) from Classical monocytes (*VCAN*⁺*S100A8*⁺*S100A9*⁺). We discovered three novel TRM subsets: 1) *LYVE1*⁺*FOLR2*⁺ *TRMs* (*FOLR2*⁺ *LYVE1*⁺ *MARCO*⁺ *SLC40A1*⁺ *SEPP1*⁺), and 2) *C3*⁺ *TRMs* (*C3*⁺*CX3CR1*⁺), in addition to four previously-identified subsets (14,15,22), including 4) *NLRP3*⁺ *TAMs* (*NLRP3*⁺ *IL1B*⁺), 5) *SPP1*⁺ *TAMs* (*SPP1*⁺*CHI3L1*⁺*MT1G*⁺), 6) *CXCL9*⁺ *TAMs* (*CXCL9*⁺*IL4I1*⁺), and 7) *ISG15*⁺ *TAMs* (*ISG15*⁺*CXCL10*⁺*CXCL11*⁺) (**Fig 1D**).

The existence of two distinct *FOLR2*⁺ *TRM* subsets has not been previously reported. Differential gene expression to compare these two subsets showed that *FOLR2*⁺*LYVE1*⁺ *TRMs* were enriched in scavenger receptors (*MARCO*, *CD36*, *MRC1*), metabolic enzymes (*BLVRB*, *PK4*), and immunoglobulins (*IGHA1*, *IGKC*, *IGLC2*). On the other hand, the *FOLR2*⁺*LYVE1*⁻

subset was enriched in phagocytosis and antigen presentation gene signatures, further supporting the distinct phenotypes of the two *FOLR2*-positive populations (**Fig 1E**).

To explore the distribution of these subsets between CRC and BC, we computed a ratio of their average frequency across samples with more than 35 myeloid cells. *CXCL9*⁺ TAMs were the most abundant TAM population in both BC and CRC, and *NLRP3*⁺ TAMs were enriched in CRC, with about 3.5 log₂ fold higher frequency than in BC (**Fig 1F, S1B-D**). We also observed fundamental cluster segregation between benign colon and tumor tissue: *NLRP3*⁺ TAMs and *SPP1*⁺ TAMs were almost exclusively confined to colon tumors, whereas *LYVE1*⁺ TRMs were most enriched in benign colon (**Fig S1E-G**). Guided by the above scRNAseq analysis (**Fig 1D, S1E-G**), we built a panel of commercially available FFPE-compatible antibodies for six macrophage markers to resolve both TAM and TRM populations on the basis of *IL411*, *NLRP3*, *SPP1*, *FOLR2*, *LYVE1*, and *MARCO* protein expression (**Fig 1G**). The following sections describe how we used these markers to discriminate spatial macrophage niches (**Fig 2-3**) and to define their cellular composition and propose their functional roles (**Fig 4-7**). For clarity, we italicized the names of scRNAseq subsets and refer to cell subsets defined based on protein expression with regular non-italic font through the text.

FOLR2, IL411, NLRP3, and SPP1 mark spatially distinct macrophage niches in the TME

To study the spatial distribution of macrophage markers in the TME of breast and colon cancer, we used *CD68* and *CD163* as canonical macrophage markers, *IL411*, *NLRP3*, and *SPP1* to differentiate TAM populations, and *FOLR2* to highlight TRMs. ScRNAseq indicated that *NLRP3* is a specific *NLRP3*⁺ TAM marker, *SPP1* is a specific *SPP1*⁺ TAM marker, but *IL411* has a broader expression, highlighting *SPP1*⁺ TAMs, *CXCL9*⁺ TAMs, and *ISG15*⁺ TAMs. Nevertheless, the combination of *IL411*, *SPP1*, and *NLRP3* antibodies was sufficient to detect and discriminate *NLRP3*⁺ TAMs, *SPP1*⁺ TAMs, and *IL411*⁺ TAM groups (encompassing *ISG15*⁺ and *CXCL9*⁺ TAMs that we could not resolve) that together labels all scRNA TAMs subsets. In addition, scRNAseq suggested that *SPP1* and *IL411* expressing populations are enriched in *CD68* gene expression and *FOLR2*⁺*LYVE1*⁻ and *FOLR2*⁺*LYVE1*⁺ TRM populations are enriched in *CD163* gene expression (**Fig 2A**).

We quantified distance from every detected macrophage to its closest tumor cell across a large number of regions and patient samples (**Fig 2B, C**), and the four panels in **Fig 2D-G** show staining results of macrophage distribution in a single representative 1.5 mm² tissue region of human BC and CRC. Each panel shows 1) an IF image of the discussed markers (*left*), 2) a corresponding dotplot representing the spatial macrophage distribution in the TMA core as revealed by the IF (*top right*), and 3) a corresponding distance quantification from each detected macrophage to the closest tumor cell in that specimen (*bottom right*). We started by analyzing the spatial distribution of *CD68* and *CD163* (**Fig 2B, 2D, v2D**). A commonly held view is that *CD163*-positive macrophages are of M2-type that help tumor growth and metastasis (24) and are expected to localize close to tumor cells. Surprisingly, contrary to this view, we found that macrophages with higher *CD163* expression localized further away from the tumor nests (**Fig 2B, 2D i**, with an average distance of 74.5 μm) compared to macrophages with higher *CD68*

expression that infiltrated and tightly surrounded tumor nests (**Fig 2B, 2D ii**, with an average distance of average 35.9 μm).

Next, we interrogated the spatial distribution of FOLR2, IL4I1, NLRP3, and SPP1 in BC and CRC. We found remarkable and unexpected segregation of these markers where FOLR2 expression was associated with benign tissue localized further away from the tumor (**Fig 2E-G**) spatially corresponding to the macrophages with higher CD163 expression (**Fig 2D i**). In contrast, macrophages expressing IL4I1 (**Fig 2E, v2E, vBC1t, vBC1b, vBC2**), NLRP3 (**Fig 2F, v2F**), and SPP1 (**Fig 2G, v2G, vBC1t, vBC1b, vBC2, vBC3, vBC4l, vBC4r, vCRC1, vCRC2, vCRC3, vCRC4l, vCRC4r, vNC**) were concentrated immediately adjacent to tumor cells, spatially reflecting the macrophages with higher CD68 expression (**Fig 2D ii**). This was confirmed by a distance comparison that analyzed 36,041 macrophages spanning 60 distinct 1.5 mm^2 tissue fragments derived from 14 CRC and 13 BC cases. This analysis showed that IL4I1⁺ TAMs were located an average of 38.3 μm away from the closest tumor cell, NLRP3⁺ TAMs 47.4 μm , SPP1⁺ TAMs 36.4 μm , while in contrast, FOLR2⁺ TRMs located 109 μm from the nearest tumor cell (**Fig 2C**).

Since we found remarkable spatial segregation of IL4I1⁺ TAMs and FOLR2⁺ TRMs in the TME (**Fig 2C, E**) in primary tumors, we sought to investigate whether this pattern is conserved in metastatic lesions. We compared IF staining of a CRC invasive front and a lymph node CRC and BC metastasis. Similar to the invasive front of the CRC tumor (**Fig S2A-B, vS2A, vS2B**), in the LN CRC metastasis (**Fig S2C-D, vS2C, vS2D**) and BC metastasis (**Fig S2E-F, vS2E, vS2F**), IL4I1⁺ macrophages were present in the desmoplastic stroma surrounding the tumor nests, and FOLR2⁺ macrophages were present further away in the surrounding benign tissue. This suggests that the presence of the tumor shapes macrophage phenotype and distribution in the TME in a similar way independent of the tumor type (BC and CRC share the same TAM populations) or whether the tumor is primary or metastatic. In addition, we report that a thin buffer zone of macrophages co-expressing both FOLR2 and IL4I1 existed in both primary tumor and metastasis specimens (**Fig S2A-F**).

To verify whether the spatial separation of IL4I1⁺ TAMs and FOLR2⁺ TRMs in the TME occurs across other human tumors we extended our analysis to additionally profile two major cancer subtypes (adenocarcinomas and squamous cell carcinomas) spanning eight different organs of origin: cheek skin, oral mucosa, larynx, esophagus, lung, stomach, pancreas, and bladder (**Fig S2G-N, vLung, vStomach, vPancreas, vBladder, vSkin, vOralM, vLarynx, vEsophagus**). Remarkably, we found that the spatial separation of the FOLR2⁺ TRMs (embedded in the adjacent benign tissue) and IL4I1⁺ TAMs (located in and around tumor nests) was present in all tumors analyzed.

Our results suggest that spatially segregated macrophage populations may serve different functions. We show that FOLR2⁺ TRMs are embedded in the normal tissue and are spatially segregated from IL4I1⁺, NLRP3⁺, and SPP1⁺ macrophages, which are tumor-associated. This is an important finding as revealing markers distinguishing TRMs from disease-associated

macrophages is a crucial step that enables the study of individual macrophage subset functions and their relevance to disease progression (25).

IL411, FOLR2, LYVE1, and MARCO label spatially segregated TRM niches in benign colon and breast

Next, we sought to learn whether the spatially segregated macrophage distribution we found in the TME was conserved in benign tissue. Previous reports have shown that TRMs govern tissue-specific roles driven by distinct gene expression programs in different normal tissues (8). Using our subset-specific markers, we found three distinct layers of TRMs in benign colon mucosa (**Fig 3A, left**). Surprisingly, the IL411⁺ macrophages, which we previously discovered to infiltrate tumor nests, were also present in the normal colon mucosa, where they localized at the top of the colon lamina propria (LP) (luminal aspect). The second layer in the middle and bottom of the LP contained FOLR2⁺ TRMs (**Fig 3B, v3B, NC1, NC2, NC4, NC6, NC7, NC8, NC9, NC12**). The third TRM layer was localized in the colon submucosa and marked by FOLR2, LYVE1, and MARCO (**Fig 3C-D, v3C, v3D**). As the gastrointestinal submucosa is rich in blood and lymph vessels, the submucosal FOLR2⁺LYVE1⁺MARCO⁺ TRM population likely corresponds to previously reported murine peri-vascular (PV) TRMs (26). The striking spatial segregation of TRMs in the bowel wall is consistent with the scRNAseq data that indicates the existence of two distinct FOLR2⁺ TRMs populations: one that is positive for *FOLR2* alone (*FOLR2⁺LYVE1⁻ TRMs*) and one that co-expresses *FOLR2*, *LYVE1*, and *MARCO* (*FOLR2⁺LYVE1⁺ TRMs*, **Fig 1D**).

In comparison, we found two spatially segregated TRM populations in benign breast stroma. Consistent with a recent report (17), the TRMs surrounding benign breast lobules and ducts were FOLR2 positive (**Fig 3E, v3E**). We called these cells Lobular TRMs and found they express a low level of LYVE1 and MARCO (**Fig 3E-F i**). Furthermore, we discovered that TRMs localized in the highly vascularized connective tissue that is further removed from the breast lobules co-expressed high levels of FOLR2, LYVE1, and MARCO (**Fig 3E-F ii, v3F**).

Taken together, these results support the single-cell transcriptomic (**Fig S1F**) and mIHC (**Fig 2C, E-G**) findings indicating that NLRP3⁺ and SPP1⁺ macrophages are associated with the TME and FOLR2⁺ and LYVE1⁺ macrophages are located in normal tissues. Interestingly, we reveal the presence of IL411⁺ macrophages in both normal colon and CRC, showing that they are present in both healthy and cancerous colon tissue.

IL411 marks phagocytosing macrophages

IL411 localizes in the lysosomes of antigen-presenting cells (27), suggesting a role in phagocytosis. A close inspection of the IF-stained invasive front of colon tumor revealed the presence of pan-cytokeratin (CK)-positive granules within the cytoplasm of IL411⁺ TAMs. We hypothesize that the pan-CK granules are apoptotic bodies derived from tumor cells that are being phagocytosed by the IL411⁺ TAMs (**Fig 4A, v4A**). The invasive front of the tumor is an area where intense tissue remodeling takes place. To invade the adjacent normal tissue, tumor

cells need to make their way through the wall of tightly joined cells and the extracellular matrix. This process is likely to cause cell death and correlates with a rich presence of IL411⁺ TAMs in the CRC invasive front. We also found that the IL411⁺ macrophages on the top of the lamina propria in normal colon, but not the FOLR2⁺ TRMs in the middle and bottom of the crypt, contain apoptotic bodies of the intestinal epithelial cells (**Fig 4B**, [v4B](#), [NC1p](#), [NC2p](#), [NC5p](#), [NC6p](#), [NC8p](#), [NC10p](#), [NC10.2p](#)). This is consistent with the longstanding finding that macrophages ingest dying intestinal epithelial cells (IEC) at the top of the intestinal lamina propria (28) but now provides a novel marker for the macrophage subset that executes this activity.

To further support the hypothesis that IL411 marks phagocytosing population of macrophages, we asked whether another specialized body phagocyte type, tingible body macrophages (TBMs), shows IL411 positivity. The TBMs localize in germinal centers where they remove apoptotic B cells (29) and thus are expected to have a high expression of phagocytic markers. TBMs contain apoptotic cellular debris at different degradation stages and are named after apoptotic nuclear debris ('tingible bodies') that can be observed in their cytoplasm. We found that the TBM in the LN germinal centers displayed very bright IL411 staining (**Fig 4Ci**, [v4C](#)) compared to the interfollicular macrophages that were FOLR2 positive (**Fig 4Cii**). The presence of TBMs is also a hallmark of Burkitt's lymphoma, a tumor characterized by fast cell turnover (30). We examined two Burkitt's lymphoma cases and found that TBMs in this tumor display high IL411 expression (**Fig 4D**, [v4D](#)).

