

Deterministic reprogramming of neutrophils within tumors

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Abstract: Neutrophils are increasingly recognized as key players in the tumour immune response, and are associated with poor clinical outcomes. Despite recent advances characterizing the diversity of neutrophil states in cancer, common trajectories and mechanisms governing the ontogeny and relationship between these neutrophil states remain undefined. Here, we demonstrate that immature and mature neutrophils that enter **tumors** undergo irreversible epigenetic, transcriptional and proteomic modifications to converge into a distinct, terminally differentiated dcTRAIL-R1⁺ state. **dcTRAIL-R1⁺ reprogrammed neutrophils predominantly localize to a glycolytic and hypoxic niche** at the **tumor** core, and **exert** pro-angiogenic **function** that favors tumoral growth. We find similar trajectories **in neutrophils present** across tumor types and in humans, suggesting that targeting this deterministic program may provide an effective way to enhance cancer immunotherapy. (123 words)

One-Sentence Summary: Deterministic reprogramming of neutrophils drives their convergent differentiation in the tumoral niche

Main Text:

Neutrophils play significant roles in the immune response to infection and injury. As one of the first cells to enter a damaged site from the circulation, the rapid recruitment of large numbers of neutrophils into the tissue is key for their [protective](#) function (1). Notably, this process is co-opted in pathological settings such as cancer, where persistent neutrophil infiltration into the tumour has been consistently associated with poorer patient outcomes (2). Many studies have associated pro-tumoral functions to neutrophils, which in this context, have been referred to as granulocytic myeloid-derived suppressor cells (3). This uniform view has since been disrupted by the identification of a wide spectrum of neutrophil states in cancer, with differences in density (4, 5), surface marker (6–10), and transcript expression (11–13).

Neutrophil heterogeneity also exists in the bone marrow in the form of various maturation stages. Numerous studies have shown that granulocytic progenitor cells differentiate sequentially into precursor, immature and finally mature neutrophils, and each subset of cells possesses unique functional capabilities. (14–19). Notably, tumour-induced chronic inflammation triggers the premature egress of these precursors into the circulation and subsequently into the tumour (4, 14, 17, 19, 20), and extramedullary sites such as the spleen, (13, 14) have been proposed to be priming sites of pro-tumoral neutrophils (12, 21, 22). Collectively, this spectrum of neutrophil states, encompassing phenotypic and maturation differences, is proposed to make up the functional diversity of neutrophils in cancer (23–26).

Here, we utilize multi-omics approaches in pancreatic tumours at single-cell transcriptional and spatial resolution to examine neutrophil functional diversity in the context of their ontogenetic order, origin and influence from tissue signals. We find, unexpectedly, that although various populations of neutrophils enter the tumour, it is only inside the tumour that neutrophils enter a convergent trajectory that directs them towards a unique pro-tumoral state. Our findings challenge current models and [reveal the potential of targeting neutrophils](#) for [cancer](#) immunotherapy.

Intratumoral neutrophils converge into a distinct transcriptional state

To dissect neutrophil heterogeneity in cancer, we used an orthotopic model of pancreatic ductal adenocarcinoma (PDAC). [Using a pancreatic cancer cell line](#) previously [established](#) from [a tumor in the Pdx1^{Cre}; Kras^{G12D/+}; Trp53^{R172H/+} \(KPC\)](#) genetically modified mouse model (23), [cultured cells were orthotopically transplanted into the pancreas, which grow *in situ* to form a tumor](#) characterized by copious neutrophil infiltration (14). Single-cell RNA sequencing (scRNAseq) was performed on total CD11b⁺CD115⁺Ly6G⁺ cells from the bone marrow (BM), spleen, blood, and tumors (n=2, **Fig. 1A, fig. S1A and 1B**) to characterize neutrophil heterogeneity in the context of their ontogenetic order and origin. Uniform Manifold Approximation and Projection (UMAP)

analysis revealed that neutrophils from the bone marrow, spleen, and blood clustered according to their developmental states, from pre-neutrophils (preNeus) to immature neutrophils (IMM 1-2) and mature neutrophils (MAT 1-5) (**Fig. 1B**, **fig. S1C**). Integration with a previous dataset containing healthy mouse neutrophils from matching tissues (19) showed that despite potential perturbations from the tumor, no new developmental clusters or trajectories emerged in neutrophils from tissues of the tumor-bearing mice (**fig. S1D and E**). This thus demonstrated a robust adhesion of neutrophils to a single developmental trajectory within the tissues where they develop and circulate. In contrast, neutrophils acquired unique transcriptional profiles in the tumor (**Fig. 1B and fig. S1C**) and formed three distinct clusters (T1-T3) separate from the other compartments.

Since immature and mature neutrophils can infiltrate tumoral tissue (14,15), we investigated the relationship between the maturation and functional states of the T1-T3 neutrophil populations utilizing a neutrophil maturation gene signature (**table S1**) curated by Xie et al (19), where each cluster in our dataset was graded by a maturation score. T1 neutrophils had a maturation score comparable to immature neutrophils (i.e. IMM 2 cluster), whereas T2 neutrophils had a maturation score similar to mature neutrophils (MAT 1-5 clusters) (**Fig. 1C**). Next, we sought to uncover more potential links between neutrophil clusters in the tumor and the other tissue compartments by utilizing a diffusion map approach, which orders cells based on transitional probabilities and better preserves differentiation trajectories (24, 25). We found that tumor neutrophils deviated from the steady-state neutrophil developmental trajectory (**Fig. 1D**). Specifically, T1 and T2 neutrophils progressed along the tumor-specific branch and converged at the T3 state, suggesting that T1 and T2 may both give rise to the T3 population, which is predicted to be the most terminally differentiated. Interestingly, RNA velocity (26) indicated a pseudotime progression from immature neutrophils to T1, and mature neutrophils to T2, suggesting that T1 and T2 neutrophils are transitory states from immature and mature neutrophils that have migrated into the tumor (**Fig. 1E**). In contrast, T3 neutrophils exhibited a maturation score that was in between T1 and T2 neutrophils (**Fig. 1C**), representing a potential admixture of T1 and T2 cells. Accordingly, we observed that velocity vectors originating from both T1 and T2 populations terminated in the T3 cluster (**Fig. 1E**). These findings thus corroborate earlier studies demonstrating that both mature and immature neutrophils infiltrate tumor tissue (4), and become T1 and T2 neutrophils, respectively. More importantly, our data predicts that these stages are transitional, as both T1 and T2 neutrophils remain amendable for further differentiation into T3 neutrophils.

Epigenetic reprogramming of tumor-infiltrating neutrophils

To confirm that T3 neutrophils can be reprogrammed from both immature (T1) and mature (T2) tumor neutrophils, we performed ATACseq (Assay for transposase-accessible chromatin followed by sequencing) on sorted CD101⁻ (immature) and CD101⁺ (mature) neutrophils (14) from various tissue compartments of control and tumor-bearing mice. Principal component analysis (PCA) revealed that both immature and mature tumor neutrophils clustered separately from corresponding subsets of neutrophils from other tissues (**Fig. 1F**), indicating that signals from tumor

microenvironment imprint specific chromatin accessibility changes within infiltrating neutrophils. We then identified all open chromatin regions (OCRs) that are differentially accessible in immature and mature tumor neutrophils relative to non-tumor neutrophil subsets (table S2). Increased chromatin accessibility for genes upregulated in T3 neutrophils (T3 genes) in both immature and mature tumor neutrophils (Fig. 1G) included genes involved with hypoxia, glycolysis, and angiogenesis, such as *Vegfa* and *Hk2* (fig. S2A). These changes in chromatin were undetectable in neutrophils from other tissues, which still shared similar accessibility for canonical neutrophil genes such as *Cebpa* and *Gfi1* (fig. S2B).

To elucidate putative upstream regulators of the T3 program, we utilized pySCENIC (27) to identify transcription factor regulons predicted in T3 neutrophils that had minimal presence in the other neutrophil subsets (Fig. 1H). This was then correlated to motifs found in the bulk ATACseq analysis (fig. S2C), which revealed T3-specific transcription factors with enriched motifs in immature and mature tumor neutrophil differentially accessible OCs (fig. S2D and E). Among the T3-specific transcription factors, *Mafk*, *Nfe2l2* and *Atf3* were implicated in regulating metabolic and oxidative stress typical of the tumor microenvironment (fig. S2D and E). Correlation of the underlying chromatin accessibility with transcription factors predicted to govern the T3 state suggested that these epigenetic changes initiated within T1 and T2 neutrophils are reinforced by transcription factor activity as they transition towards a T3 state. These findings indicate that tumor-infiltrating neutrophils can be reprogrammed by the tumor microenvironment regardless of their stage of maturity by converging transcriptional and chromatin trajectories towards a distinct T3 state.

CD101 and dcTRAIL-R1 discriminate tumor neutrophil states

Having shown that immature T1 and mature T2 neutrophils in the tumor undergo transcriptional and epigenetic reprogramming towards the T3 neutrophils, we next assessed if we could distinguish these subsets by protein expression. This was particularly important, since increases in RNA expression in neutrophils typically precedes protein expression, especially for genes tightly regulated in neutrophil development such as granule proteins (14). T1, T2 and T3 neutrophils had differential expression of surface marker genes, suggesting that a combination of surface marker protein staining could be used to identify them (fig. S3A). Using a multiparametric flow cytometry approach, live CD45⁺ cells of the PDAC tumor were screened for 249 surface markers. We then clustered all captured cells with InfinityFlow (28, 29), and evaluated co-expression patterns of all tested markers (fig. S3B) which revealed two distinct neutrophil clusters (Fig. 2A). Interestingly, high levels of immune-modulatory/suppressive markers such as dcTRAIL-R1, PD-L1 (CD274) (6), CD14 (13), CD371 (30), VISTA (31), and CD39 (32) distinguished Cluster 2 neutrophils from those of Cluster 1 (Fig. 2B). These markers correlated with surface marker genes enriched in T3 neutrophils (fig. S3C), indicating that they may serve to identify the T3 state. Indeed, we found that dcTRAIL-R1 was expressed mainly by a population of tumor neutrophils (Fig. 2C) while having minimal expression in tumor-infiltrating monocytes and macrophages and in neutrophils

in the bone marrow, spleen, and blood (Fig. 2C and fig. S3D). In contrast, staining for other markers such as VISTA and CD14 showed expression across multiple neutrophil subsets, and was less restricted to tumor neutrophils (Fig. 2D and fig. S3D). dcTRAIL-R1 expression in tumor-infiltrating neutrophils was also conserved across several other tumor types, including orthotopic breast cancer (median: 20.4%, interquartile range, IQR: 18.0-23.1%) and orthotopic lung cancer (median: 12.1%, IQR: 4.54 – 19.5%) models (fig. S3E). Across all experimental tumor models examined, dcTRAIL-R1 expression was similarly limited to a subset of tumor neutrophils, indicative of a promising candidate marker for T3 neutrophils.