We used gene set enrichment analysis to further investigate the association between phagocytosis and the IL411⁺ TAMs. We found that compared to all other scRNA macrophage subtypes, the *CXCL9*⁺ TAMs (a subset of *IL411*⁺ TAMs) were most enriched in Phagosome, Lysosome, Endocytosis, and Antigen Processing and Presentation gene sets expression (**Fig 4E**). To evaluate the possible clinical relevance of this finding, we next asked whether *IL411*⁺ TAMs express known phagocytosis checkpoints (like CD47 and PD-L1) and might thus be targets of phagocytosis-modulating cancer therapies, including anti-CD47 and anti-PD-L1 treatment (31). Indeed, using our integrated myeloid scRNAseq atlas (**Fig 1C**) we found that *SIRPA* (which encodes the CD47 ligand) and *CD274* (encoding PD-L1) were both enriched in *IL411* expressing scRNA myeloid clusters, including *SPP1*⁺ TAMs, *ISG15*⁺ TAMs and *CXCL9*⁺ TAMs (**Fig 4F**). In addition, we show that in comparison to all cells detected in the tissue, macrophages and mast cells are major sources of *CD274* in the TME (**Fig S3A-D**). Together, this indicates that of all macrophages present in the TME it is the IL411 TAMs that likely constitute an indirect target of anti-CD47 immunotherapies, and a direct and major target of anti-PD-L1 immunotherapies.

Recent reports demonstrated that PD-L1 expression on TAMs, but not tumor cells, predicts response (32) and patient survival (33) in the context of patients receiving anti-PD-1/PD-L1 therapy. Thus, an important question is whether IL411 could be used as a predictive marker of response to PD-1 axis blockade. To address this question, we used the scRNA monocyte and macrophage transcriptomes from Bassez et al. (23) (**Fig 1C, S1A**), that contains samples of advanced breast cancer patients taken before and after pembrolizumab treatment. We found that the frequency of IL411 expressing scRNA TAMs, both pre- and post-treatment, was

increased in patients that responded to the therapy (**Fig 4G-H**). This important finding suggests IL411 as a promising marker for response to PD-1 axis therapy.

In addition to their decreased frequency in the non-responder group the *SPP1*⁺ TAMs, *ISG15*⁺ TAMs, and *CXCL9*⁺ TAMs displayed decreased *CD274* gene expression compared to their counterparts in responders (**Fig 4I**). Similar to IL411⁺ transcriptional macrophage subsets in Bassez et al. dataset (23), the *SPP1*⁺ TAMs, *ISG15*⁺ TAMs, and *CXCL9*⁺ TAMs from one breast and two separate scRNAseq CRC datasets displayed increased *CD274* gene expression compared to all other monocyte and macrophage subsets (**Fig 4J**).

These results 1) demonstrate that IL411 is a marker associated with active phagocytosis of individual cells in BC and CRC, 2) suggest that IL411⁺ TAMs are targets of the anti-CD47 and anti-PD-L1 immunotherapies (**Fig 4K**) that may affect IL411⁺ TAMs phagocytosis potential, and 3) indicate IL411 as a potential novel predictive marker of PD1–PD-L1 axis blockade. Importantly, these results demonstrate that IL411⁺ macrophage population is shared between healthy and diseased tissue with high cell turnover, including the top of the normal colon lamina propria, germinal center TMB, Burkitt's lymphoma, and the TME.

CODEX multiplexed imaging reveals spatial cellular interactions in macrophage niches within colon and breast cancer tissues

Having identified the spatial segregation of the IL411, NLRP3, SPP1, FOLR2, and LYVE1 macrophage populations, we sought to elucidate the cellular compositions of the spatially segregated niches where these populations occur. We used CO-Detection by indEXing (CODEX) multiplexed tissue imaging to simultaneously visualize 36 protein markers on a single tissue microarray section of breast and colon benign and tumor tissue (34–36). This panel allowed us to recognize major immune, epithelial and stromal cell types. Our CODEX antibody panel contained four general myeloid markers (CD16, CD68, CD163, CD206) and three macrophage subset-specific markers (SPP1, LYVE1, and FOLR2). Using the CODEX computational pipeline (i.e., imaging processing, single-cell segmentation, and unsupervised clustering) (37), we identified two epithelial cell types, seven stromal cell types and fifteen immune cell types (**Fig 5A, Fig S4A**). Among the immune cell types, we discriminated five macrophage populations: CD68⁺ TAMs, SPP1⁺ TAMs, CD163⁺ TRMs, FOLR2⁺ TRMs, and LYVE1⁺ TRMs (**Fig S4B**). We could not add IL411, NLRP3, and MARCO antibodies to the CODEX panel for technical reasons. Based on their spatial tissue distribution (discussed below and illustrated in **Fig S4C-E**) the CODEX-identified CD68⁺ TAMs likely corresponded to IL411⁺ TAMs and NLRP3⁺ TAMs populations we identified by IL411 and NLRP3 immunostaining in our IF studies using TMA sections immediately adjacent to the sections used for CODEX profiling (**Fig 2, S5A**). The CODEX-identified CD163⁺ TRMs likely represent LYVE1⁺ TRMs and FOLR2⁺ TRMs for which FOLR2 and/or LYVE1 staining was not detected.

CODEX imaging showed that the distribution of CD68 and CD163 is different between the five macrophage populations, with CD68⁺ and SPP1⁺ TAMs enriched in CD68 expression while CD163⁺, FOLR2⁺, and LYVE1⁺ TRMs were enriched in CD163 expression (**Fig S4B**).

Consistent with the scRNAseq and 4-color IF results (**Fig 1D, Fig 3A, 3C-F**), CODEX imaging confirmed the existence of two FOLR2-positive macrophage populations: FOLR2⁺LYVE1⁻ and FOLR2⁺LYVE1⁺ (**Fig S4B**). Moreover, we validated that SPP1⁺ TAMs (average distance 28.4 μ m) localize more closely to tumor cells than FOLR2⁺ macrophages (average distance 65.8 μ m) (**Fig S4C**). In addition, CODEX data showed that similar to FOLR2⁺ TRMs, the CODEX LYVE1⁺ TRMs are localized further away from the tumor (average distance 106 μ m) (**Fig S4C**). In agreement with our scRNAseq and IF analysis (**Fig 2B,D**) - and in opposition to prior dogma-, CD68⁺ TAMs were again closer to the tumor nest than CD163⁺ counterparts (**Fig S4C**).

To uncover the cellular composition of the different macrophage niches, we next performed cellular neighborhood analysis on the CODEX multiplexed imaging data (38–40). We clustered cells based on the identity of their ten closest neighboring cells and identified 14 cellular neighborhoods, of which nine were enriched in macrophages (**Fig 5B**). We grouped the nine macrophage-containing neighborhoods into four neighborhood types, each named after the primary macrophage subtype it contained: 1) a single CD68⁺ TAM neighborhood, 2) three SPP1⁺ TAM neighborhoods, 3) four FOLR2⁺ TRM neighborhoods, and 4) a single LYVE1⁺ TRM neighborhood (**Fig 5B, S4D**). The single CD68⁺ TAM neighborhood was localized inside the tumor nests and co-enriched with the tumor cells (**Fig 5B, S5B, vS5B**); we called it the **Intra-tumoral TAM neighborhood**. The three discrete SPP1⁺ TAMs neighborhoods were all enriched with SPP1⁺ TAMs and the tumor cells but differed in cellular composition. The **Peri-tumoral SPP1⁺ TAM neighborhood** contained CD68 macrophages (**Fig 5B, S5C, vS5C**), the **Inflamed SPP1⁺ TAM neighborhood** contained neutrophils (**Fig 5B, S5D, vS5D**), and the **Hypoxic SPP1⁺ TAM neighborhood** held hypoxic tumor cells marked by CA9 expression (**Fig 5B, S5E, vS5E**). The four discrete FOLR2⁺ neighborhoods were co-enriched in FOLR2⁺ TRMs and CD163⁺ TRMs but had different cell compositions and tissue locations. The **Plasma Cell (PC) enriched FOLR2⁺ TRM neighborhood** was co-enriched with PCs and located close to the blood vessels and in the normal gastrointestinal (NGI) LP (**Fig 5B, S5F, vS5F**). The **Smooth Muscle FOLR2⁺ TRM neighborhood** labeled the bowel muscle wall (**Fig 5B, S5G, vS5G**). The **Trapped Fibrous FOLR2⁺ TRM neighborhood** was enriched in FAP fibroblasts and marked fibrous bands entrapped between growing tumor nests (**Fig 5B, S5H, vS5H**). The **Lymphoid FOLR2⁺ TRM neighborhood** contained CD4T, CD8T, Tregs, DCs, and FOLR2⁺ TRMs (**Fig 5B, S5I, vS5I**). The LYVE1⁺ TRM neighborhood was co-enriched with LYVE1⁺ TRMs, FOLR2⁺TRMs, CD163⁺ TAMs, PDGFR β fibroblasts, mast cells, and blood and lymph vessels. We called it the **Peri-Vascular LYVE1 TRM neighborhood (Fig 5B, S5J, vS5J)**.

Next, we used two approaches to map each CODEX-macrophage neighborhood tissue distribution relative to the tumor. First, we computed the distance of every macrophage, labeled by the neighborhood it belongs to, to the closest tumor cell (**Fig 5C**). Second, we calculated the fraction of tumor cells in every macrophage-enriched neighborhood (**Fig 5D**). We interpret the distance to the tumor and the fractional enrichment in tumor cells as an indicator of how closely the given neighborhood is associated with the tumor. These analyses revealed a remarkable spatial macrophage neighborhood segregation and a 3-tier distribution of closeness to the tumor. Specifically, we show that TAMs in the **Hypoxic SPP1⁺ neighborhood** and the **Intra-tumoral neighborhood** were located the closest to the tumor cells with an average distance of

9.37 and 10.6 μm to the nearest tumor cell (**Fig 5C**) and that those two neighborhoods had the highest fraction of tumor cells (**Fig 5D**). In contrast, TRMs in *the Lymphoid FOLR2⁺*, the **PCs enriched FOLR2⁺**, the **Peri-Vascular LYVE1⁺** and the **Smooth Muscle FOLR2⁺ neighborhoods** lay the farthest from the tumor with an average distance of 55.2, 57.5, 74.8, and 76.0 μm from the closest tumor cell (**Fig 5C**). In agreement, they also contained the smallest percentage of tumor cells (**Fig 5D**). Macrophages in the **Peri-tumoral SPP1⁺**, the **Inflamed SPP1⁺**, and the **Trapped Fibrous FOLR2⁺ neighborhoods** localized at an intermediate distance between the two extremes.

To better visualize the spatial distribution of the macrophage neighborhoods in benign and tumor tissues, we plotted the neighborhood frequency by anatomic location. We show that the **Peri-Vascular LYVE1⁺ TRMs neighborhood** was most enriched in benign breast, while the **PCs enriched FOLR2⁺ TRMs neighborhood** was most enriched in NGI mucosa. The **Smooth Muscle FOLR2⁺ TRMs neighborhood** labels bowel wall and was thus specific to gut samples, and it could be detected in benign, in the invasive front and center of the tumor samples. This is consistent with the fact that CRC invades the bowel wall. In turn, the **Intra-tumoral TAM neighborhood**, the **Inflamed SPP1⁺ TAM neighborhood**, the **Peri-tumoral SPP1⁺ TAM neighborhood**, the **Hypoxic SPP1⁺ TAM neighborhood**, and the **Trapped Fibrous FOLR2⁺ neighborhood** were enriched in ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), in the IF of CRC and the CRC center of the tumor (CT), further supporting that they are tumor-associated (**Fig 5E, S4E**).

Taken together, the CODEX data (**Figs 5, S4, S5**) allowed us to identify spatial associations between macrophage subtypes and other cell types in benign and tumor tissues. We showed that SPP1⁺ TAMs were co-enriched with CD68⁺ TAMs close to the tumor cells, localized in hypoxic tumor areas, and associated with neutrophilic infiltration. In contrast, CD163⁺ TRMs, FOLR2⁺ TRMs, and LYVE1⁺ TRMs were co-enriched in adjacent benign tissue located further away from the tumor. We showed that FOLR2⁺ TAMs constituted a tissue-resident macrophage population in the bowel muscle wall and were associated with PCs in the intestinal lamina propria and connective breast tissue. We found that FOLR2⁺ TRMs from the breast connective tissue or muscle bowel wall can be trapped within growing tumor nests and thus become a part of the TME (**Fig 5F**).

FOLR2⁺ TRMs spatially colocalize with plasma cells and may maintain long-lived plasma cell tissue niche

To further explore the association between CODEX-identified FOLR2⁺ TRM with PCs, we used IHC and multicolor IF. Single color IHC showed that in the tumor-adjacent stroma FOLR2⁺ TRMs were in direct contact with PCs, which can be histologically identified by their nuclear chromatin condensation pattern and asymmetric cytoplasmic 'hof' where antibodies are produced and stored (arrowheads, **Fig S6A**). Multicolor IF additionally revealed that FOLR2⁺ TRMs and CD38⁺ PC occupied the same space in the middle and bottom layers of the colon lamina propria (**Fig S6B, vS6B**), corroborating the CODEX results. Furthermore, we found Lobular FOLR2⁺ TRMs were immediately adjacent to PCs around benign breast glands (**Fig**

S6C, [vS6C](#)). Previous studies showed that CD163⁺ macrophages surround PCs in the extrafollicular foci in the tonsil (41). Here we show that it was the FOLR2⁺ TRM subtype that localized directly next to PCs in the LN interfollicular zone (**Fig S6D**, [vS6D](#)).

To unequivocally demonstrate that the cells spatially co-enriched with FOLR2⁺ TRMs were PCs, we used 4-plex IF staining and showed that cells localized directly next to FOLR2⁺ TRMs were marked by overlapping expression of CD38 and a prototypical PC marker - CD138 (**Fig S6E**, [vS6E](#)).

To demonstrate that the association between PCs and FOLR2⁺ TRMs was specific, we computed the distance from every IL411⁺ TAM and FOLR2⁺ TRM to their closest PC across seven different tissue regions. As anticipated, PCs were localized closer to FOLR2⁺ TRMs than the IL411⁺ TAMs (**Fig S6F-G**).

To gain insight into the possible molecular mechanism governing the contact between the FOLR2⁺ TRMs and PCs, we next performed scRNAseq-based ligand-receptor interaction analysis. In BC(23), the highest probability interactions were found between APRIL (TNFSF13) and BAFF (TNFSF13B) on the *FOLR2⁺ TRMs* and BCMA (TNFRSF17) on the *PCs* (**Fig S6H**). APRIL and BAFF are known to drive PC infiltration and their long-term survival in the tissue (42,43). Similarly, using the *IgA⁺PC*, *IgG⁺PC*, and *FOLR2⁺ TRMs* scRNAseq transcriptomes from benign colon and CRC (21), we also identified BAFF (TNFSF13B) and BCMA (TNFRSF17) interaction as the highest probability interaction between *IgA⁺PC* and *FOLR2⁺ TRMs* (**Fig S6I**). Our results provide a marker for the type of macrophage described in previous literature that suggests antigen-presenting cells maintain the PC niche in human tonsils (41), murine bone marrow (44), and human lamina propria (45). These observations suggest that FOLR2⁺ TRMs may play a key role in recruiting and maintaining PCs in inflamed benign tissue adjacent to tumors and the lamina propria of benign colon (**Fig S6J**).

Prior work reported the existence of HES1⁺/FOLR2⁺ macrophages in normal human breast and lung, and human cancers of breast, liver and lung (14,17,46). In addition, Chakarov et al. found Lyve1 expression to discriminate between two murine TRM populations, Lyve1^{lo}MHCII^{hi}CX3CR1^{hi} surrounding nerves, and Lyve1^{hi}MHCII^{lo}CX3CR1^{lo} surrounding the blood vessels (10). As mentioned above, the murine Lyve1^{hi}MHCII^{lo}CX3CR1^{lo} population likely corresponds to the FOLR2⁺LYVE1⁺ human macrophages we describe, as they display the same marker expression and share spatial tissue association with blood vessels. In contrast, the murine Lyve1^{lo}MHCII^{hi}CX3CR1^{hi} described by Chakarov et al. (10), and human FOLR2⁺LYVE1⁻ macrophages we describe here are not likely to correspond to each other as: 1) human FOLR2⁺LYVE1⁻ do not express *CX3CR1* gene (**Fig 1D**) that was used by Chakarov et al. to define the murine Lyve1^{lo}MHCII^{hi}CX3CR1^{hi} population (10), and 2) we find human FOLR2⁺LYVE1⁻ macrophages to spatially interact with plasma cells and not nerves (**Fig 5B**, **Fig S6**). In fact, the murine Lyve1^{lo}MHCII^{hi}CX3CR1^{hi} population is more likely to correspond to the C3⁺ TRMs subset we found in scRNAseq analysis since the C3⁺ TRMs is the only macrophage subset expressing the *CX3CR1* gene (**Fig 1D**). Therefore, our work significantly extends upon previous reports by showing that not one but two distinct FOLR2⁺ TRM populations exist in

humans, by providing a detailed topography of their distribution, and revealing their spatial cell interactions in human colon breast and lymph nodes that were not studied in mice. Importantly, we also, for the first time, revealed that a subset of FOLR2⁺LYVE1⁻ macrophages is embedded in the plasma cell tissue niche where it expresses signals promoting long-term plasma cell retention in the tissue.

SPP1⁺ TAMs are enriched in hypoxic and necrotic tumor areas

CODEX neighborhood analysis revealed a spatial co-enrichment of SPP1 TAMs with neutrophils in the **Inflamed SPP1⁺ TAM niche**. Notably, we also found NLRP3⁺ TAMs to be enriched in neutrophil-infiltrated tumor areas (**Fig 6A**). However, unlike NLRP3⁺ TAMs, which were spatially co-enriched with live neutrophils in viable areas, SPP1⁺ TAMs were associated with areas containing necrotic tissue (**Fig 6B**). This observation prompted us to compare *NLRP3⁺* and *SPP1⁺* TAMs' transcriptomes. Differential gene expression showed that *NLRP3⁺* TAMs expressed high levels of neutrophil chemoattractant cytokines (*CXCL1*, *CXCL2*, *CXCL8*). In contrast, the most upregulated genes in *SPP1⁺* TAMs were associated with phagocytosis and lipid metabolism, including apolipoproteins (*APOC1*, *APOE*), lipid scavenger receptors (*TREM2*, *MARCO*), lipid transporter *FABP5*, cathepsins (*CTSB*, *CTSD*, *CTSZ*), and matrix metalloproteinase (*MMP9*, *MMP12*) (**Fig 6C-D**). Interestingly, SPP1 itself has been implicated in phagocytosis (47,48) and lipid metabolism (49). To further interrogate the association of SPP1⁺ TAMs with necrosis, we used a publicly available 10x Visium FFPE Human Breast Cancer sample to show that necrotic tumor areas in this specimen were enriched in *SPP1⁺* rather than *NLRP3⁺* gene expression (**Fig 6E**). These results suggest that NLRP3⁺ TAMs likely contribute to neutrophil recruitment in the TME and that the SPP1⁺ TAMs may play a role in the phagocytosis of necrotic tumor areas. It is important to note that although both IL4I1⁺ TAMs and SPP1⁺ TAMs are associated with phagocytosis and the SPP1⁺ TAMs are a subset of IL4I1⁺ TAMs, the IL4I1⁺ macrophages are enriched in viable tissue areas with cells undergoing individual cell death, whereas the SPP1⁺ TAMs are enriched in areas with large regions of hypoxic and necrotic tissue that is characterized by the presence of deceased neutrophils.

NLRP3⁺ TAMs activate NLRP3 inflammasome in the TME

NLRP3 is a pathogen- and danger-associated molecular pattern receptor known to form an intracellular complex called the inflammasome, leading to proteolytic pro-IL1 β activation and release. IL1 β is known to play a role in neutrophil recruitment in infection (50) and cancer (51). Inflammasome activation results in the assembly of proteins forming an inflammasome into a micrometer-sized protein complex called a speck (52). Speck formation can be used as a simple readout for inflammasome activation (53). Interestingly, we observed that in breast and colon cancer, the NLRP3 expression could be either seen as a diffuse expression within the macrophage cytoplasm (**Fig 6F i**, **v6Fi**) or aggregated in a single speck (**Fig 6F ii**, **v6Fii**). We found that speck-like NLRP3 aggregation, which we interpret as activated inflammasome complexes, was linked to neutrophil infiltration (**Fig 6F ii**). To confirm, we stratified BC and CRC NLRP3⁺ TAM-positive regions by whether they were enriched in NLRP3⁺ TAMs with diffuse staining or NLRP3⁺ TAMs with NLRP3 specks, and quantified the number of neutrophils. The

presence of NLRP3 specks in the cytoplasm of macrophages was associated with neutrophil tissue infiltration (**Fig 6G**). Thus, we hypothesize that assembly of the inflammasome in NLRP3⁺ TAMs likely induces IL1 β activation and secretion, which drives neutrophil infiltration (**Fig 6H**).

To extend our findings beyond cancer, we investigated whether we could detect NLRP3 inflammasome activation in Crohn's disease (CD), a type of inflammatory bowel disease associated with neutrophil infiltration. Indeed, the examination of three cases of advanced CD showed that regions with high macrophage infiltration 1) contained macrophages with NLRP3 specks and 2) were highly infiltrated by neutrophils (**Fig S7A-B**, [vS7B](#), [CD2](#), [CD3](#)). The most convincing human studies implicating inflammasome involvement in human cancer are based on SNP associations and a report that IL1 β blockade in atherosclerosis correlated with reduced incidence of lung cancer (54,55). We are the first to provide histologic evidence demonstrating inflammasome formation in human BC, CRC, and CD in human FFPE tissue sections and to demonstrate an association of the NLRP3 inflammasome formation with neutrophil infiltration.

To further validate the inflammatory properties of the NLRP3⁺ TAMs in comparison to SPP1⁺ TAMs, we profiled 31 0.5 mm² tissue regions (17 CRC, 10 BC, 4 CD) across 21 patients (13 CRC, 6 BC, 2 CD) using CosMx SMI 64-plex Human Immuno-Oncology Protein Panel. In support of pro-inflammatory polarization of the NLRP3⁺ TAMs we found that they express significantly more proteins involved in tissue inflammation including IL-18, ICAM1, IDO1 and iNOS, compared to the SPP1⁺ TAMs. In contrast, we found that the SPP1⁺ macrophages express higher levels of immune-inhibitory receptors (B7-H3, LAG3, TIM3) (**Fig 6I**, **S7C-D**, [vS7Ci](#), [vS7Cr](#), [vS7Di](#), [vS7Dr](#)).

Taken together, these results suggest that NLRP3⁺ TAMs may be involved in the onset of inflammation by activating the NLRP3 inflammasome and may be driving neutrophil infiltration in human cancer and Crohn's disease. In contrast, SPP1⁺ TAMs seem to be playing a role in tissue repair by clearing necrotic lesions in hypoxic tumor areas.

High IL4I1⁺ TAM numbers predict good while high SPP1⁺ TAM numbers predict bad patient outcomes in CRC

IF and scRNAseq studies of IL4I1⁺ TAMs (**Fig 4A,E,K**) and SPP1⁺ TAMs (**Fig 6C,D**) suggest that both cell types perform phagocytosis in the TME. We next sought to elucidate the biological impact IL4I1⁺ TAMs and SPP1⁺ TAMs have on the tumor biology by correlating their abundance with clinical outcome in CRC patients. Since scRNAseq suggested that *IL4I1* is also expressed in SPP1⁺ TAMs, we first stained for both IL4I1 and SPP1 simultaneously to learn to what extent the IL4I1 and SPP1 are co-expressed in a cell on the protein level (**Fig 7A-D**, [v7A](#)). By analyzing 254 CRC regions from 135 patients we found that 56% of cells detected by IL4I1 and SPP1 staining were IL4I1 single positive (IL4I1⁺ TAMs), 35% were SPP1 single positive (SPP1⁺ TAMs), and 7% displayed a co-expression of IL4I1 and SPP1 (IL4I1⁺SPP1⁺ TAMs) (**Fig 7B-D**).

SPP1 and IL4I1 can be also expressed in epithelial cells, thus we stained with CD68, and IL4I1 or SPP1 antibodies simultaneously to quantify infiltration of IL4I1⁺ TAMs and SPP1⁺ TAMs in

CRC (**Fig 7E, 7F, v7E, v7F**). Previous reports showed that macrophage subtype signatures, including that of SPP1⁺ TAM (13,21,56) and FOLR2⁺ TRM (17), are predictive of clinical outcome in cancer. However, the association of IL411⁺ TAMs with clinical outcomes remains unexplored and existing reports of SPP1⁺ TAMs associations with outcome in CRC are based on RNA expression (13,21). Since our data suggests that both IL411⁺ TAMs and SPP1⁺ TAMs are involved in tumor phagocytosis, we expected to see that high numbers of both TAM populations would be associated with favorable outcomes. This would be also in line with previous reports showing that CD68⁺ macrophage infiltration in CRC is associated with a good outcome (4,20). However, our analysis revealed that while IL411⁺ TAMs are strong predictors of favorable outcome in two independent CRC cohorts (**Fig 7G-H, S8A-D**), the SPP1⁺ TAMs are associated with poor outcomes in CRC (**Fig 7I, S8E-F**). This is consistent with the spatial associations of SPP1⁺ TAMs and tumor necrosis and hypoxia (**Fig S5E, 5B, 6B, 6E**), and the fact that both tumor necrosis and hypoxia are hallmarks of tumor aggressiveness (57–59). To the best of our knowledge, this is the first protein-based evidence showing opposite clinical outcome correlations between two TAM populations in any solid tumor. This suggests a division of labor between different TAM populations, which may contribute differently to patient outcomes.