Consistent with its protein expression pattern, gene expression and RNA velocity (indicative of active gene transcription) for the gene encoding dcTRAIL-R1 (*Tnfrsf23*) were highest in the T3 neutrophil cluster (fig. S4A). Therefore, we attributed the T3 population to the dcTRAIL-R1⁺ cluster (cluster 2), while the T1 and T2 populations are likely contained within the dcTRAIL-R1⁻ cluster (cluster 1) identified in our InfinityFlow analysis (Fig 2A). Since T1 and T2 neutrophils were transcriptionally identified as immature and mature in our scRNAseq data (Fig. 1D), we next evaluated CD101 expression, which separates immature neutrophils from mature neutrophils (14). Cluster 1 exhibited a continuum of CD101 expression (Fig. S4B), suggesting that CD101 can be used to distinguish T1 and T2 neutrophils within the dcTRAIL-R1⁻ population. Using CD101 and dcTRAIL-R1 to identify the different neutrophil types by flow cytometry (Fig. 2E), we isolated putative T1, T2, and T3 neutrophils and discovered that putative T1 neutrophils possessed a toroidal nuclear morphology resembling immature neutrophils from the bone marrow (Fig. S4C). Putative T2 neutrophils had hyper-segmented nuclei similar to mature BM neutrophils (Fig. S4C) while putative T3 neutrophils contained cells with hyper-segmented or toroidal nuclear morphology (Fig. S4C), congruent with our findings that T3 neutrophils are reprogrammed from both immature and mature neutrophils. Next, we found that dcTRAIL-R1⁺ neutrophils showed the highest expression of T3 genes (Table S3), whereas dcTRAIL-R1⁻CD101⁻ and dcTRAIL-R1⁻CD101⁺ populations were strongly enriched for genes associated with T1 and T2 neutrophils respectively (Fig. 2F and fig. S4D). Finally, all three neutrophil populations were identified in all experimental tumor models examined (fig. S4E), and the frequencies of T1-T3 populations in PDAC as determined by scRNAseq were comparable to the median frequencies of putative T1-T3 populations isolated by flow cytometry (fig. S4E and F). Thus, dcTRAIL-R1 and CD101 expression phenotypically divides tumor neutrophils into three distinct populations and successfully recapitulates the T1-T3 populations defined in our transcriptomic and epigenetic approaches.

Spatial compartmentalization of neutrophils in tumors

Spatial mapping of immune microenvironments in tumors have been highly informative by providing insights into their diversity and functionality based on their localization within the tumor (33). To gain a better understanding of how tumor neutrophils are impacted by their localization in the tumor, we first dissected possible functional differences between the three tumor neutrophil

subsets by performing Gene Ontology (GO) analysis on differentially expressed T1, T2, and T3 genes (**table S4**). T1 neutrophils were enriched for pathways relating to transcription and translation (including genes governing ribosomal biogenesis such as *Npm1*), and oxidative phosphorylation (proton membrane transport) (**Fig. 3A**), which are consistent with the immature phenotype of T1 neutrophils (14). T2 neutrophils were enriched in pathways related to transcriptional regulation, amide and reactive oxygen species metabolism, and immune responses as well as type I interferon genes including *Ifit1*, *Ifit2*, *Ifit3*, and *Isg15* (**Fig. 3B**), which likely reflect neutrophil activation upon tumor infiltration. Finally, T3 neutrophils featured pathways of cell stress and survival, including response to hypoxia, oxidative stress, and glycolysis, (**Fig. 3C**), suggesting adaptation to the tumor environment. Interestingly, T3 neutrophils were also enriched for angiogenic genes, including *Vegfa*, *Thbs1* (34), and *Lgals3* (35) that combined, were suggestive of a strong pro-tumoral role.

We next characterized how neutrophils with distinct transcriptional states would interact with the neighboring tumor microenvironment. Using DAPI and pan-cytokeratin (panCK) co-staining, we noted that our orthotopic tumors were mainly comprised of tumor cell regions (PanCK^{high}DAPI^{high}), fibrotic/necrotic regions (panCK^{high}DAPI^{low}), and sparse stromal regions along the edges (panCK^{low}DAPI^{high}) (**fig. S5A and B**). We performed spatial transcriptomics on four cryo-sections of three different PDAC tumor samples (**Fig. 3D**). As expected, necrotic regions with low numbers of DAPI+ cells contained low quality counts, which were manually annotated and filtered to mitigate RNA contamination from nearby spots and improve downstream analyses (**fig. S5C and D**). We next assessed the tumor architecture by utilizing BayesSpace (36) to identify transcriptionally similar neighborhoods within the tumor (**fig. S5E**). Clustering and UMAP embedding revealed 10 clusters shared across all four sections (**fig. S5E**). Indicative of the complexity of the tumor environment, the distributions of spatial clusters identified was different in the two cryosections (region of interest, ROI1, ROI2) taken from different regions of the tumor (**fig. S5E**). Each spatial cluster possessed specific functional pathways associated with their distribution (**fig. S5F**). For example, regions enriched for pathways related to epithelial-to-mesenchymal transition (EMT, clusters 1 and 10), typically associated with the invasive tumor front (37), were present closer to the tumor periphery, while regions associated with hypoxia (cluster 6) and cell cycle progression (G2-M checkpoint, cluster 2) were located close to the core of the tumor (**fig. S5E and F**).

Utilizing Cell2location (38) with a pre-annotated scRNAseq dataset (**fig. S6A**) allowed us map the full transcriptional signature of each cell cluster within the tumor and estimate cell type abundances within each spot. To ensure the accuracy of neutrophil assigned spots, we ascertained the presence of Ly6G immunofluorescence staining falling within the spot as a selection cutoff (**fig. S6B**). Cell2location revealed that T1, T2 and T3 neutrophils mapped to different regions within the tumor (**Fig. 3E**), with visualization of T3 enriched spots on the UMAP embedding indicating that T3 neutrophils had the highest enrichment in spatial cluster 6, which was associated with hypoxia (**Fig. 3F**). T3 neutrophils were also observed to be mostly distributed adjacent to

PanCK^{high}DAPI^{-low} necrotic zones, (**Fig. 3G**), consistent with their localization to cluster 6 (**fig. S5E**). In contrast, T1 neutrophils were highly enriched in cluster 1 (EMT), 7 and 8 (IL-2 and STAT5 signaling) regions, while T2 neutrophils were mostly found in cluster 3 associated with p53 signaling (**Fig. 3F**). Both T1 and T2 annotated spots were generally localized closer to the tumor periphery compared to T3 neutrophils (**Fig. 3G**). Our data thus reveals that neutrophils are spatially organized within the tumor, and T3 neutrophils occupy a tumor niche that is distinct from that of T1 or T2 neutrophils.

Reprogrammed neutrophils occupy a hypoxic and glycolytic tumor niche

Since T3, but not T1 or T2, neutrophils had transcriptional and epigenetic profiles that were enriched for hypoxia, glycolysis and angiogenesis pathways, we evaluated whether this was reflected in their localization to hypoxic, glycolytic or angiogenic regions within the tumor. All spots within the tumor region were first scored for GO pathways corresponding to glycolysis (**fig. S6C**), hypoxia (**fig. S6D**) and angiogenesis (**fig. S6E**), with a 50th percentile cut-off employed to determine low and high regions. We then determined the frequency of neutrophil-containing spots falling into low and high regions for each tumor neutrophil subset. Notably, T3 neutrophils were found at greater frequencies in high scoring regions for glycolysis ($68.8\% \pm 19.7$), hypoxia ($53.3\% \pm 4.65$) and angiogenesis ($62.2\% \pm 14.2$) (**Fig. 3H**). In contrast, the majority of T2 (**fig. S6F**) and T1 (**fig. S6G**) neutrophil-containing spots fell into regions with low glycolysis, hypoxia and angiogenesis scores, indicating that partitioning of the neutrophil subsets potentially results from T3 neutrophils occupying a specific regional hypoxic and glycolytic tumor niche.

We next set out to validate these observations at single-cell resolution, since the current Visium spatial transcriptomic resolution is limited to 55um spots. We utilized MACSima imaging cyclic staining (MICS) (**Fig. 4A**), where iterative immunofluorescence staining and photobleaching allows for evaluation of large numbers of antibody targets within the same tissue slice (39). Optimization was first carried out to determine working markers within orthotopic pancreatic tumor tissues (**fig. S7A and B**), given that MICS staining times were relatively short at 10 minutes. Testing revealed that CD29, Galectin-3 and CD44, which typically mark fibroblasts (40–42) displayed the greatest staining at peripheral stromal/desmoplastic (panCK^{-low}DAPI^{hi}) regions (**fig. S7C**), while CD31 and CD105 marked vessels within the stromal and tumor region (panCK⁺). Notably, CD105 was able to better resolve intra-tumoral vessels (**fig. S7C**), consistent with their role in marking tumor-associated endothelium (43). To identify hypoxic regions within the tumor, Hif1a, CD39 and CD73 staining was evaluated. CD73, an adenosine monophosphate ectoenzyme, has been found to be directly induced by hypoxia and Hif1a in cancer (44–47). Immunofluorescence analysis revealed diverse staining patterns of CD73 in both stromal/desmoplastic and tumor (panCK⁺) regions (**fig. S7C**), resembling the spatial distribution of hypoxia gene signature scores observed in our 10x Visium analysis (**fig. S6B**). In contrast, Hif1a did not stain within the MICS timeframe, while CD39 mostly identified vessels (**fig. S7C**). Similarly, GLUT1 (glucose transporter 1) staining showed regional restriction (**fig. S7C**) similar

to glycolysis signature scores (**fig. S6B**). Thus, CD73 and GLUT1 representative staining was utilized to define the hypoxic and glycolytic tumor niche. T1, T2 and T3 neutrophils were identified by staining for Ly6G, CD101 and dcTRAIL-R1 based on our flow cytometric strategy (**Figure 2D**) and successfully annotated in both confocal (**fig. S7D**) and MICS (**fig. S7E**).

We carried out MICS for five different ROIs across two different orthotopic pancreatic tumors at 4-6 weeks post injection. ROIs were selected to unequivocally cover an entire region from the left to right margin encompassing the core of the tumor (**fig. S8A-B**), and we observed staining across the full panel for all markers surveyed (**fig. S9**). We next specifically evaluated staining for the tumor vasculature (CD105), hypoxic (CD73) and glycolytic (GLUT1) across all five ROIs (**Fig. 4C and fig. S8A-B**). CD105⁺ blood vessels were present as a diffuse network scattered throughout the tumor (**Fig. 4C and fig. S8A-B**). A gradient of CD73 staining was apparent throughout the tumor, while GLUT1 staining was more tightly localized, however GLUT1^{hi} areas strongly co-localized with CD73^{hi} regions marking a distinct hypoxic and glycolytic niche (**Fig. 4D and fig. S8A-B**). Annotation of T1 (Ly6G⁺CD101⁻dcTRAIL-R1⁻) and T2 (Ly6G⁺CD101⁺dcTRAIL-R1⁻) neutrophils (**fig. S8C**) revealed that T1 and T2 neutrophils were distributed throughout the tumor parenchyma in two different patterns reflective of their likely routes into the tumor – bordering the tumor edges in CD73⁻ stromal regions or diffused throughout GLUT1⁻CD73⁺ regions, both of which contained a substantial network of CD105⁺ vessels (**Fig. 4E and fig. S8A-B**). In contrast, T3 (Ly6G⁺CD101^{+/+}dcTRAIL-R1⁺) neutrophils across all ROIs were more likely to cluster together at the CD73^{hi}GLUT1^{hi} hypoxic and glycolytic niche (**Fig. 4E**), thus giving rise to a clear spatial segregation of the three neutrophil populations as suggested by our 10x Visium analysis (**Fig. 4F and fig. S8A-B**).