Discussion

This work reveals a rich landscape of human macrophage populations, and their spatially segregated niches, conserved across healthy and cancerous human breast and colon tissue. These macrophage populations were not randomly distributed across the tissue; rather, each macrophage subset occupied a discrete multicellular niche. For example, we found IL411⁺ macrophages are embedded in areas enriched in cell death in the desmoplastic stroma at the invasive front of the tumor across ten different tumor types, in the upper lamina propria of colonic mucosa, and in LN germinal centers. Thus, our findings indicate that macrophage populations, and the unique niches they occupy, are fundamental tissue “building blocks” reiteratively repeated within and across tissues. In addition, we uncover some of the incoming and outgoing signals governing the macrophage niche. For example, we are the first to histologically identify NLRP3 inflammasome aggregation in human cancer and to show that it is associated with neutrophil recruitment.

It has been recognized that TRMs across different organs exhibit specialized functions reflecting local tissue physiology (8). However, we are the first to uncover the existence of distinct functional spatial niches harboring discrete macrophage populations and cellular compositions within a single organ system in humans. In particular, we reveal the existence of four separate macrophage niches in the bowel wall, including a phagocytic IL411⁺ TAM niche, a novel FOLR2⁺ TRMs plasma cell niche, a perivascular LYVE1⁺FOLR2⁺ TRMs niche in the bowel submucosa, and a smooth muscle FOLR2⁺ TRMs niche in the muscularis propria.

Notably, our results reveal that IL411⁺, SPP1⁺, and NLRP3⁺ TAM niches are closely associated with tumor nests and implicated in the response to cancer, including individual tumor cell death, hypoxia and diffuse tissue necrosis, and acute inflammation, respectively. Surprisingly, we

found opposite clinical outcome associations of IL411⁺ TAMs and SPP1⁺ TAMs in CRC: high IL411⁺ TAM numbers are associated with better overall survival, whereas high SPP1⁺ TAM numbers are associated with worse overall survival, hinting that these TAM populations might function dichotomously. Our results inform future TAM-directed immunotherapies, implying that targeting specific TAM populations might be therapeutically important, in contrast to current therapies that monolithically repress all TAMs.

In addition, IL411⁺ TAMs might be implicated in response to anti-CD47 and anti-PD-L1 therapy as they express the CD47 ligand- *SIRPA* and *CD274* encoding PD-L1, and correlate with anti-PD1 treatment response. Moreover, we show that NLRP3 inflammasome activation correlates with acute inflammation in BC, CRC, and CD. These findings nominate the NLRP3 inflammasome as a novel therapy target where its specific small molecule inhibitor - MCC950 (60) could function as a novel therapeutic agent in solid tumors and CD.

Collectively, our findings elucidate a landscape of discrete human macrophage niches, uncover unexpected cell interactions and mechanisms governing the macrophage niche biology, explore the prognostic and predictive significance, and suggest novel therapy targets (**Fig S9**). Importantly, since the antibody tools we present are FFPE-compatible, they enable the use of archival clinical material and provide a framework for the entire field to study human macrophage function in health and disease. All images acquired during this work are publicly accessible via an interactive browser ([https://magdalenamat.github.io/Spatial MAC Map/](https://magdalenamat.github.io/Spatial_MAC_Map/)), thus providing a resource for human macrophage research.

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Author contributions

M.M., R.W., and M.v.d.R conceived of the study. M.M., J.W.H., G.L., D.P., G.P.N., B.L. and A.M.N. designed and performed experiments with assistance from S.W.B., S.Z., D.R.C.C. M.M. analyzed the data with assistance from J.W.H., B.L., G.L., L.K., D.G.P.v.IJ., D.K.O., K.M.L. and A.M.N. M.M. and M.V.d.R wrote the paper. G.C., J.S., R.W., M.v.d.R procured tissue specimens and assisted in data interpretation. All authors commented on the manuscript at all stages.

Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contacts Magdalena Matusiak (mmatusia@stanford.edu) and Matt van de Rijn (mrijn@stanford.edu).

Materials Availability

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Patient Samples

All clinical specimens in this study were collected from patients with written informed consent for research use. The studies were conducted in accordance with the Declaration of Helsinki and approved by the Stanford University Institutional Review Board.

Breast and colon cohorts FFPE samples

This study used FFPE samples from 36 invasive breast cancer (IBC) and 32 colon carcinoma (CRC) cases to identify differential spatial distribution of the FOLR2⁺, LYVE1⁺, IL4I1⁺, SPP1⁺, and NLRP3⁺ macrophages in benign and malignant breast and colon tissue.

Crohn's Disease FFPE samples

We performed the analysis in **Fig 6A-B**, using three advanced Crohn's Disease patient FFPE samples.

Various solid tumor samples

The images in **Fig S2G-N** were generated using a single FFPE case sample per indication.

IF and CODEX Tissue microarrays

The tissue microarrays used in IF and CODEX experiments in this study were constructed from 36 1.5 mm² regions from 19 CRC cases, and 29 1.5 mm² regions from 18 IBC cases. Regions were selected based on differential spatial staining observed on full section staining with IL4I1, SPP1 and FOLR2 antibodies.

CosMx Tissue microarray

The CosMx tissue microarray was constructed from 31 1mm² tissue regions from 21 CRC, BC and CD cases. Regions were selected based on differential spatial staining observed on full section staining with SPP1, NLRP3, IL4I1, and CD68 antibodies.

METHOD DETAILS

External datasets

Single-cell RNA-seq tumor atlases

Publicly available scRNA Seq datasets analyzed in this study are available under following links: Qian et al. (22) and available under <https://lambrechtslab.sites.vib.be/en/pan-cancer-blueprint-tumour-microenvironment-0>, CRC data from Lee et al. (21) available in the NCBI Gene Expression Omnibus (GEO) database under the accession codes GSE132465, GSE132257 and GSE144735, and data from Bassez et al. (23) available at <https://lambrechtslab.sites.vib.be/en/single-cell>.

We obtained preprocessed scRNA-seq count data from four datasets covering breast carcinoma (BC), and colon carcinoma (CRC). For each dataset, we extracted monocytes, macrophages, and dendritic cells by clustering SCTransformed count data using Seurat and subsetting clusters expressing *AIF1* (encoding IBA1), *CST3*, *CD68*, *CD163*, *ITGAX*, and *HLA-DRA*.

Next, we integrated the myeloid clusters from the 4 datasets using the reciprocal PCA workflow with Seurat. We used log normalization. To clean the data we excluded dying cells, stressed cells, and cell duplets. We identified dying cells' clusters by inspecting the distribution of $\log_2(\text{nCount_RNA}+1)$ per cell. Stressed cells were identified based on high expression levels of HSP genes. Cell duplets were identified based on the coexpression of non-myeloid cell markers as follows: myeloid-epithelial cell (*TFF3*, keratins), myeloid-Tcells (*CD3D*), myeloid-stromal cells (*SPARCL1*, *SPARC*, *COL1A1*), and myeloid-plasma (immunoglobulin genes). Since we intended to focus exclusively on monocytes and macrophages, we excluded neutrophils and dendritic cell clusters identified based on the following gene enrichment: neutrophils (*SOD2*, *GOS2*, and low detected number of counts per cell), cDC1s (*CLEC9A*), cDC2s (*FCER1A*, *CD1C*, *CD1E*, and *CLEC10A*), migratoryDC (*BIRC3*, *CCR7*, *LAMP3*), follicular DC (*FDCSP* and immunoglobulin genes), plasmacytoid DC (*JCHAIN*, *LILRA4*, *IRF7*), CD207⁺ DC (*CD1A*, *CD207*, *FCAR1A*). Next, we re-clustered the integrated and cleaned Seurat object containing only monocytes and macrophages with resolution = 0.6 in the *FindClusters()* function. We obtained 15 clusters and annotated them based on the most differentially expressed genes in each cluster. Monocytes have been identified by *FN1*, *FCGR3A*, and *VCAN*. Macrophages were identified based on *C1QA*, *APOE*, and *TREM2* expression. We merged clusters 0 and 12 into ISG15 TAMs, clusters 1, 6, and 14 into CXCL9 TAMs, and clusters 11 and 13 into LYVE1⁺FOLR2⁺ TRMs. The resulting myeloid object is presented in **Fig 1C**.

Spatial transcriptomics

We obtained pre-processed spatial transcriptomic data from Human Breast Cancer: Ductal Carcinoma In Situ, Invasive Carcinoma (FFPE) sample data from 10x website <https://www.10xgenomics.com/resources/datasets/human-breast-cancer-ductal-carcinoma-in-situ-invasive-carcinoma-ffpe-1-standard-1-3-0> (Fig 6E).

Enrichment of monocyte and macrophage scRNA Seq populations

For the analysis in **Figs 1F, S1C-D,F-G**, we selected samples with more than 35 monocyte and macrophage cells and computed the frequency of the different scRNA subsets in each sample. **Figs S1C-D,F**, we present these frequencies stratified by tumor type and anatomical location. In addition, for **Fig 1F** and **S1F**, we computed a mean frequency for every scRNA subset and calculated a ratio of its frequency between BC and CRC (**Fig 1F**) and normal colon and CRC (**Fig S1F**).

Average cluster gene expression

The average gene expression dotplots per scRNA monocyte and macrophage clusters in **Fig1D, 2A, 4F, 6D** were plotted using the aggregated myeloid object from Fig 1C.

Spatial transcriptomics dataset processing and visualization

For the analysis in **Fig 6E**, we used STUtility r package to normalize, annotate and visualize the pre-processed spatial transcriptomic data. Specifically, we used the SCTransform function for normalization and the ManualAnnotation function to annotate data based on the H&E image.

Immunohistochemistry

For the analysis in **Fig S6A** and **6A-B**, 4 μ m tissue sections were deparaffinized and rehydrated. Subsequently, antigen retrieval was performed in EDTA pH 9 buffer for 5 min at 95 °C in a pressure cooker. Slides were next stained with FOLR2, SPP1 or NLRP3 antibodies listed in Table S1, and imaged with a Keyence BZ-X800 microscope at 20' magnification.

Immunofluorescence (IF)

For the analyses shown in Fig 1G, 2D-G, S2A-N, 3B-F, 4A-D, S6B-EI, 6F, S7A,C, 7A,E-F 4 μ m full tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed using EDTA pH 9 buffer at 95 °C for 10 min. Sections were blocked for 20 min with horse serum and stained for 1h with primary antibodies. Sections were subsequently stained with secondary antibodies for 1 h. A list of primary and secondary antibodies used in this work can be found in Table S1. Sections were then mounted in ProLong Gold Antifade reagent with DAPI and cover-slipped. Stained sections were imaged with a Keyence BZ-X800 microscope at 20' or 40' magnification. Of note, LYVE1 is expressed on both TRMs and lymphatic endothelial cells. Yet, lymphatic endothelial cells can be readily differentiated from TRMs as they are organized in tubes, display much higher LYVE1 expression than TRMs, and do not express FOLR2 and MARCO (Fig 3C,D,F).

IF images dearraing

IF images were acquired with a Keyence BZ-X800 microscope at 20' magnification. Next, the TMA core coordinates were extracted using the dearray functionality in QuPath (61).

Subsequently, the TIFF TMA images were dearrayed using QuPath extracted core coordinates with vips crop function in Linux command line.

IF images cell segmentation and immunofluorescence signal quantification

Cell nuclei on the dearrayed TMA cores were segmented using Mesmer (62). Subsequently, IF signal was quantified for each detected nuclei by computing staining intensity within 3-pixel distance from the nuclear border. We consider a nucleus and its accompanying IF signal within 3-pixel distance from the nuclear border as a cell. In effect, each cell is described by its x and y pixel coordinate and IF staining intensity.

Clustering and annotation of IF data

Each individual IF staining was clustered separately. First, IF staining intensity was z normalized using zscore function from scipy.stats python module. Next, cells were clustered using Leiden clustering implementation in scanpy python package. All clusters were individually visually inspected on the dearrayed TIFF images by indicating location of cells attributed to a given cluster. Cell clusters were annotated based on morphology, location, and staining intensity.

For Fig S6C, we clustered and annotated cells from 7 1.5 mm² tissue regions including 6 BC and 1 CRC cases. We used FOLR2, IL4I1, and CD138 staining intensity to discriminate FOLR2 TRMs, IL4I1 TAMs and PCs, respectively.

Distance quantification of IF and CODEX data

For every TMA core, the distance between every cell and every other cell present in the core was computed using cdist function from scipy.spatial.distance python module. Next, for every macrophage, the shortest distance to a Tumor Cell was selected from the matrix of all cell distances. This shortest distance is reported as the distance to the closest Tumor Cell. For CODEX data, normal breast and gastrointestinal tract samples were excluded.

Significance assessment within one tissue region

Wilcox test was used to assess the significance in Figures 2D-G.

Significance assessment across multiple tissue regions

Linear mixed-effect models were used to assess significance in Figures 2B-C, 5C, S4C, S6G. We used the lmer function from package lme4 (v1.1.21), and took the tissue region intercept as a random effect. The pairwise p-values were derived from t-ratio statistics in the contrast analysis using the lmerTest (v3.1.2) and corrected for multiple hypothesis testing using the Holm Bonferroni method implemented in the modelbased (v0.1.2) package(github.com/easystats/modelbased).

CODEX macrophage distance quantification by niche

For distance quantification in Fig 5C, macrophages were stratified by the macrophage niche they belong to.

CODEX antibody panel

The antibody panel in this study was constructed by selecting antibodies targeting epithelial and stromal tumor compartments, with a focus on the myeloid compartment. Detailed information on the included antibodies can be found in Table S2. Each antibody was first conjugated to a unique oligonucleotide tag. Next, antibody-oligonucleotide conjugates were tested in low-plex fluorescence assay to determine whether their staining patterns match patterns established in IHC and IF experiments and to establish the best staining concentration and exposure time. Subsequently, all antibody conjugates were tested together in a single test CODEX imaging multicycle to evaluate optimal concentration, exposure time, and imaging cycle.

CODEX imaging

CODEX imaging was performed as previously described (34). BC and CRC tissue microarrays were simultaneously stained with a previously validated cocktail of antibody-oligonucleotide conjugates and sequentially subjected to CODEX multiplexed imaging using the optimized conditions established during the test run. Metadata with detailed information on each CODEX run can be found in Table S3.

CODEX data processing

CODEX imaging data was processed using a software tool called RAPID (Lu G, et al. Manuscript under review, 2022), which included 3D GPU-based deconvolution, spatial drift correction, image stitching, and background subtraction (available at <https://github.com/nolanlab/RAPID>). Next, cell nuclei segmentation on the processed images was performed using a neural network-based segmentation algorithm called CellVisionSegmenter. CellVisionSegmenter has been shown to work well with segmenting both dense and diffuse cellular tissues with CODEX data (63). CellVisionSegmenter is an open-source, pre-trained nucleus segmentation and signal quantification software based on the Mask region-convolutional neural network (R-CNN) architecture. The only parameter that was altered was the growth pixels of the nuclear mask, which we found experimentally to work best at a value of 3.

CODEX data clustering, visualization, and cell type assignment

Cell clustering and annotation were performed according to a previously published protocol (37). First, nucleated cells were selected by subsetting cells with positive Hoechst signal imaged in 2 separate CODEX cycles. Next, marker signal intensity was z-normalized, and data was overclustered using Leiden clustering in scanpy Python package. Each cluster was visually

examined by mapping a location of cells attributed to a given cluster to processed CODEX images and inspecting its marker staining. ImageJ was used to view processed CODEX images. Cell clusters were annotated based on cell morphology, tissue location, and marker staining intensity.

CODEX niche analysis

Niche analysis was performed as described earlier by Schurch et al. (38) with $k = 10$ nearest neighbors and 30 clusters. The cell clusters were annotated and grouped into 13 Niches based on location in the tissue and cell type enrichment score.

Ligand-Receptor interaction analysis

Ligand-Receptor analysis was performed using CellChat R package workflow with default settings and using `netVisual_bubble` function to extract all identified significant ligand-receptor interactions between FOLR2 TRMs and Plasma Cells (PCs). For the analysis in Fig S6I, IgA⁺ and IgG⁺ PCs annotation was extracted from Lee et al. (21). For Fig S6H, PCs were identified using `FindClusters` Seurat function with $res = 0.4$, and selecting cluster #19 with high CD38 and JCHAIN expression. Fig S6H shows all detected significant interactions between FOLR2 TRMs and PCs. Fig S6I shows 10 top significant interactions detected between FOLR2 TRMs and IgA⁺ and IgG⁺ PCs.

Gene Set Enrichment Analysis

KEGG pathway gene set enrichment analysis from Fig 4E was performed using `clusterProfiler` R package. The KEGG enrichment was performed on the list of differentially enriched genes between the 11 transcriptional MAC scRNA Seq populations. Next, enrichment results of Antigen processing and presentation, Phagosome, Lysosome, and Endocytosis gene sets were plotted to compare enrichment of phagocytosis-related pathways between the scRNA MAC populations.

Pembrolizumab response analysis

For the analysis in Fig 4G-H, was performed on scRNA myeloid transcriptomes from Bassez et al. (23), that we subsetted from the aggregated myeloid object from Fig 1C. The patient samples were stratified by the authors of the original publication based on whether the T cell repertoire, as assessed by TCR sequencing, expanded (E) or not (NE) after the pembrolizumab administration. We labeled patients with expanded T cell repertoire as responders (R) and patients with non-expanded T cell repertoire as non-responders (NR).

For the analysis in Fig 4H, we used scRNA monocyte and macrophage transcriptomes of responders and non-responders pre pembrolizumab treatment. We first computed scRNA cluster frequencies in patients with more than 35 monocyte and macrophage cells. Next we compared the mean scRNA cluster frequencies with Chi-squared test using `chisq.test` function

from stats R package and used `chisq.posthoc.test` function from `chisq.posthoc.test` R package to assess significance. p values were adjusting using Bonferroni correction.

Neutrophil infiltration quantification in BC, CRC, and Crohn's Disease

For the analysis in Fig 6G, we counted the number of neutrophils present in 1.5 mm² tissue microarray(TMA) cores. The IF-stained TMA cores were evaluated by a pathologist and stratified into cores containing CD68 positive macrophages with diffuse NLRP3 staining or cores that contained CD68 positive macrophages with NLRP3 aggregated in a speck. Cores that contained both diffused and aggregated NLRP3 were classified as cores with NLRP3 speck, as we assumed that the NLRP3 aggregation contains an active inflammasome complex that projects the inflammatory signaling. For the analysis in Fig S7B, we counted the number of neutrophils in 1mm² tissue regions selected from whole slide sections. We selected areas that contained CD68 positive macrophages containing NLRP3 aggregated in a speck. Since we didn't detect any macrophages with NLRP3 diffuse staining in the Crohn's disease tissue sections, we compared the neutrophil numbers in Crohn's disease patients to benign colon submucosa. CD68 and NLRP3 signals were used to identify NLRP3 TAMs, and Calprotectin was used to identify neutrophils.

CosMx Protein profiling

For the analysis in Fig 6I and S7D, CosMx protein data were produced by NanoString company as a service using the CosMx SMI 64-plex Human Immuno-Oncology Protein Panel. Images were processed and data generated using NanoString proprietary pipelines. Raw data were analyzed using Seurat package in R. Regions to profile were selected based on SPP1, NLRP3 and CD68 staining on the TMA section immediately adjacent to the section profiled in the CosMx assay (Fig S7C).

Survival analyses

For the analyses in Fig7 F-H and FigS8, we stained 2 CRC tissue microarrays with a combination of CD68 and IL4I1 (TA118 and TA379), and CD68 and SPP1 (TA118). The IL4I1 TAM and SPP1 TAM counts detected on each tissue core were normalized by multiplying by 100 and dividing by the total cell number detected in each core. TA118 and TA379 contain 2 tissue cores per patient. For this analysis we selected a core with a higher number of detected total cells per core. Next we used R packages *survival* (v 3.5.3) and *survminer* (v 0.4.9) to fit and plot Kaplan-Meier survival curves. Survival curves were compared using the Log-Rank test.

QUANTIFICATION AND STATISTICAL ANALYSIS

Wilcoxon test was applied for group comparisons. Linear mixed effect models were applied when groups contained multiple observations from the same tissue region (for instance, when comparing the distance of macrophages to tumor cells across multiple tissue regions). Results with $P < 0.05$ were considered significant. Data analyses were performed with R and python. The investigators were not blinded to allocation during experiments and outcome assessment.

No sample-size estimates were performed to ensure adequate power to detect a pre-specified effect size.

Data and Code Availability

All IF and CODEX images presented in the figures are available for viewing and download under https://magdalenamat.github.io/Spatial_MAC_Map/#/.

The myeloid scRNAseq object, CODEX and IF images, associated immunofluorescence single cell data and annotations in tabular format, as well as all Python and R scripts used in this work can be found https://magdalenamat.github.io/Spatial_MAC_Map/#/files.

Interactive Histological Image Viewer

Our project's histological images, accessible via [GitHub](#), can be investigated using an interactive image viewer we developed. This viewer is based on the [OpenSeadragon Viewer](#), allowing high-resolution, deep-zoom image viewing. The user interface was constructed with [Vue.js](#). All images were tiled to enable fast loading using the [ImmunoViewer python package](#). Image data is hosted on the Google Cloud Platform. The viewer's source code is also available on our [project's GitHub page](#):

https://github.com/MagdalenaMat/Spatial_MAC_Map/tree/main/browser

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Figure Legends

Fig 1. Identification of macrophage subset markers by single-cell RNA-sequencing (A and B) Flow charts of experimental design. **(C)** UMAP projection of monocyte and macrophage scRNA transcriptomes from 4 studies colored by annotated populations (*left*) and a breakdown of cells, samples and patient numbers by study (*right*). **(D)** Dotplot of average marker gene expression per scRNA myeloid population. Highlighted in bold are 6 markers for which FFPE-compatible antibodies were identified. **(E)** Volcano plot shows top differentially expressed genes between FOLR2⁺, LYVE1⁻ and FOLR2⁺, LYVE1⁺ TRMs. **(F)** Barplot of the ratio of log₂ average fractional scRNA myeloid population enrichment between CRC and BC in tumor samples with more than 35 monocytes and macrophages detected. **(G)** Immunofluorescence images show overlap of the established FFPE antibodies and CD68, confirming their reactivity with macrophages.

Fig 2. FOLR2, IL4I1, NLRP3, and SPP1 mark spatially distinct macrophage niches in the TME (A) Dotplot shows average macrophage marker expression in scRNA macrophage populations and indicates which scRNA macrophage populations are detectable in 4-color IF staining by anti-NLRP3, -SPP1, -IL4I1, -FOLR2, and a combination of anti-FOLR2, -LYVE1 and -MARCO antibodies. **(B)** Distance (μm) of CD68 and CD63 macrophages to the closest tumor cell. **(C)** Distance (μm) of IL4I1 TAMs, NLRP3 TAMs, SPP1 TAMs, FOLR2 TAMs to the closest

tumor cell. **(B,C)** Cells were identified on CODEX images, *P values* were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. **(D-G)** *Left*: CODEX image **(D)** or Immunofluorescence (IF) images **(E,F,G)** show the distribution of CD68 and CD163 **(D)**, or FOLR2 and IL4I1 **(E)**, NLRP3 **(F)**, SPP1 **(G)** protein expression in representative cases of CRC **(D,E,G)** and BC **(F)**. PanCK marks tumor cells. Close-up images on the bottom correspond to boxed regions on the top. *Top right*: Scatterplots show the distribution of CD68 Macs, CD163 Macs, FOLR2 TRMs, IL4I1 TAMs, NLRP3 TAMs, SPP1 TAMs corresponding to IF images on the left. *Bottom right*: Boxplots show the distance quantification of each macrophage to the closest tumor cell corresponding cells identified on IF images on the left. Pairwise comparisons were determined using a two-sided Wilcoxon rank-sum test on 1092 **(D)** 580 **(E)**, 739 **(F)**, and 203 **(G)** cells.

Fig 3. IL4I1, FOLR2, LYVE1, and MARCO label spatially segregated TRM niches in normal Colon and Breast **(A)** The schematic shows the distribution of TRM populations in normal colon mucosa and submucosa (*left*) and around normal breast glands (*right*). **(B-C)** IF images show 3 TRM layers marked by IL4I1, FOLR2, and LYVE1 in normal colon mucosa and submucosa. Note that LYVE1 also stains normal lymph vessels. **(D)** IF image shows that FOLR2⁺, LYVE1⁺ TRMs in normal colon submucosa are MARCO⁺. **(E-F)** IF images show TRMs in normal breast marked by FOLR2, LYVE1, and MARCO, depending on whether they are Lobular (i) or Peri-vascular (ii). **(B-F)** Close-up images correspond to boxed regions. The scale bar of 10 μm is identical for all close-up images.