Next, we agnostically quantified the three neutrophil subgroups' relative locations within the tumor. Using marker intensities on segmented cells, we excluded CD45⁺ immune and CD105⁺ endothelial cells, and subdivided the remaining cells into three distinct regions – stromal (CD73⁻GLUT1⁻), tumor parenchyma (CD73⁺GLUT1⁻) and the hypoxic^{high}glycolytic^{high} tumor niche (CD73^{hi}GLUT1^{hi}) (**Fig. 4G and fig. S8C**). In all five ROIs, T3 neutrophils had the shortest average minimum distance to the hypoxic^{high}glycolytic^{high} niche compared to T1 and T2 neutrophils (**Fig. 4H**). We then assessed normalized mixing scores, which quantify proportion of direct touches between the target regions and reference neutrophil cells (target-reference) against reference-reference interactions (48). T3 neutrophils had the greatest mixing scores within hypoxic^{high}glycolytic^{high} tumor regions, which was conserved with each radius of area investigated (**Fig. 4H**). Scores for the stromal region showed a trend for highest scores in T1, followed by T2, with T3 being minimally detected within the stroma (**fig. S8D**). Finally, T2 neutrophils trended towards higher proportions of interspersions within the tumor parenchyma compared to T1 and T3 neutrophils (**fig. S8D**). These observations suggest that, T1 and T2 neutrophils are mostly located away from the hypoxic^{high}glycolytic^{high} regions in contrast to T3 neutrophils. As such, our spatial data implies that T3 neutrophils likely migrate into and occupy the specialized hypoxic-glycolytic tumor niche. This is consistent with our finding that transcription factor regulons enriched in T3

neutrophils (Fig. 1H) include those upregulated in response to hypoxia (*Hif1a*, *Bhlhe40*) and to metabolic and ER stress (*Atf4*, *Ddit3*, *Atf3*, *Nfe2l2*). As such, epigenetic and transcriptional upregulation of these pathways in T3 neutrophils could confer an added survival advantage within these tumor microenvironments. Collectively, our data provides a spatial representation by which tumor neutrophils converge upon the occupancy of a specialized tumor niche upon reprogramming.

Deterministic reprogramming of neutrophils in the tumor

Because both mature and immature neutrophils were predicted to give rise to T3 neutrophils inside the tumor, we examined the impact of the maturation state on the acquisition of the T3 signature. We first isolated immature and mature neutrophils from wild-type mice and cultured them with tumor conditioned media (TCM), which allowed us to mimic the infiltration of immature and mature neutrophils into the tumor microenvironment (Fig. 5A). We then measured upregulation of dcTRAIL-R1 on cultured neutrophils across multiple timepoints as a proxy to estimate the acquisition of the T3 profile (Fig. 5A). After three days, a substantial proportion of bone marrow immature and mature neutrophils cultured in TCM were dcTRAIL-R1⁺ but not those cultured in control media (cDMEM) (Fig. 5A). Specifically, exposure to TCM could trigger dcTRAIL-R1 expression 24h hours into culture and prolonged survival up to three days in comparison to culture in cDMEM (fig. S10A-B). We obtained similar results regardless of tissue origin or maturation state, such that neutrophils isolated from the bone marrow, spleen and circulation cultured in TCM all exhibited dcTRAIL-R1 upregulation and concurrent increased survival (Fig. 5B, fig. S10A-B). Furthermore, exposure to TCM induced the upregulation of the T3 gene signature in immature and mature neutrophils (Fig. 5C). We then assessed if culture in hypoxic conditions would enhance neutrophil survival or dcTRAIL-R1 upregulation of wild type neutrophils. Culture in hypoxic conditions slightly augmented neutrophil survival *in vitro*, especially for mature neutrophils (fig. S10C), but did not induce dcTRAIL-R1 expression (fig. S10D), indicating that hypoxic conditions are not the main driver of T3 reprogramming. Adoptive transfer of CD45.1⁺ immature and mature neutrophils into tumor-bearing mice (Fig. 5D) further confirmed that exposure to tumor microenvironment was necessary for their upregulation of dcTRAIL-R1 (Fig. 5E), and dcTRAIL-R1 upregulation *in vivo* followed the same kinetics as *in vitro* (Fig. 5F, fig. S11A). Hence, exposure to tumor-derived stimuli supports extended survival and the acquisition of the T3 phenotype in wild-type neutrophils.

Both TCM-cultured immature and mature neutrophils upregulated dcTRAIL-R1 expression and the T3 gene signature to an equal degree, indicating that immature neutrophils could directly be reprogrammed into T3 neutrophils (Fig. 5A-C). RNA velocity analysis suggested that this occurs in a stepwise fashion as immature neutrophils transit into T1 neutrophils, and subsequently differentiate into T3 neutrophils (Fig. 1E). As expected, transfer of CD45.1⁺ immature neutrophils resulted in the appearance of dcTRAIL-R1⁺CD101⁺ T1 neutrophils within the tumor one day post-transfer (fig. S11B). In contrast, only dcTRAIL-R1⁺CD101⁺ T2 neutrophils, and not T1

neutrophils, were observed in the tumor with CD45.1⁺ mature neutrophil adoptive transfer (**fig. S11B**). Both T1 and T2 populations derived from transferred cells were undetected after three days, as the majority of these transferred cells completed their reprogramming into dcTRAIL-R1⁺ T3 neutrophils (**fig. S11C**). We further corroborated the transitory nature of T1 and T2 subsets by *in vitro* culture (**fig. S11D-E**). Isolated T1 and T2 neutrophils upregulated dcTRAIL-R1 expression after 24 hours in culture. Unlike tumor-naïve neutrophils, dcTRAIL-R1 upregulation was independent of the media utilized for both T1 (**fig. S11F**) and T2 neutrophils (**fig. S11G**). As such, T1 and T2 neutrophils reflect immature and mature neutrophil populations captured in the process of T3 differentiation, and thus do not require any further input from tumor soluble factors. Taken together, our findings uncover a deterministic program within neutrophils that enables them to acquire the T3 phenotype independent of their maturation stage.

T3 neutrophils are long-lived, terminal effectors within the tumor microenvironment

To better understand the temporal regulation of neutrophil differentiation into the T3 state within the tumor microenvironment *in vivo*, we administered BrdU intravenously at selected timepoints prior to harvest, pulse-labelling all proliferating neutrophil precursors (**Fig. 5G**). This approach defines a timestamp at the point of labelling, allowing us to evaluate all timepoints simultaneously at the time of harvest, thereby minimizing batch effects. Modelling the disappearance rate from the peak of BrdU⁺ signal (**fig. S12A-B**) and incorporating the entire temporal interval measured allowed the prediction the neutrophil half-life and lifespans (**fig. S12C**), reflecting their dwell time within each tissue compartment (**Fig. 5G**).

The earliest peak of BrdU neutrophils was observed in the bone marrow and spleen, due to active granulopoiesis within these organs in the tumor-bearing state, while recruitment of BrdU-labelled neutrophils into the blood and subsequently the tumor placed the peak at roughly 4-5 days post labelling (**Fig. 5G**). As the dwell times of neutrophils in the bone marrow and spleen are complicated by continual neutrophil production, we focused our comparison between the blood and tumor neutrophils. Blood neutrophils had a half-life of 31.4 hours, reflecting an increased transit time in circulation compared to previously predicted times for wild type blood neutrophils (49). Even so, tumor neutrophils had an even longer half-life (41.8h, 95% C.I. [39.6, 46.6]) and a predicted lifespan of 135 hours (up to 5.625 days, 95% C.I. [126.5, 142.4]) (**Fig. 5G, fig. S12C-D**), representing a remarkable extension of neutrophil lifespan within the tumor microenvironment. Notably, when compared to the unmarked BrdU⁻ fraction, the proportion of BrdU⁺ dcTRAIL-R1⁺ neutrophils increased over time, surpassing baseline levels at day 6 post labelling (**fig. S12E and F**). Newly recruited BrdU⁺ tumor neutrophils already expressed dcTRAIL-R1 at 1-day post-labelling (**fig. S12E**), suggesting that T3 reprogramming is initiated upon tumor entry. dcTRAIL-R1 expression steadily increased and was the highest in neutrophils at 15 days post-labelling, (**Fig 5H and I**), confirming that acquisition of the T3 phenotype was associated with their extended lifespans *in vivo*. Our results show that a notable increase in the duration of neutrophil half-life and residence in the tumor, as compared to those in non-tumor tissues (**fig. S12D**) – indicating that

neutrophils can survive long enough within the tumor to undergo reprogramming and sustained persistence within the tumor as long-lived dcTRAIL-R1⁺ T3 neutrophils.

To examine if the T3 state is transitory or is stably maintained once it has been acquired, we isolated and cultured T3 neutrophils overnight, and up to 3 days in the presence or absence of TCM (Fig. 5J). Survival of T3 neutrophils in culture was not affected by the media type (fig. S12G), and dcTRAIL-R1 expression was maintained despite the absence of tumor-derived factors (Fig. 5K) with expression levels comparable to freshly isolated T3 neutrophils at both timepoints (fig. S12H). Additionally, T3 neutrophils did not downregulate their gene signature in culture (Fig. 5L). This *in vitro* evidence indicates that once neutrophils have been reprogrammed to T3 neutrophils, they do not revert their phenotype in the absence of supportive tumor factors and that they represent the terminally differentiated neutrophil population within the tumor.

T3 neutrophils are pro-angiogenic and promote tumor growth

Prior studies have identified key roles for neutrophils in tumor progression, especially via the promotion of tumor angiogenesis (50–52). We therefore sought to determine the functional specialization of T1-T3 neutrophils in promoting tumor growth. Given their phenotypic stability, distribution near angiogenic regions, and the expression of a pro-angiogenic transcriptional signature (Fig. 3C), we speculated that T3 neutrophils promoted angiogenesis from their hypoxic-glycolytic niche to support continual tumor growth. In contrast, we anticipated that the transient T1 and T2 neutrophil states would not yet feature pro-angiogenic ability. T3 neutrophils had the highest transcript (Fig. 6A) and protein expression (Fig. 6B-C) of *Vegfa* (vascular endothelial growth factor a) in comparison to the other neutrophil subsets in the tumor and periphery. We then evaluated whether T3 neutrophils had a greater capacity to induce blood vessel formation *in vivo* using a modified Matrigel plug assay and measured angiogenesis by the rate of vascular flow measured by Doppler imaging (53) (fig. S13A). Matrigel plugs co-injected with T3 neutrophils showed the greatest flux intensity when compared to control WT BM neutrophil plugs within the same mouse (fig. S13B) and this increase in vascularization, while modest, was consistent across all mice observed in the assay (fig. S13C). In contrast, co-injection of T2 and T1 neutrophils did not enhance vascular flow (fig. S13B-C), indicating that T3 population contains the most potent angiogenic ability (Fig. 3C).