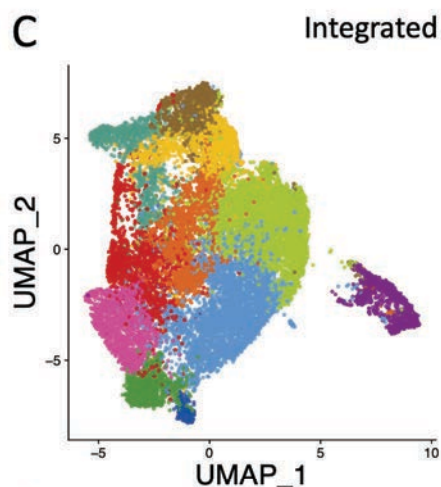
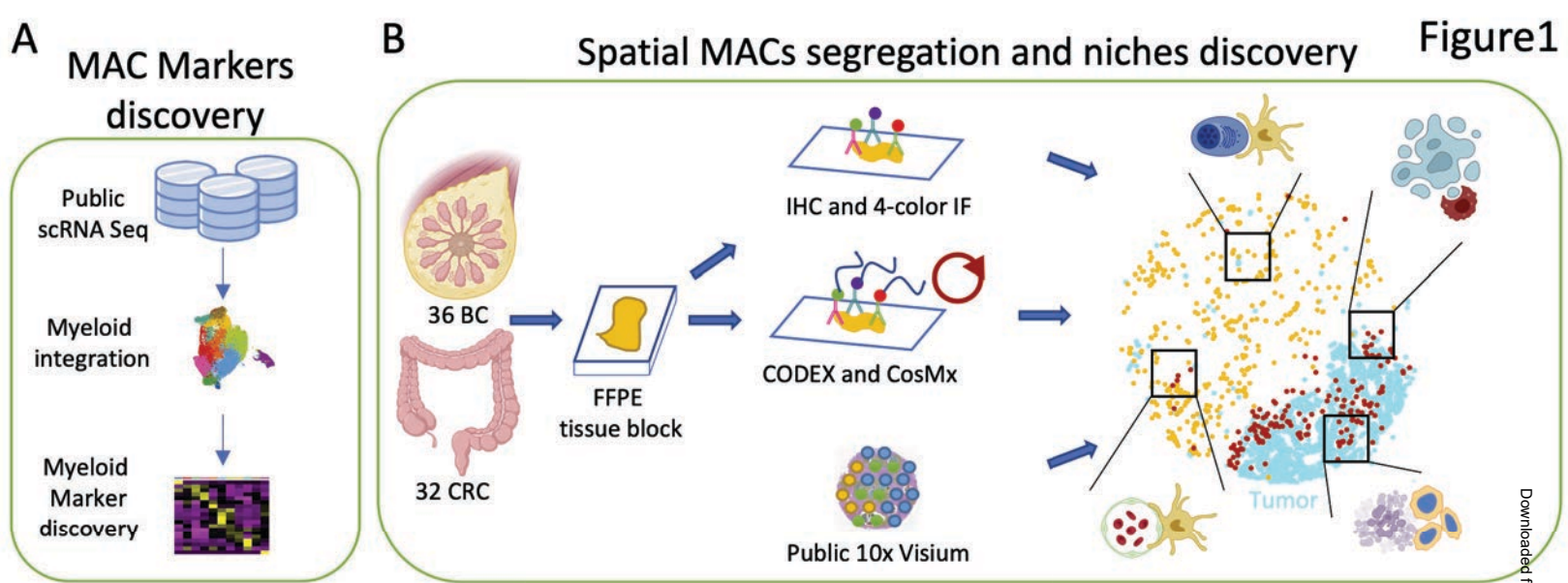
Fig 4. IL4I1 marks phagocytosing macrophages **(A)** IF images of invasive front of CRC stained with IL4I1, IBA1, panCK, and DAPI show the presence of panCK⁺ material within IL4I1 macrophages. **(B)** Same as **(A)** but stained with IL4I1, CD68 and panCK in normal colon mucosa. **(C)** IF images of normal Lymph Node stained with IL4I1, FOLR2, IBA1 and DAPI. (i) is a close-up image of a germinal center tingible body macrophage (TBM), (ii) is a close-up image of interfollicular FOLR2 TRMs **(A-C)** Close-up images on the right correspond to the boxed region on the left. **(D)** Images of TBMs in Burkitt's lymphoma stained with *left*: H&E and *right*: IL4I1 and DAPI. **(E)** *Top*: KEGG pathways enrichment analysis of phagocytosis-related pathways across scRNA macrophage populations. Populations with no significantly enriched pathways were omitted. *Bottom*: average IL4I1 gene expression across scRNA macrophage populations with enriched phagocytosis-related gene sets. **(F)** Dotplot shows average gene expression in scRNA macrophage populations. **(G)** Barplots show frequency of scRNA monocyte and macrophage clusters in dataset from Bassez et al. (23), stratified by response to pembrolizumab and time of sample collection. **(H)** Boxplots show frequency of scRNA monocyte and macrophage clusters pre pembrolizumab treatment from Bassez et al. (23) **(I-J)** Same as **F** but from **(I)** Bassez et al. dataset (23), and **(J)** across Qian et al. (22) and Lee et al. (21) datasets. **(K)** Schematic illustrating IL4I1 TAM association with cell death and efferocytosis and highlighting IL4I1 TAMs as potential anti-CD47 (indirect as IL4I1 TAMs express CD47 ligand-SIRP1 α) and anti-PD-L1 (direct) therapy targets.

Fig 5. CODEX reveals spatial cellular interactions in macrophage niches within colon and breast cancer tissues (A) Schematic shows CODEX imaging and cellular neighborhood analysis workflow. (B) Heatmap shows CODEX cell types (x axis) enrichment (color) in the identified cellular neighborhoods (y axis). (C) Boxplot shows distance (μm) to the closest tumor cell for every macrophage identified by CODEX labeled by the neighborhood it belongs to. (D) Barplot shows a percentage of the epithelial cells occupied in each CODEX macrophage neighborhood. (E) Barplot presents the frequency of CODEX macrophage neighborhoods grouped by anatomical location. NB-normal breast, DCIS-ductal carcinoma in situ breast, IDC-invasive ductal carcinoma breast, NGI-normal GI tract, IF invasive front CRC, CT-center of tumor CRC. (F) Schematic shows cellular macrophage neighborhood organization and closeness to the tumor.

Fig 6. SPP1 TAMs are enriched in hypoxic and necrotic tumor areas and NLRP3 TAMs activate NLRP3 inflammasome in the TME (A) Immunohistochemical image shows NLRP3 TRMs surrounded by neutrophils (arrowheads). (B) Immunohistochemical image shows SPP1 TRMs surrounded by karyorrhectic debris in necrotic material (arrowheads). (C) Volcano plot shows differential gene expression between scRNA transcriptomes of SPP1 TAMs and NLRP3 TAMs. (D) Dotplot of average expression of genes associated with neutrophil chemoattraction, lipid metabolism and phagocytosis across scRNA macrophage populations. (E) Dotplot shows the annotation of Tumor (green) and Necrotic (brown) areas (*top left*) and normalized expression of SPP1 (*top right*) and NLRP3 (*bottom right*) on the 10x Visium FFPE Human Breast Cancer sample, and barplot shows normalized log₂ SPP1 expression in Tumor and Necrosis regions (*bottom left*). (F) Immunofluorescence (IF) shows a representative BC region stained with NLRP3, CD68, Calprotectin (CPTN) and DAPI. Scale bar of 10 μm is identical for all close-up images. (G) Quantification of the number of neutrophils present on 9 BC 1.5 mm² tissue regions stratified by whether they contained diffuse NLRP3 (3 regions) or NLRP3 specks (6 regions). *P* value was computed using a two-sided Wilcoxon's rank-sum test. (H) Schematic of a possible mechanism through which NLRP3 TAMs can contribute to the recruitment of neutrophils in the TME. (I) Sina plots show marker protein expression profiled using CosMx SMI 64-plex Human Immuno-Oncology Protein Panel.

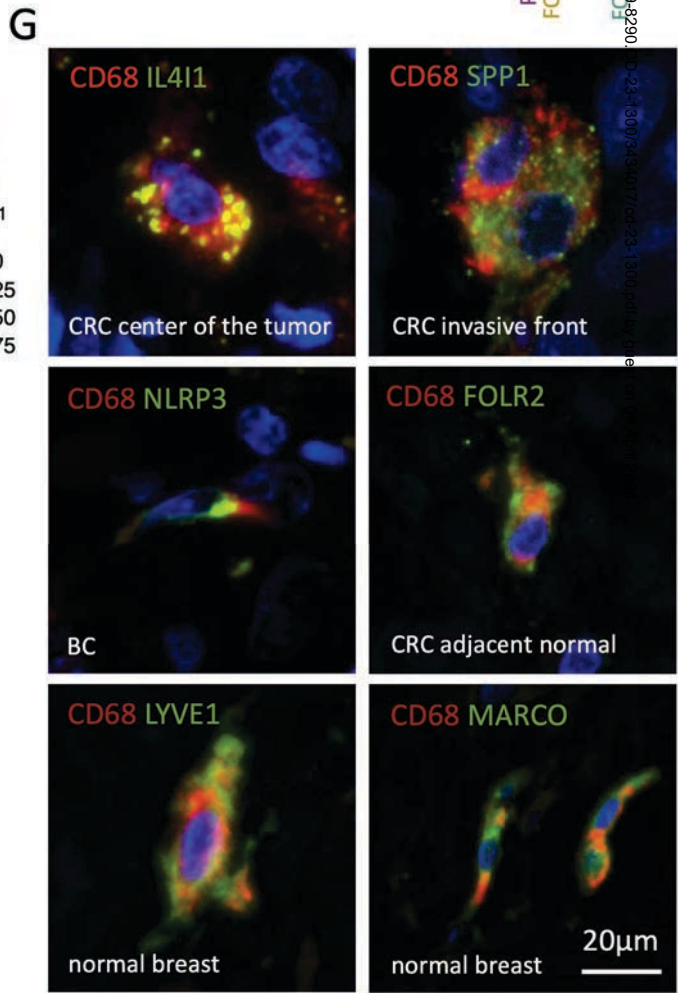
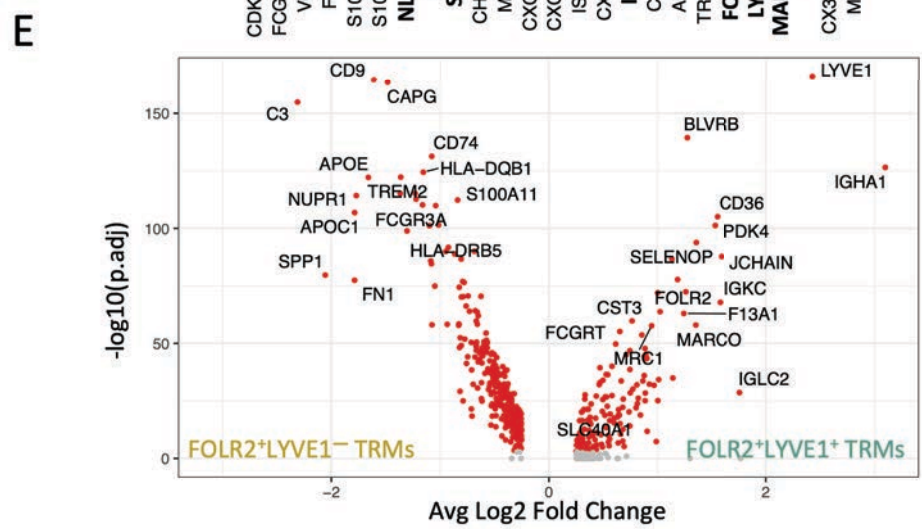
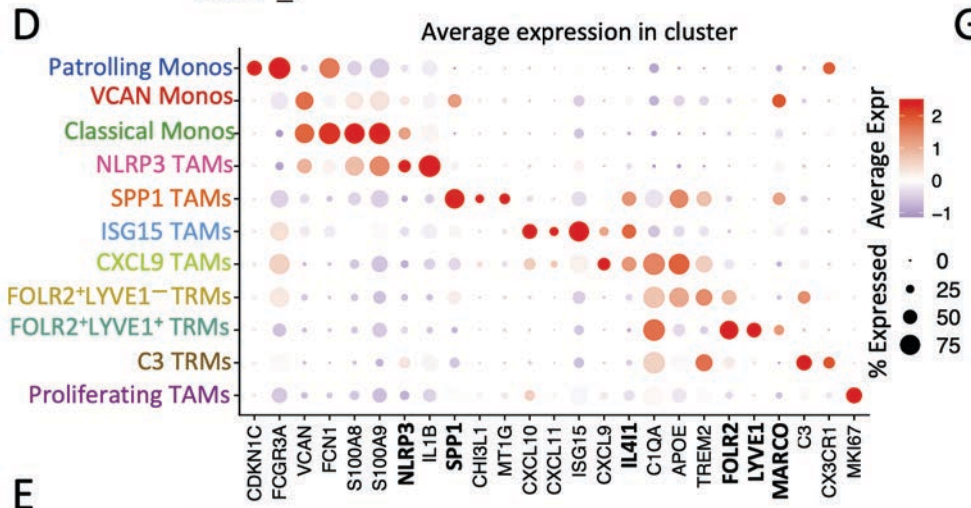
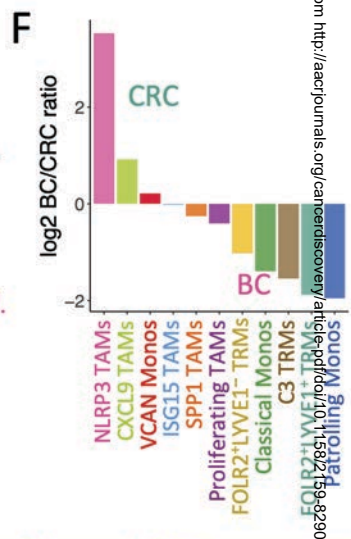
Fig 7. IL4I1 TAM infiltration predicts good and SPP1 TAM infiltration predicts bad outcome in CRC

(A) IF image shows spatial distribution of SPP1 and IL4I1 staining in CRC. (B) Heatmap shows normalized IF intensity in cells annotated as IL4I1 TAMs, SPP1 TAMs and IL4I1⁺SPP1⁺ double positive TAMs. (C-D) Total proportion (C) and proportion by tissue region (D) of IL4I1 TAMs, SPP1 TAMs and IL4I1⁺SPP1⁺ double positive TAMs detected in 254 0.5 mm² tissue regions spanning 135 CRC patients. (E) IF image of a single tissue core from TA118 used for outcome analysis in H, shows CD68 and IL4I1 protein expression in cells annotated as IL4I1 TAMs. (F) IF image of a single tissue core from TA118 used for outcome analysis in I, shows CD68 and SPP1 protein expression in cells annotated as SPP1 TAMs. (G-I) Kaplan-Meier plot of CRC patient samples stratified by normalized count of IL4I1 TAMs (G,H), or SPP1 TAMs (I). Presented are 40% top and 40% bottom cases.

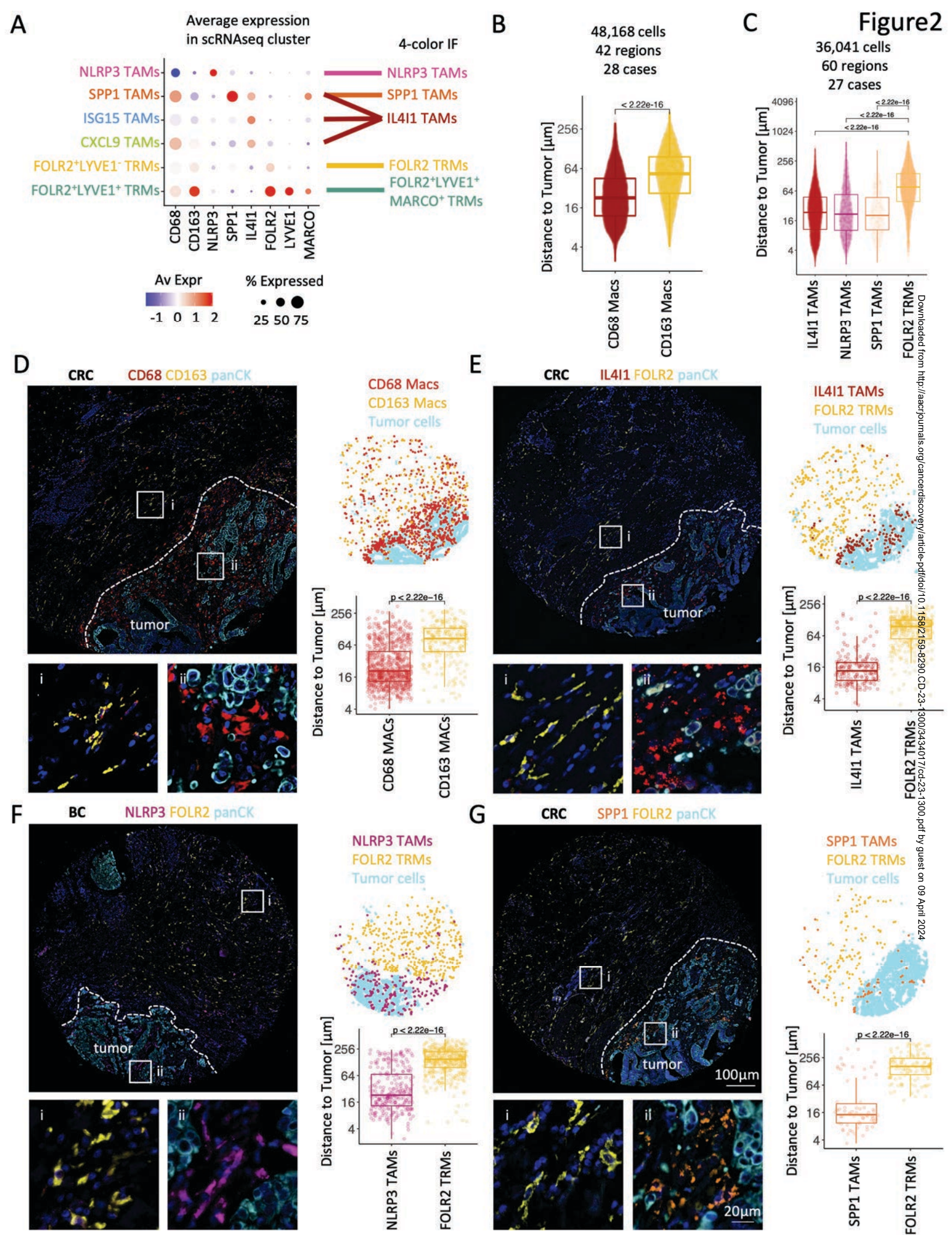


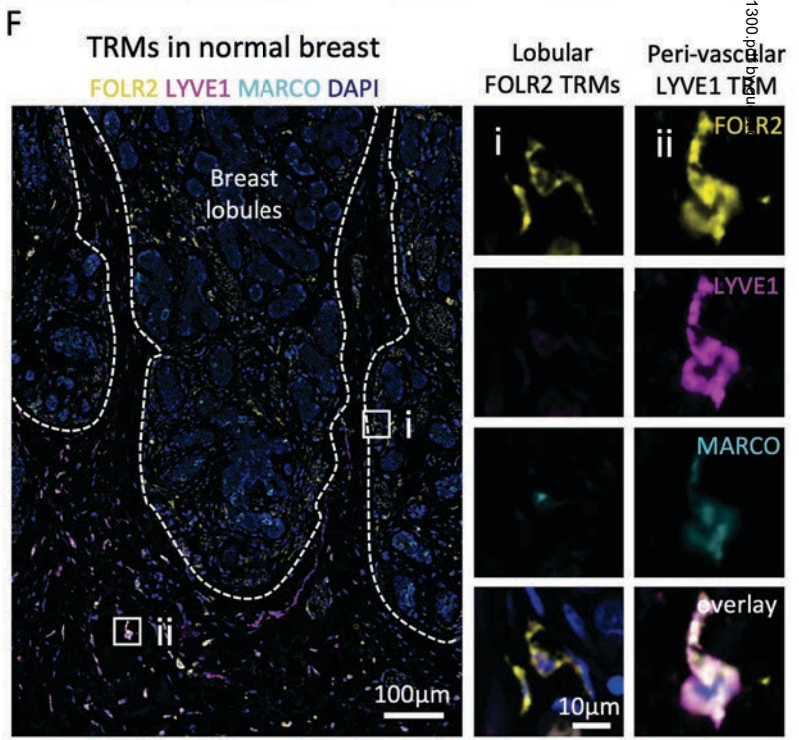
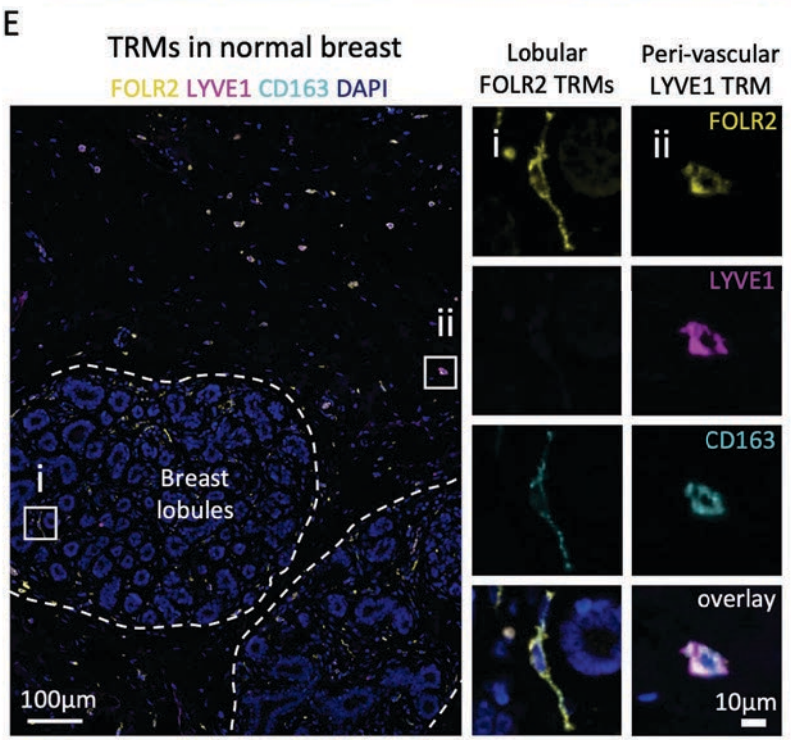
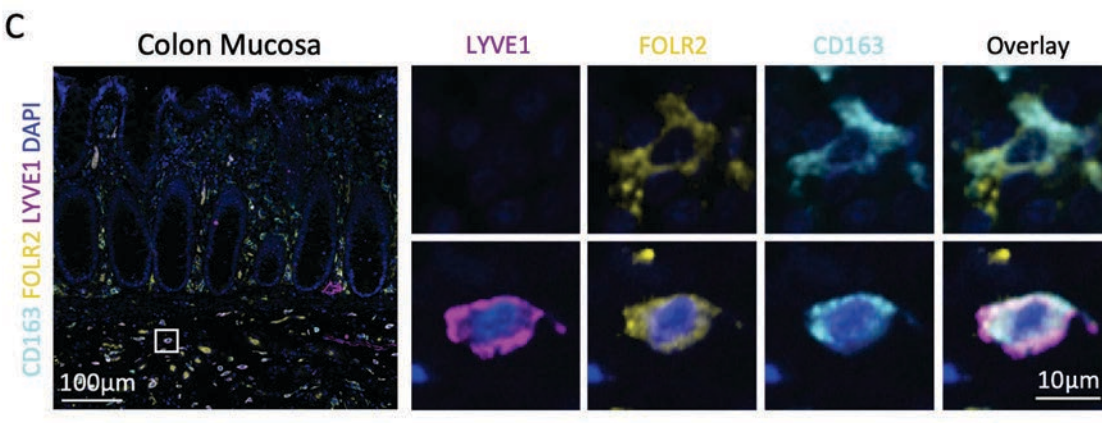
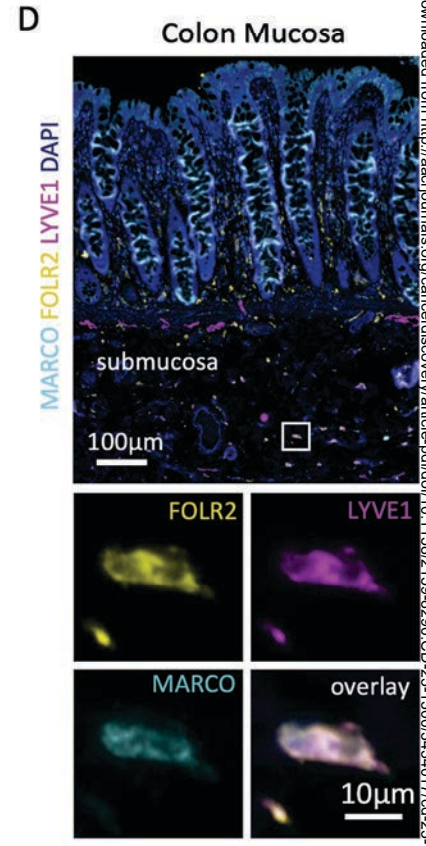
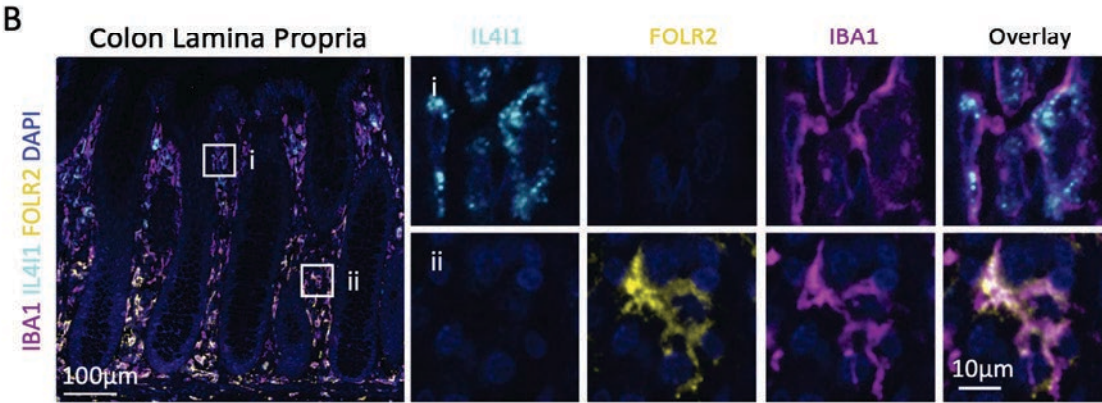
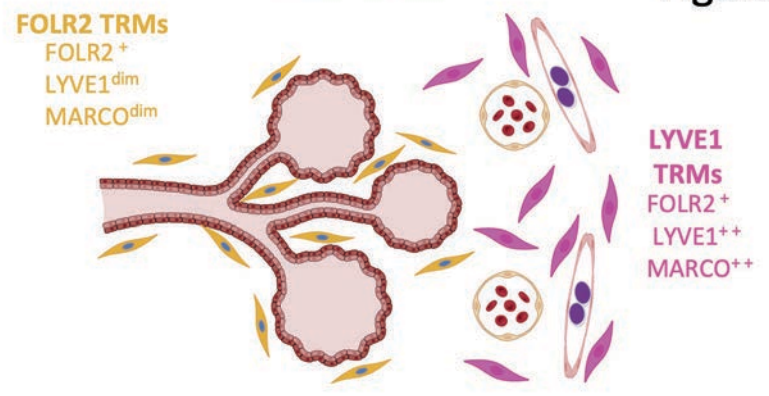
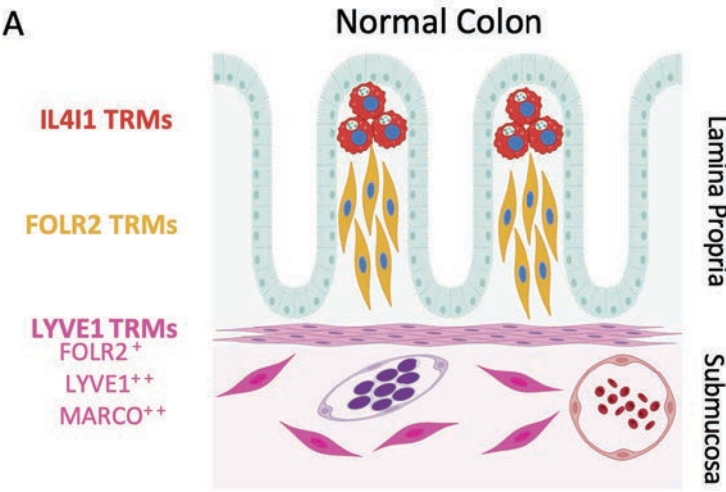
- C3 TRMs
- FOLR2+LYVE1+ TRMs
- FOLR2+LYVE1- TRMs
- SPP1 TAMs
- CXCL9 TAMs
- ISG15 TAMs
- VCAN Monos
- NLRP3 TAMs
- Classical Monos
- Patrolling Monos
- Proliferating TAMs