The tumor triggers angiogenesis in order to maintain the supply of oxygen and other nutrients to the core of the tumor to sustain continual growth (54). To test whether T3 neutrophils residing in their hypoxic-glycolytic niche at the tumor core support this angiogenic switch, we modified our PDAC model by implanting PDAC tumors subcutaneously to enable longitudinal tumor size measurements. We then assessed whether injection of neutrophils within the tumor affected their subsequent growth (Fig. 6D). PDAC cells co-injected with T3 neutrophils formed tumors that grew rapidly (Fig. 6D), while co-injection with other neutrophil types had little influence in tumor growth. Notably, T3 co-injected tumors had a 100% tumor growth rate (Fig. 6E, as evaluated in

fig. S13D), and had the greatest mass at the endpoint (**Fig. 6F**). T2-coinjected tumors showed the lowest tumor incidence rates (5/12) (**Fig. 6F**), suggestive of increased tumor rejection, consistent with the expression of pro-inflammatory genes in T2 neutrophils detected in our scRNAseq dataset (**Fig. 3B**). T3-coinjected tumors continued to grow rapidly for up to 3 weeks past the last injection of neutrophils, suggesting that even after their disappearance, transferred T3 neutrophils generated long-lasting effects that sustained this accelerated growth rate. Neutralization of VEGF α in our model resulted in the reduction of growth in T3 co-injected tumors, but had no impact on the growth of WTBM MAT co-injected tumors (**Fig. 6G**). To assess if this growth inhibition was due to increased angiogenesis driven by T3 neutrophils, we optically cleared T3- and WTBM MAT co-injected tumors (**fig. S13E**), and visualized the intratumoral 3D vessel network via CD31 staining (**Fig. 6H**). Notably, T3 co-injected tumors had greater CD31 staining density towards the tumor core (**Fig. 6H, Movie S1**), while the vessel network in WTBM MAT co-injected controls showed greater distribution along the tumor edges (**Fig. 6H, Movie S2**). Normalization for tumor size revealed that both T3 and WTBM MAT co-injected tumors had similar densities of CD31⁺ blood vessels (**fig. S13F**) but drastically differed in their distribution (**Fig. 6I**). Although T3 neutrophils were the highest expressors of *Vegfa* in the pancreatic tumor, *Vegfa* expression was also detected in macrophage populations (**fig. S13G**), indicating that neutrophils may not be the sole pro-angiogenic contributor for tumor growth support. Nonetheless, blockade of T3 neutrophils with an anti-dcTRAIL-R1 antibody decreased tumor growth in T3 co-injected tumors compared to isotype controls (**Fig. 6J**), indicating that T3 neutrophils are the predominant cells responsible for increasing the tumor growth rate, most likely through vascular remodeling.

Evidence for conserved neutrophil reprogramming across tumor types and human PDAC

Phenotypic differences in tumor-infiltrating neutrophils have been observed across mouse and human cancers, and multiple subsets have been characterized by differential surface marker or transcriptome expression (6–13). Whether such neutrophil profiles are conserved across tumor types and species remains unclear. We therefore assessed if T1-T3 neutrophil states can be detected in [previously](#) published mouse cancer scRNAseq datasets containing annotated neutrophils (11, 13). We projected these datasets onto a reference UMAP embedding, mapping each cell on the same UMAP space as our dataset (**fig. S14A**). As in the PDAC model, neutrophils within Lewis lung carcinoma (LLC) tumors (13) could be assigned to the T1-T3 states, while spleens from wild type or tumor-bearing mice mostly contained non-tumor neutrophil clusters (**fig. S14B**). An intermediate neutrophil subset identified in cancer-bearing individuals (PMN2 in (13)) was enriched in the preNeu and IMM 1-2 clusters (**fig. S14C**), while T1-T3 clusters scored highly for the tumor-specific neutrophils (PMN3; **fig. S14D**). Similarly, re-analysis of [a different scRNAseq dataset](#) showed that neutrophils from [their KP1.9 tumor-bearing lung model](#) (11) mapped as T1-T3 clusters, while the normal lung tissue was mostly enriched for mature neutrophils (**fig. S14E**). Likewise, neutrophil types identified as tumor-specific neutrophil clusters in this model (mN3-6 in (11)), corresponded to T1-T3 clusters (**fig. S14F**), confirming that the tumor microenvironment induces a prototypical transcriptional trajectory that is deterministic in nature. In contrast, the

signature of neutrophils from healthy lungs (49) was largely independent of tumor-induced transcriptional changes (fig. S14G). Taken together, these data suggest that reprogramming of tumor neutrophils is conserved across different tumor types, and that T3 neutrophils represent terminal differentiated tumor neutrophils.

To examine cross-species conservation of the neutrophil tumoral trajectory found in mice, we evaluated if our neutrophil classification could account for heterogeneity present in human tumors. We examined independent scRNAseq datasets (55, 56) from two human PDAC cohorts (fig. S14H and I) and mapped them to a simplified reference UMAP embedding via label transfer (fig. S14J). T3 and T2 neutrophils were amply labelled in both datasets, and a smaller cluster of T1 neutrophils was also identified (fig. S14K and L). These tumor neutrophil subsets were predominantly enriched in the pancreatic tumor and not in adjacent normal pancreatic tissue (fig. S14K) or in peripheral blood (fig. S14L). Interestingly, a pro-angiogenic neutrophil type expressing genes linked to hypoxia and glycolysis in one of these studies (referred to as TAN-1 (56)) strongly matched the T3 state in the murine tumor, indicative that our T3 classification can capture subsets independently identified to be pro-tumoral (fig. S15A). Similarly, tumor neutrophil gene signatures derived from our mouse dataset (fig. S15B) were conserved in their ability to distinguish between human tumor neutrophils, and showed strong specificity in identifying T3, T2 and T1 neutrophils within the tumor and not in the adjacent normal tissue (fig. S15B). In all, our findings suggest that tumor-induced reprogramming of neutrophils is conserved in humans.

Because T3 neutrophils promote the growth of pancreatic tumors, we hypothesized that the genetic signature associated with the T3 state might be predictive of pancreatic cancer outcomes in human patients. To test this, we performed survival analysis on two independent pancreatic cancer cohorts from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium Pancreatic Cancer-Australia (PACA-AU) by scoring patients based on high and low expression of each signature (see **Methods** for scoring criteria). Patients with high expression of the T3 neutrophil signature had poorer overall survival across both data sets, with a median of 652 (TCGA) and 427 (PACA-AU) days respectively (Fig. 6K), and this was independent of potential confounders such as patient gender, age and tumor stage (Table S5). Similarly, when disease-free survival (DFS, assessed as time to an adverse event from the initial treatment) was evaluated, patients with high T3 neutrophil signature expression had reduced DFS (Fig. 6M), with equal distribution of potential confounders across the two groups (Table S6). When T1 and T2 signatures were considered, high T2 signature expression correlated with worse OS only in the TCGA dataset (fig. S15C-D) but this was confounded by gender (Table S5), while high T1 signature expression was associated with lower DFS only in the PACA-AU dataset (fig. S15E-F). Hence, only the T3 signature was consistently associated with poorer OS and DFS across both datasets. We next considered whether the T3 neutrophil signature was also associated with poorer overall survival in other solid tumors. Within the TCGA pan-cancer database (57), higher expression of the T3 signature was associated with a significantly higher risk of death across a subset of solid cancers, including pancreatic cancer (PAAD), (Fig. 6L, Table S7 for p-values and confidence intervals). In contrast, T2 and T1

signatures were both protective against (lower hazard ratios, HRs) and associated with (higher HRs) patient death depending on the solid tumor (Fig. 6L, Table S7), in line with their nature as transitional subsets in the process of reprogramming. Taken together, these data support a model where tumor-educated T3 neutrophils drive tumor progression both in mouse and human cancers.

Discussion

Environmental cues can fine-tune immune responses by inducing cell recruitment and expansion of immune cells, generating productive responses with adequate numbers and specialized phenotypes. Unable to further proliferate, neutrophils rely on their ability to swiftly mobilize into tissues to perform their functions effectively and, in diseases like cancer, neutrophils at various maturation stages, tissue origins, and phenotypes are recruited into the tumor in large numbers (12–14, 17, 19). Given the likely co-existence of multiple tumor neutrophil states with different functional phenotypes, it is thus unclear how neutrophils exert a focused local effect in the tumor. Here, we examine how neutrophils decouple their initial maturation phenotype from their eventual pro-tumoral function by undergoing convergent reprogramming within the tumor.

Our study demonstrates that tumor-infiltrating immature and mature neutrophils acquire distinct epigenetic, transcriptomic, and proteomic phenotypes while retaining features of their initial maturation status. We find that both immature (T1) and mature (T2) tumor neutrophils within the tumor converge towards a third population (T3) expressing dcTRAIL-R1. T3 neutrophils thus show intermediate maturation scores when quantified at the population level, and have both toroidal (immature) and hyper-segmented (mature) neutrophil nuclei, representative of them being an admixture of reprogrammed neutrophils of T1 and T2 origin. Our data reveals the capacity of neutrophils to simultaneously integrate different types of signals from the environment, allowing them to layer a new functional phenotype onto their pre-existing differentiation stage. We demonstrated this plasticity with immature and mature neutrophils from tumor-naïve mice, which are both capable of acquiring phenotypic (dcTRAIL-R1 expression) and transcriptional traits of the T3 state when cultured in tumor-conditioned media or upon entering the tumor *in vivo*. By circumventing neutrophil maturation as a rate-limiting step, this adaptability allows immature neutrophils to be equally mobilized and reprogrammed within the tumor in a shorter timeframe. Subsequently, this intrinsic ability embedded in neutrophils consolidates the various functional neutrophil states into one terminal neutrophil phenotype as directed by the tissue, in this case, a tumor.

Circulating neutrophils have a predicted half-life of ~10 hours (58), while tissue-resident neutrophils persist for up to one day (49). Although studies have hinted that neutrophils persist far longer within the tumor (10, 59, 60), whether this coincides with terminal differentiation in the tumor remains an open question. Using a BrdU pulse-labelling approach, we revealed that up to 5% of the originally labelled neutrophils could remain within the tumors for as long as 5.625 days upon entry. In addition, while our curve-fit was accurate for the first labelling timepoints, there

was a noticeable underfitting at 12- and 15-days post-labelling, representing a possible underestimation of the full tumor neutrophil lifespan and indicating that a small population of neutrophils could persist even longer within the tumor. Long-lived neutrophils were predominantly of the dcTRAIL-R1^{hi} T3 phenotype, which indicates a correlation between their ability to survive within challenging hypoxic and glycolytic environments and their continued persistence within the tumor. Spatial mapping at the transcriptome and protein level placed T3 neutrophils predominantly within a hypoxic-glycolytic niche nearer to the tumor core. In contrast, T1 and T2 neutrophils were positioned at the stromal and tumor parenchyma where a large vessel network exists, supporting the notion that these cell states are in the process of reprogramming after tumor entry. Given that hypoxia is not required to trigger T3 reprogramming, which can occur in normoxic conditions, we propose that migration towards the hypoxic-glycolytic niche occurs after acquisition of T3 epigenetic and transcriptional programs is fully complete to ensure neutrophil survival. Consistent with this possibility, upstream transcription factors regulating metabolic and oxidative stress are switched on in T3 neutrophils compared to T1 or T2 populations. Deletion or inhibiting these transcription factors to trace the timeline of T3 reprogramming thus represent important goals for future studies within the field.

T3 neutrophils accelerated early tumor growth experimentally, and while we still observed tumor growth with other co-injected neutrophil subsets, this occurred at a slower rate likely due to recruitment and reprogramming of endogenous T3 neutrophils at the later timepoints. Notably, T1 and T2 neutrophils did not show statistically significant enhancement or inhibition of tumor growth compared to other control neutrophil populations or PBS, reflecting their transitional nature as opposed to a fully anti- or pro-tumoral population. In contrast, the sustained growth advantage conferred by T3 neutrophils stemmed, at least in part, from T3-dependent remodeling of the vasculature towards the core of the tumor. Given their localization within the glycolytic-hypoxic niche, an intriguing scenario would see T3 neutrophils serve as possible guide-rails to direct angiogenesis to relieve hypoxic and nutrient stress in areas that would most require it. In accordance, resistance towards anti-angiogenic therapies in human cancer has been associated with neutrophil infiltration (61), and neutrophil depletion reduces tumor vascularization and growth (51, 52, 62, 63). Studies facilitating further understanding of T3-mediated vascular remodelling would reveal new therapeutic targets against pathological angiogenesis within the tumor.

A particularly striking finding is the conservation of this differentiation program in tumor-infiltrating neutrophils across tumor type and species. When scRNAseq datasets from other tumor models were examined, T3 annotation could identify pro-tumoral clusters found both in mouse (i.e. mN5, (11)) and in human (i.e. TAN-1,(56)). T3 neutrophils promoted PDAC tumor growth in mice, while ablation of T3 neutrophils or their pro-angiogenic function removed this growth advantage. In parallel, the T3 signature consistently predicted poorer patient outcomes in two independent human PDAC cohorts, as well as in a subset of other solid tumors. Thus, different studies converge upon our identification of a terminally differentiated neutrophil state, whose signature can now be used to better understand neutrophil function in cancer and to predict tumor

progression. We propose that the reported heterogeneity of neutrophils across tumors more likely reflects transitional states derived from populations at different stages of maturation and/or reprogramming.

Collectively, by ordering neutrophils through the lens of their ontogeny, we assess global neutrophil phenotypic heterogeneity through maturation stages, extra-medullary sources, and the specialization of each neutrophil to each tissue (49). Although all of these states co-exist within the tumor-bearing mouse, the neutrophil maturation trajectory remains unchanged, consistent with findings in other pro-inflammatory settings (19, 20, 64, 65). As such, while our findings do not fully exclude the possibility of neutrophil reprogramming outside of the tumor, they suggest that it is unlikely that upstream changes in neutrophil progenitors specifically drive the formation of a pro-tumoral neutrophil population. Instead, it is the intrinsic capacity of recruited neutrophils to adapt in response to the tumor environment, regardless of their initial phenotype, that allows them to adopt convergent trajectories to settle upon a final, pro-tumoral state. [This feed-forward loop thus ensures the continued supply and differentiation of long-lived, pro-angiogenic neutrophils that support tumor growth.](#) Expanding from the results of our study, we propose that it is advantageous for tissues to induce functional homogeneity in neutrophils at the local scale to support tissue growth and function. This process is then hijacked by the tumor to favor a functional neutrophil state that promotes aberrant tumor growth. Our findings thus reveal a general mechanism by which short-lived effector cells such as neutrophils efficiently adjust their functions to meet the demands of a tissue, and suggests that local neutrophil responses can be therapeutically targeted.

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1063 **Data and materials availability:** All data needed to evaluate the conclusions in the paper are

1064 present in the paper and/or in the Supplementary Materials. scRNAseq, ATACseq and spatial

1065 transcriptomics data in the paper will be eventually deposited in the NCBI Gene Expression

1066 Omnibus.

1067

1068 **Supplementary Materials**

1069 Materials and Methods

1070 Figs. S1 to [S16](#)

1071 Tables S1 to [S7](#)

1072 References (*57–71*)

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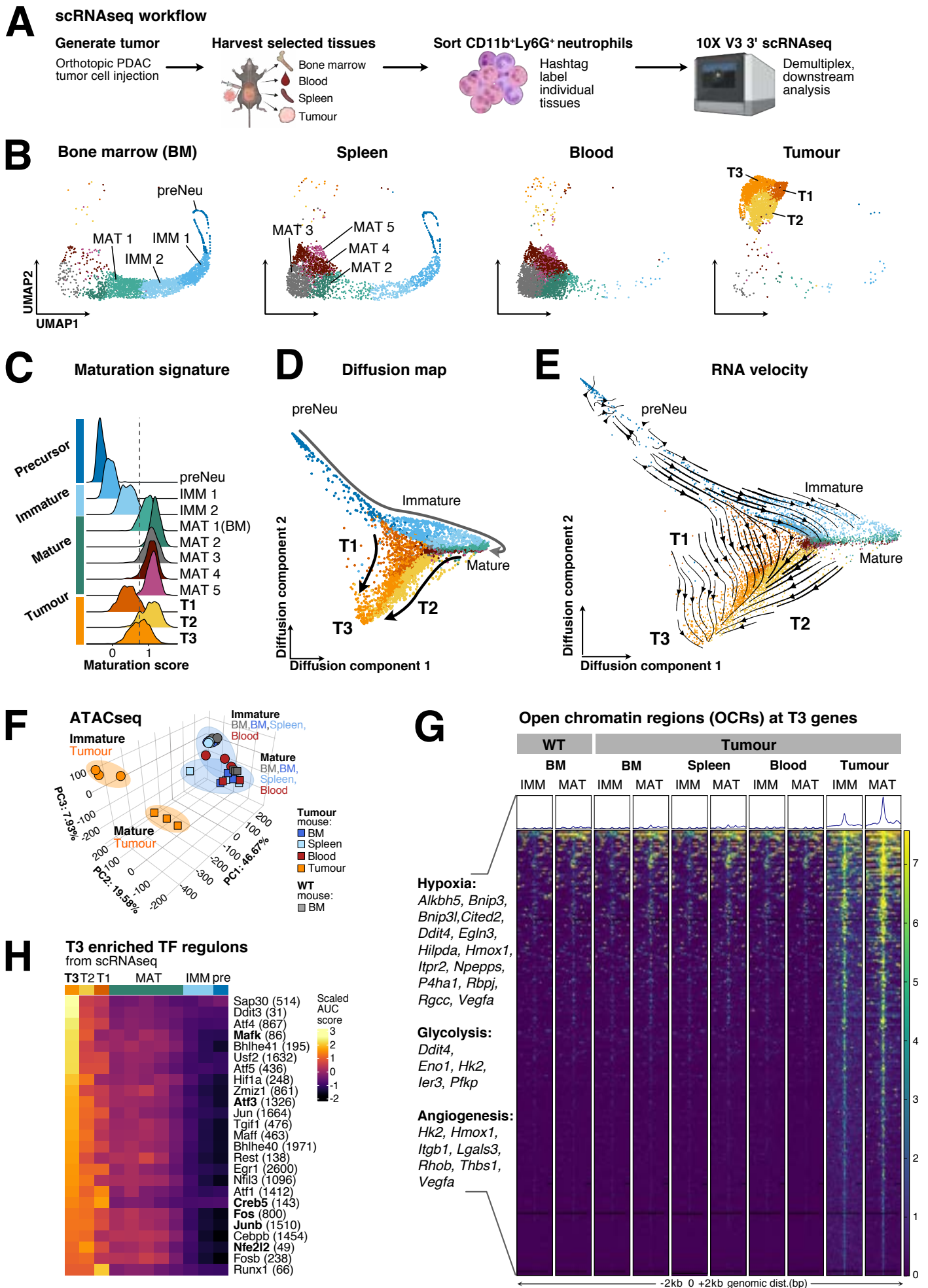


Figure 1. Neutrophils infiltrating the pancreatic tumor undergo further differentiation and converge upon a transcriptionally distinct T3 neutrophil state.

- (A) Schematic shows scRNAseq workflow. CD11b⁺Ly6G⁺ neutrophils were sorted from the bone marrow, spleen, blood and pancreatic tumor for tumor bearing mice 6 weeks post orthotopic injection (n=2). Each sample was individually tagged with cell-hashing antibodies before they were pooled for analysis with 10X V3 3' scRNAseq. (see also fig. S1A and Materials and Methods)
- (B) UMAP projection of total neutrophils in bone marrow (BM), spleen, blood and tumor show strong enrichment of three clusters (T1, T2 and T3) in the tumor. Louvain clustering was performed and colors correspond to clusters identified.
- (C) The neutrophil maturation score can be used to identify Louvain clusters along their differentiation trajectory. Histograms show the module score of the maturation gene signature for each cluster identified in (B), with scores closest to 1 being the most mature. Dotted line in grey defines the cut-off for mature neutrophils, and is set at the lower bound of the Mature 2 cluster.
- (D) Low dimensional embedding of all neutrophils using a diffusion-map approach reveals a branch point between mature and tumor neutrophils. Scatterplot shows diffusion components 1 and 2. Light grey arrows are used to indicate the trajectory from precursor, immature and mature neutrophil states. Black arrows denote the branching into T3 neutrophils from T1 and T2 states.
- (E) RNA velocity suggests the convergent differentiation of T1 and T2 into T3 neutrophils. RNA velocity vectors are projected on the diffusion map embedding with velocity vectors terminating in the tumor and mature neutrophils.
- (F) Principal component analysis of bulk ATAC (Assay for transposase accessible chromatin) sequencing (ATACseq) indicate that changes in chromatin accessibility established in tumor neutrophils cluster them away from other neutrophil subsets. ATACseq was performed for immature (circles) and mature (squares) neutrophils sorted from the bone marrow (grey and dark blue, n= 3 each), spleen (light blue, n=3 each), blood (red, n=3 mature, n=2 immature) and tumor (orange, n =3 each) in wild type (WT) or tumor-bearing (tumor) mice.
- (G) Open chromatin regions (OCRs) matched to differentially expressed T3 genes, have increased accessibility only in immature and mature tumor neutrophils. Heatmap shows intensity of fragment mapping across indicated neutrophil subsets, histograms indicate

average mapping intensity. T3 genes linked to hypoxia, glycolysis and angiogenesis are annotated. (see also table S2)

(H) Heatmap shows scaled AUC scores of the top 25 transcription factor regulons enriched in T3 neutrophils computed by PySCENIC (see also table S2). Numbers in brackets denote the number of genes assigned to the transcription factor regulon. Transcription factors that also had increased motif enrichment in tumor immature and mature bulk neutrophil populations are in bold.