	#cells	#samples	#cases	
	1952	21	7	CRC Qian et al.
	5835	51	29	CRC Lee et al.
	1642	14	14	BC Qian et al.
	9269	42	42	BC Bassez et al.
	18698	128	92	total

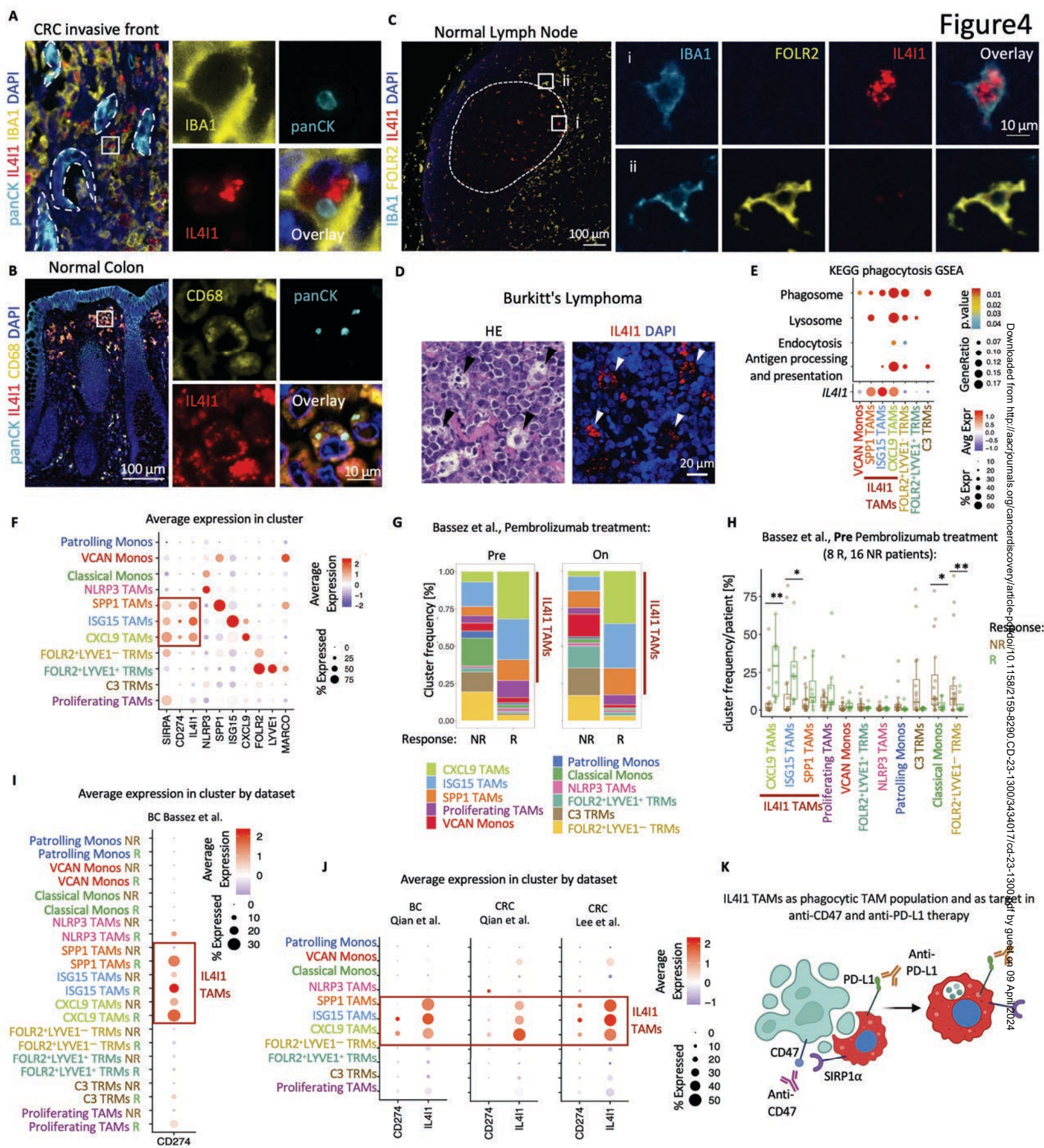


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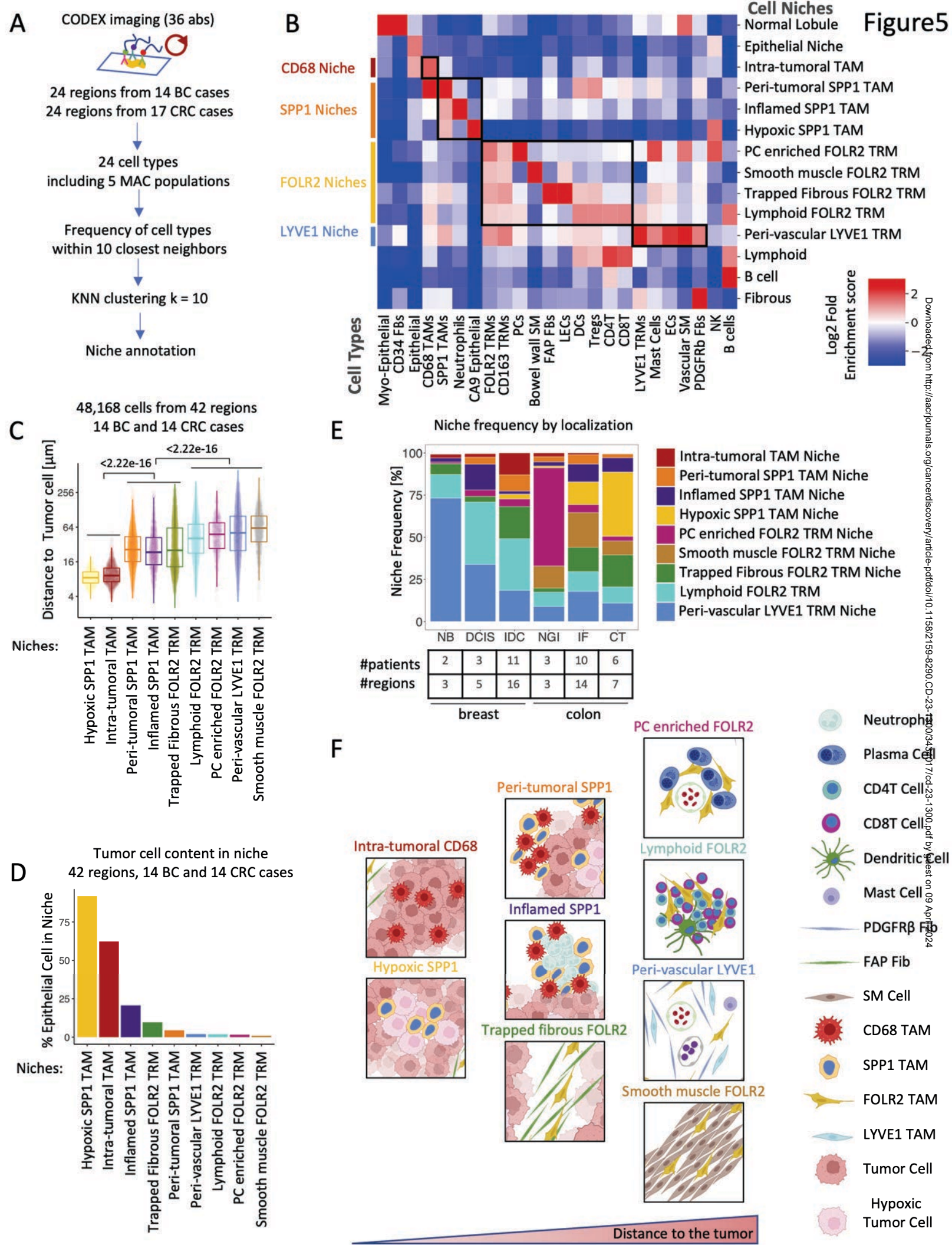


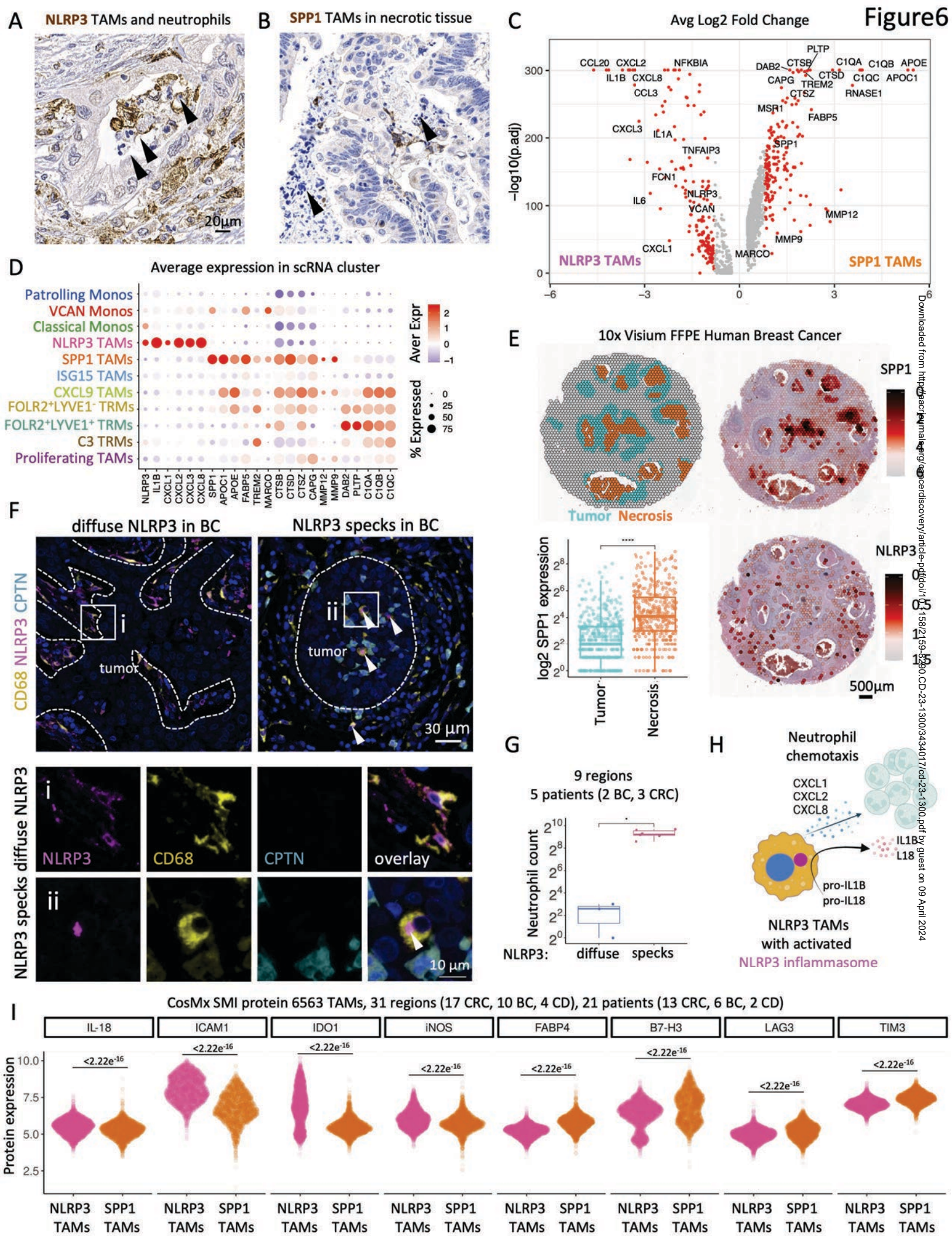


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