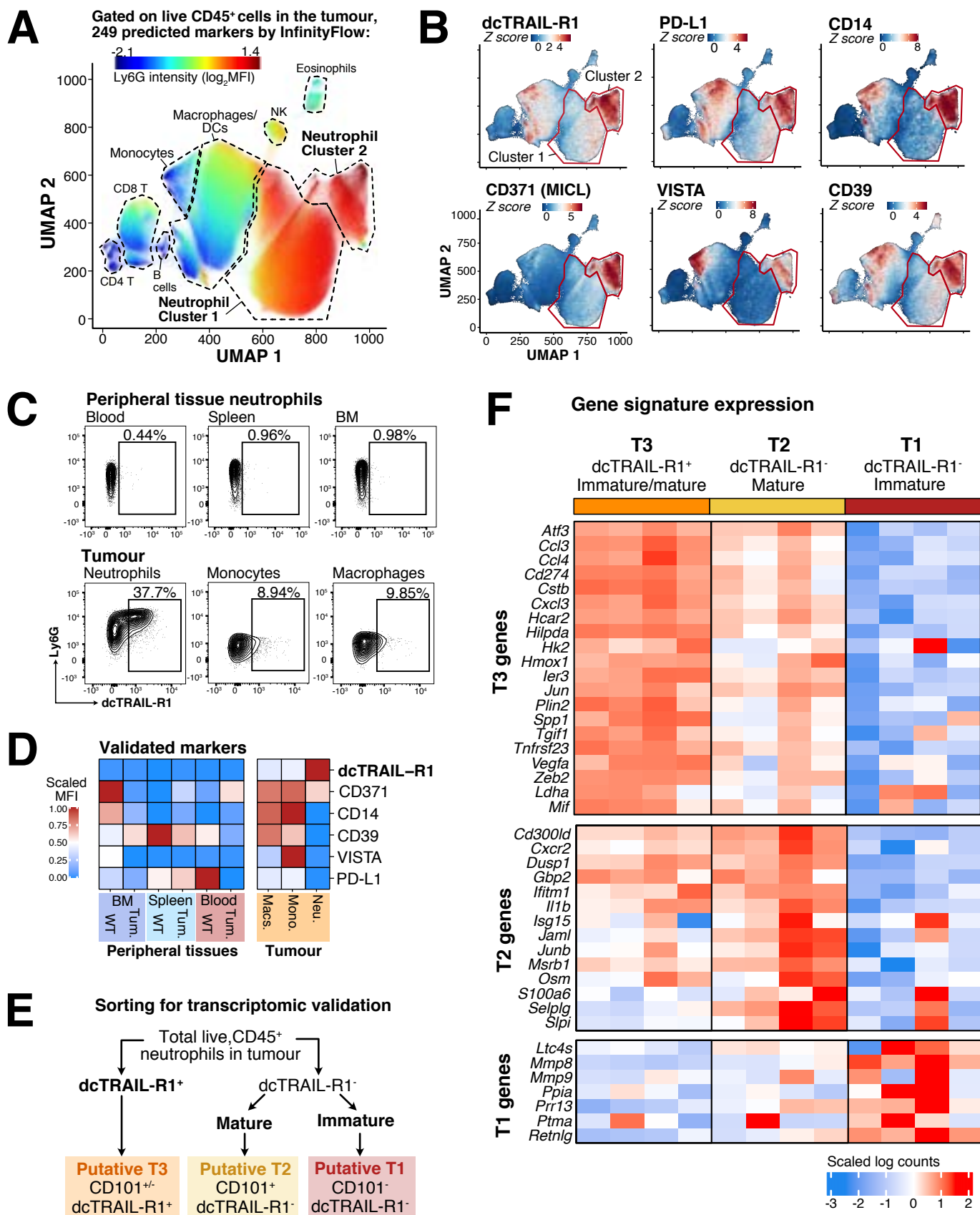


Figure 2. Combinatorial CD101 and dcTRAIL-R1 expression identifies T3, T2 and T1 neutrophils

- (A) UMAP projection of live, CD45⁺ immune cells within the pancreatic ductal adenocarcinoma tumor reveals two clusters of neutrophils present in the tumor. High parameter flow cytometry was carried on single cell suspensions of pooled tumours (n=10) using the LEGENDScreen™ panel (Biolegend). Live, CD45⁺ well-compensated FCS were first analyzed and then exported out for analysis using the InfinityFlow package in R. UMAP shows Ly6G expression intensity imputed by InfinityFlow from high (red) to low (blue). Clusters are annotated by canonical surface marker expression. (see also fig. S3B).
- (B) Neutrophil cluster 2 has increased expression of immunosuppressive/ immunomodulatory surface markers compared to cluster 1. Surface marker expression intensities are shown for curated surface markers from clockwise: dcTRAIL-R1, PD-L1, CD14, CD371, VISTA and CD39. Data are represented as a Z score based on predicted log₂ mean fluorescence intensity (MFI) from high (red) to low (blue). (see also fig. S3C)
- (C) dcTRAIL-R1 expression marks and is restricted to a separate population of tumor infiltrating neutrophils. Representative contour flow cytometry plots (top) show dcTRAIL-R1 expression against Ly6G expression in the tumor for indicated cell populations. Heatmap (bottom) shows scaled mean fluorescence intensity (MFI) for markers in (B), scaled between 0-1 across all populations, with 0 being the lowest MFI. Populations were analyzed by flow cytometry, where total neutrophils were gated as CD11b⁺CD115⁻Ly6G⁺ in the bone marrow (BM), spleen, blood and tumor. Tumor macrophages (CD11b⁺Gr-1⁻F4/80^{hi}MHCII^{hi}) and monocytes (CD11b⁺Ly6G⁻Ly6C^{hi}) were gated accordingly. (see also fig. S3D)
- (D) Proposed gating strategy to isolate T3, T2 and T1 neutrophils by dcTRAIL-R1 and CD101 expression [from the tumour](#).
- (E) Sorted dcTRAIL-R1⁺ tumor neutrophils have the highest expression of the T3 transcriptional signature. Heatmap shows scaled Nanostring gene counts (normalized against internal positive controls and housekeeping genes) for T1 (n=4), T2 (n=4) and T3 (n=4) neutrophils sorted according to (D). Genes belonging to either the T3, T2 or T1 transcriptional signature are indicated. (see also fig. S4D).

Figure 3. Spatial compartmentalization of T1, T2 and T3 neutrophils in the pancreatic tumor

- (A) Chord diagram shows differentially expressed genes (DEGs) in T1 neutrophils that are enriched for gene ontology pathways linked to transcription and oxidative phosphorylation.
- (B) Chord diagram shows DEGs in T2 neutrophils that are enriched for gene ontology pathways linked to metabolism and immune response.
- (C) Chord diagram shows DEGs in T3 neutrophils that are enriched for gene ontology pathways linked to survival and angiogenesis. (A-C) Bars associated with each gene are colored by strength of fold change of differential expression, and are sized based on the number of pathways it interacts with. (see also table S4 for DEG lists)
- (D) Spatial transcriptomic analysis workflow. PDAC tumors were isolated and cut into quarters, where the sharp edges denote the core facing regions, before flash-freezing. Fresh frozen PDAC tumors were sectioned and placed on 10X Visium slides containing spatially barcoded capture spots. After processing and sequencing, the data was clustered spatially (BayesSpace) and cell type deconvolution was performed (Cell2location). Gene signatures of various biological processes were then probed and mapped with the UCell package.
- (E) Tumor neutrophils localize to different spatial clusters. Projection of T3, T2 and T2 neutrophil enriched spots identified by Cell2location on merged UMAP derived from BayeSpace enhanced clustering analysis of tumor sections (n=4)
- (F) Merged UMAP representation of spots of tumor sections analysed, color-coded according to BayesSpace identified clusters (top). Violin plots show frequency of T1, T2 and T3 neutrophils enriched spots mapping to each cluster (bottom).
- (G) Spatial mapping of T1, T2 and T3 neutrophils across tumor sections (n=4) by Cell2location. Black lines denote the outline of the section, grey colored areas indicated the excluded DAPI-panCK^{hi} regions annotated to be fibrotic/necrotic. Spots are filtered based on Ly6G positive staining (see also fig. S6B).
- (H) Quantification of deconvoluted T3 neutrophil-enriched spots falling into high or low scoring spots for GO ontology pathways: glycolytic (GO:0061621), hypoxic (GO:001666), and angiogenic (GO:0045766). Boxplots show median (centre line) with standard deviation (whiskers). $p < 0.05^*$, by one-tailed T test.

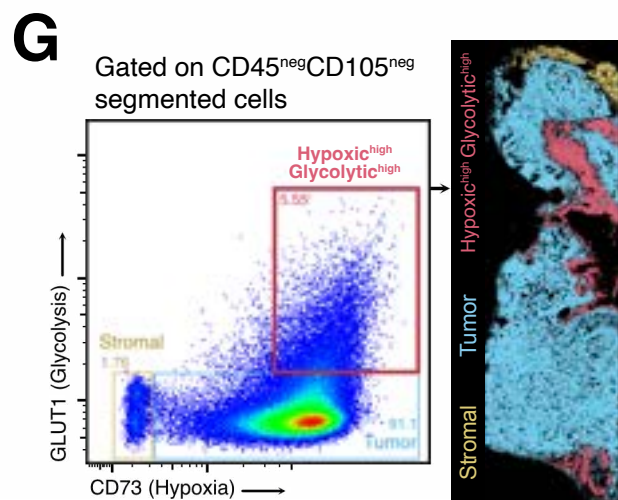
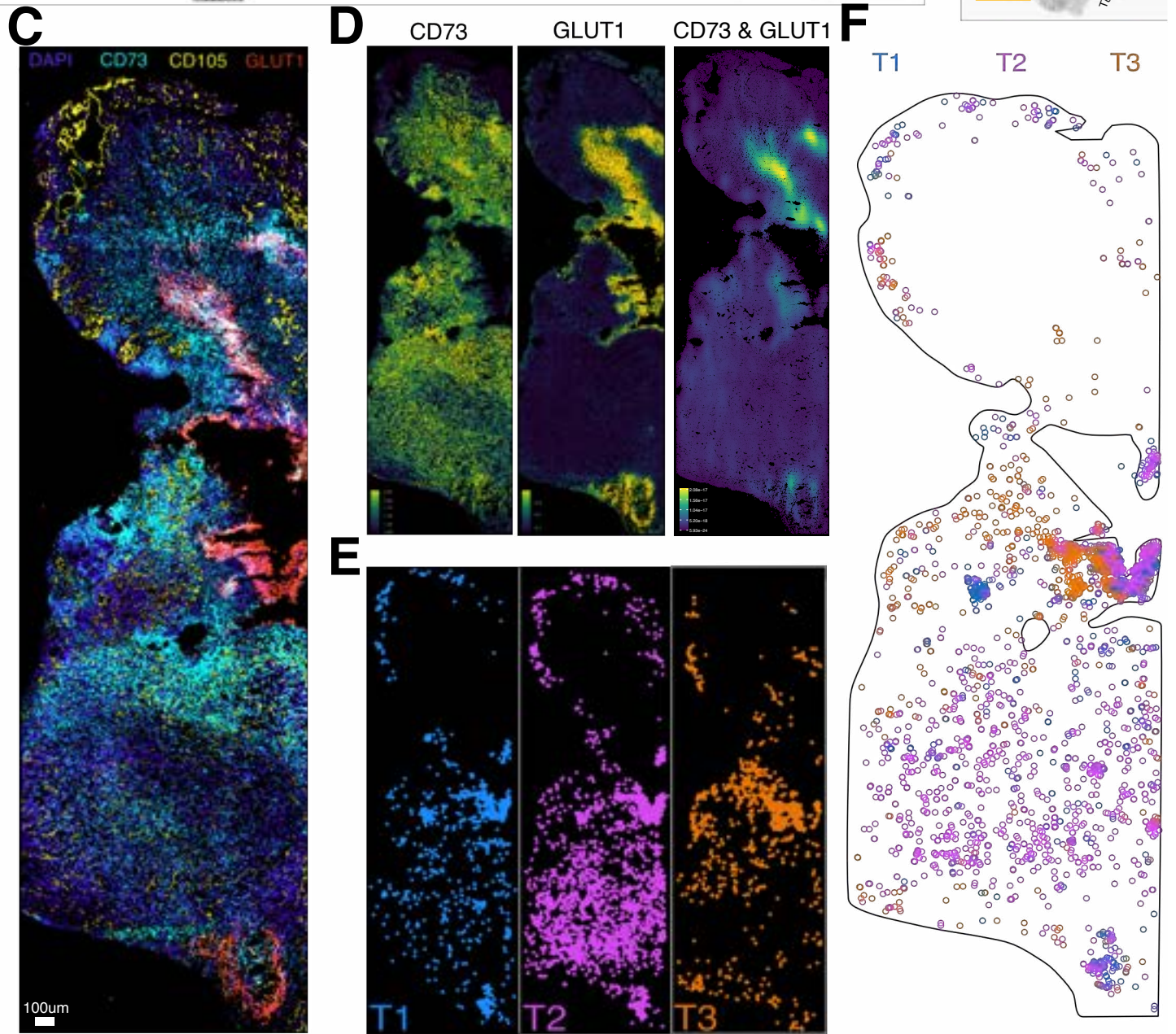
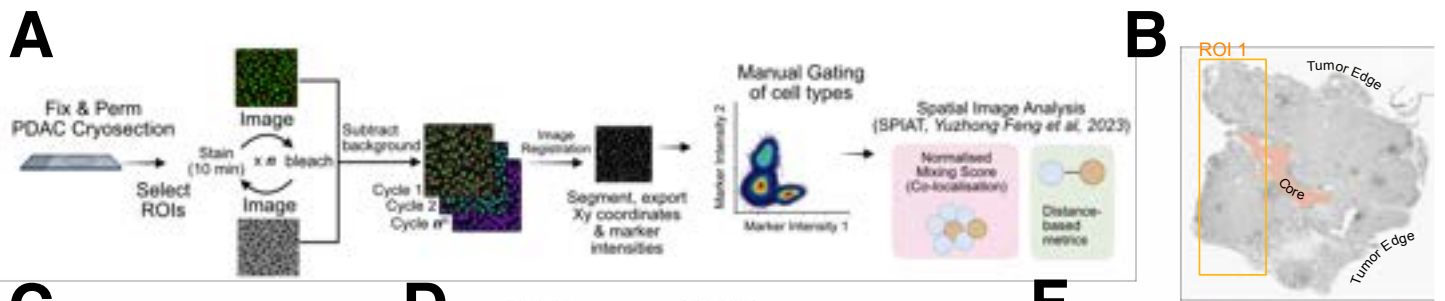


Figure 4. T3 neutrophils occupy a hypoxic-glycolytic niche in the pancreatic tumor

- (A)** Workflow diagram of multiplexed imaging using MICS technology. Cryosections of halved PDAC tumors were placed on slides, fixed and permeabilized, before region of interest (ROI) selection with DAPI staining. Sections were stained for 10 minutes per cycle containing antibodies in FITC, PE and APC. After scanning, sections are photobleached and scanned to subtract background signals. After imaging the desired cycles, images were registered, segmented and exported for conventional flow cytometric annotation of cell types which are further used for spatial statistical analysis with SPIAT.
- (B)** Tumor pictograph showing ROI1 selection area.
- (C)** Immunofluorescent image of ROI1 with indicated stain markers of respective tumor regions. Scale bar = 100um.
- (D)** (left) Expression marker intensity map of CD73 and GLUT1. (right) Co-expression plot of CD71 and GLUT1 marker intensities.
- (E)** Spatial mapping of each annotated tumor neutrophil subsets in ROI1.
- (F)** Co-mapping of neutrophil subsets in ROI1.
- (G)** (left) Gating strategy of CD45-negative, CD105-negative tumor regions demarcated by CD73 and GLUT1. (right) Mapped gated regions on segmented data of ROI1.
- (H)** Spatial statistical analysis of segmented tumor neutrophils. (left) Average minimum distance of each segmented neutrophil subset to hypoxic^{high}glycolytic^{high} regions. (right) Co-localisation of tumor neutrophils with hypoxic^{high}glycolytic^{high} regions, measured using a normalized mixing score for each radius of interaction.

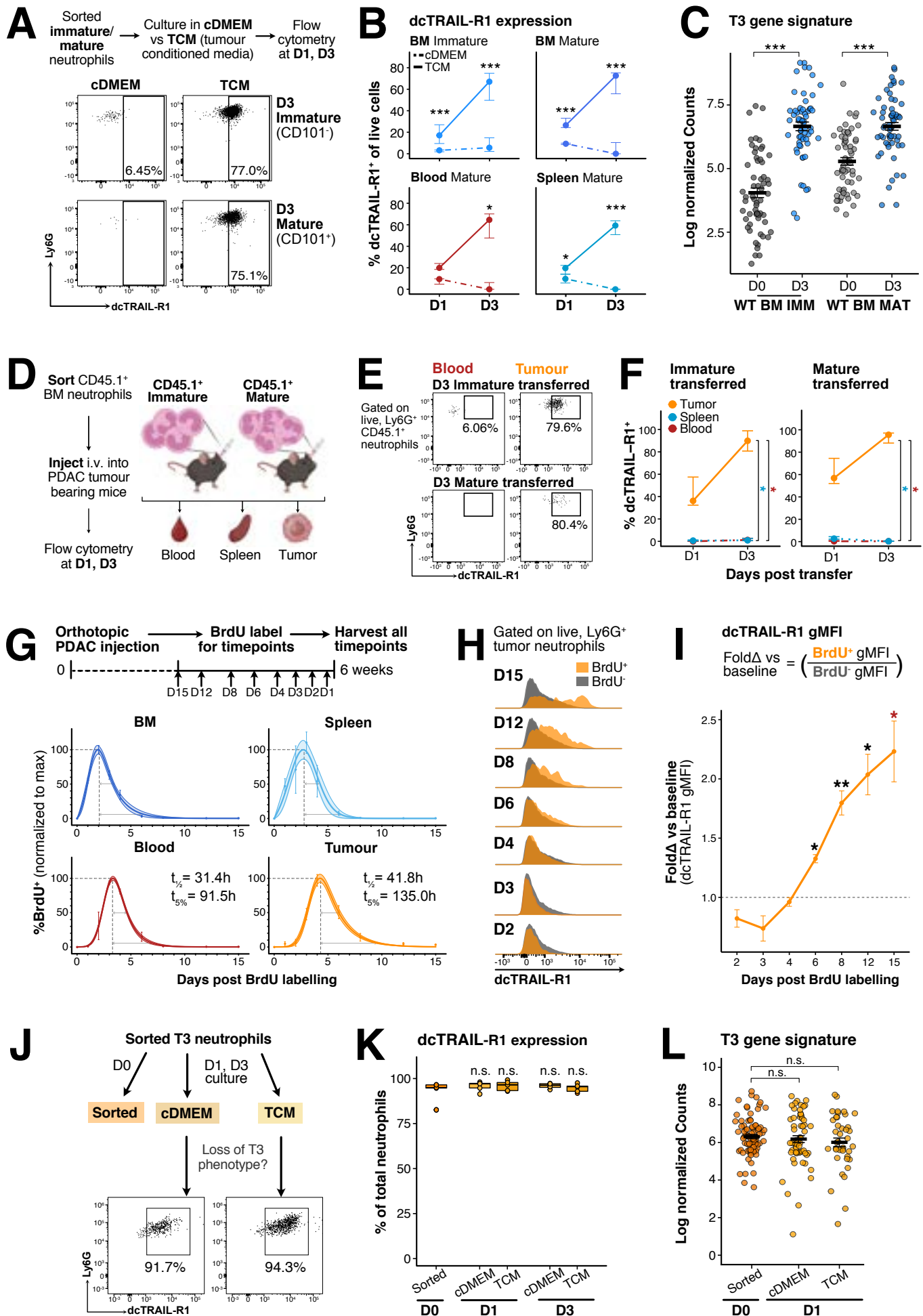


Figure 5. Reprogramming within the tumor environment results in long-lived, terminally differentiated T3 neutrophils

- (A) Experimental set-up of *in vitro* culture of sorted neutrophils from wild type (WT) mice in control media (complete DMEM, cDMEM) versus tumor conditioned media (TCM). Representative flow cytometry plots show dcTRAIL-R1 expression increases on sorted immature and mature WT neutrophils from the bone marrow (BM) after three days of culture in TCM but not in cDMEM.
- (B) Neutrophils cultured in TCM upregulate dcTRAIL-R1 over time. Lineplots show the percentage of dcTRAIL-R1⁺ cells gated as in (B), dots indicate the median, error bars indicate Q1 and Q3 intervals for neutrophil subsets cultured in cDMEM (dotted line) and TCM (solid line) over 1 and 3 days. Each group contains the following number of samples – Day 1 cDMEM: BM immature (n=8), BM mature (n=8), Spleen Mature (8), Blood Mature (3), Day 1 TCM: BM immature (n=8), BM mature (n=8), Spleen Mature (8), Blood Mature (5), Day 3 cDMEM: BM immature (n=10), BM mature (n=10), Spleen Mature (10), Blood Mature (4), Day 3 TCM: BM immature (n=10), BM mature (n=10), Spleen Mature (10), Blood Mature (5). $p<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$, by Mann-Whitney U test.
- (C) Neutrophils cultured in TCM upregulate the T3 gene signature. Scatter dot plots for T3 gene signature expression in BM immature (WT BM IMM) and mature (WT BM MAT) that were freshly sorted (D0) or cultured for 3 days (D3), n=3 for all samples. Each dot denotes a single gene, lines denote the mean, error bars show standard error mean (SEM). $p<0.001^{***}$, by Wilcoxon signed-rank test with Bonferonni's correction, comparisons indicated on graph.
- (D) Experimental set-up for transfer of CD45.1⁺ neutrophils into pancreatic tumor bearing mice. Sorted WTBM immature and mature neutrophils were intravenously injected into WT PDAC tumor-bearing mice. At 1- and 3-days post transfer, CD45.1⁺ neutrophils were evaluated within the blood, spleen and tumour for dcTRAIL-R1 expression by flow cytometry.
- (E) Upregulation of dcTRAIL-R1 expression is restricted to the tumor. Representative flow plots show dcTRAIL-R1 expression present on transferred CD45.1⁺ immature and mature neutrophils present in the blood or tumor at 3-days post transfer.
- (F) Lineplots show proportion of WT BM CD45.1⁺ immature (n=4 at day 1, n=3 at day 3) or mature neutrophils (n=3 at both timepoints) expressing dcTRAIL-R1. Each dot denotes a single gene, lines denote the mean, error bars show standard error mean (SEM). $p<0.05^*$, by Kruskal-Wallis test with Dunn's post-test.

- (G)** Experimental set-up for BrdU pulse-labelling in tumor-bearing mice. WT mice received orthotopic injection of the pancreatic ductal adenocarcinoma (PDAC) cells, and the tumor was allowed to grow. At days 15 (n=4), 12 (n=4), 8 (n=5), 6 (n=3), 4 (n=4), 3 (n=4), 2 (n=3), and 1 (n=4) prior to the harvest, mice were injected with BrdU, thus labelling proliferating neutrophils within the bone marrow and spleen. At day 42 (6 weeks) post injection, mice were harvested, and BrdU⁺ neutrophils within the bone marrow, spleen, blood and tumor were quantified. BrdU percentages at all timepoints were then normalized to the maximal BrdU⁺ percentage value for each tissue, which was set to 100%. Dots shows mean expression with error bars denoting 95% confidence intervals. A mathematical model capturing the full temporal window was fitted to estimate half-life ($t_{1/2}$) and lifespan ($t_{5\%}$) for each organ, denoted on each plot in hours (see also Methods and fig. S12C).
- (H)** BrdU labelled neutrophils upregulate dcTRAIL-R1 over time. Histograms show geometric mean fluorescence intensity (gMFI) of dcTRAIL-R1 within BrdU⁺ (orange) and BrdU⁻ (grey) neutrophils over day 2,3,4,6,8,12 and 15 timepoints post BrdU labelling.
- (I)** Quantification of gMFI in **(H)**. Line plots fold change of dcTRAIL-R1 gMFI of BrdU⁺ against BrdU⁻ neutrophils. BrdU⁻ neutrophils served as a measure of baseline dcTRAIL-R1 gMFI within the tumour. Each dot denotes the mean, with error bars showing SEM. $p < 0.05^*$, $p < 0.01^{**}$, by Mann-Whitney U test, one-tailed, alternative = “greater”.
- (J)** Experimental set-up of *in vitro* culture of sorted T3 neutrophils from PDAC mice in cDMEM or TCM at 1 or 3 days. Representative flow cytometry plots show dcTRAIL-R1 expression is retained on T3 neutrophils after 1 day of culture in both cDMEM and TCM.
- (K)** Boxplots show quantification of frequency of dcTRAIL-R1⁺ neutrophils (n=5 each) in **(I)**, where lines denote the mean. $p = \text{n.s.}$ (non significant), by Kruskal-Wallis followed by many-to-one U test comparing against the D0 timepoint.
- (L)** T3 neutrophils cultured overnight do not downregulate the T3 gene signature. Scatter dot plots for T3 gene signatures in sorted, cDMEM or TCM cultured neutrophils (n=3 each), each dot denotes a single gene, lines denote the mean, error bars show standard error mean. $p = \text{n.s.}$, by Kruskal-Wallis followed by Dunn’s post-test.

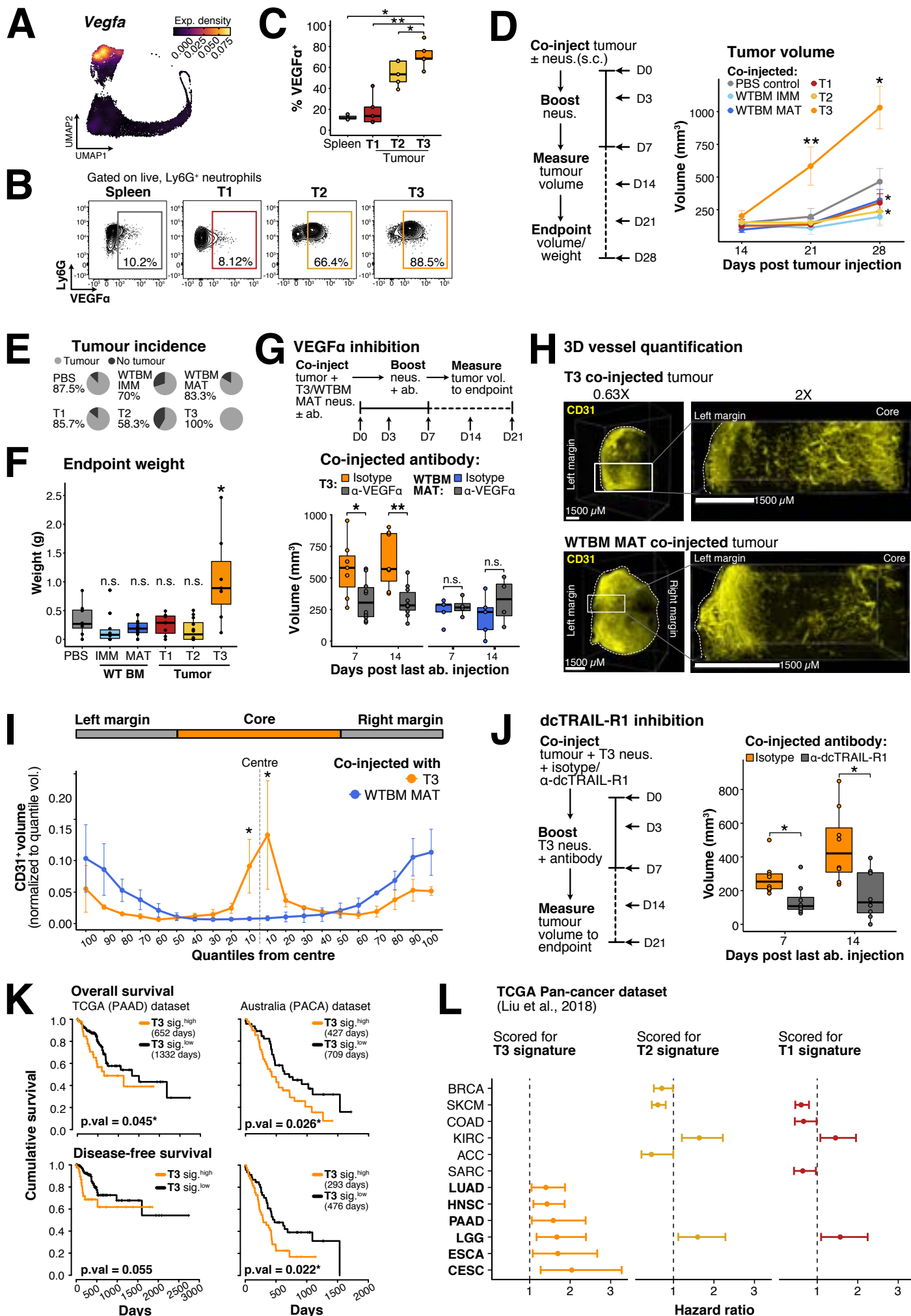


Figure 6. Pro-tumoral T3 neutrophils promote tumor growth and associate with poorer patient outcomes

- (A) T3 neutrophils have the highest expression of *Vegfa* transcripts. UMAP projection of total neutrophils in bone marrow (BM), spleen, blood and tumor shows expression density of *Vegfa*.
- (B) T3 neutrophils have the highest expression of VEGF α . Representative flow plots show intracellular VEGF α protein staining in neutrophils from the spleen and T1, T2 and T3 neutrophils in the pancreatic tumor.
- (C) Boxplots quantify VEGF α expression as shown in (B). Boxplots show median (centre line) with standard deviation (whiskers). $p<0.05^*$, $p<0.01^{**}$, by Kruskal-Wallis followed by many-to-one U test comparing against T3 neutrophils.
- (D) T3 neutrophils promote rapid tumor growth. Schematic of experimental set-up to determine the ability of neutrophils to promote tumor growth *in vivo*. Equal numbers of neutrophils and pancreatic ductal adenocarcinoma cells (PDAC) were mixed in Matrigel before subcutaneous injection into the right flank. Neutrophils in the tumor was boosted on day 3 (D3) and D7, by direct subcutaneous injection into the visible matrigel plug/tumor. Tumor growth was measured by calipers weekly until the day 28 endpoint. Line plots show volume of measured subcutaneous tumors co-injected with PBS (n=8), WT BM immature (WTBM IMM, n = 10), WT BM mature (WTBM MAT, n=12), T1 (n = 7), T2 (n=12), and T3 (n = 7) neutrophils. Dots show mean with error bars indicating standard error of mean (SEM), over all three measurement timepoints. $p<0.05^*$, $p<0.01^{**}$, by Kruskal-Wallis test followed by many-to-one U test comparing all other conditions to PBS co-injection.
- (E) Quantification of PDAC subcutaneous tumor incidence after neutrophil co-injection (see **fig. S13D** for quantification method). Piecharts show frequency of mice with tumours (light grey) or no tumor growth (dark grey) at day 28 endpoint in (D).
- (F) Tumors co-injected with T3 neutrophils had the biggest weights compared other conditions. Each dot represents one biological replicate, with boxplots showing median with interquartile range, and whiskers indicating lowest and highest measurement. $p<0.05^*$ by Kruskal-Wallis test followed by many-to-one U test comparing all other conditions to PBS co-injection.
- (G) Antibody neutralization of VEGF α reduces T3-mediated acceleration of tumor growth. Schematic of experimental set-up with sorted T3 neutrophils are co-injected with PDAC tumor with anti-VEGF α targeting antibody (n=10) or isotype control (n=7). WTBM mature neutrophils were utilized as controls (anti-VEGF α targeting antibody, n=4, isotype, n=5).

Neutrophils were further injected on D3 and D7 with the corresponding antibody added, and tumour growth was measured at D14 and D21, 7 and 14 days post tumor injection. Boxplots show median tumour volume with whiskers representing the interquartile range. $p < 0.05^*$ by Mann-Whitney U test.

(H) Visualization of CD31 vessels within T3 and WTBM MAT co-injected subcutaneous tumours. Subcutaneous tumours from **(A)** for T3 (n=3) and WTBM MAT (n=3) were dissected, optically cleared and permeabilized, stained with anti-CD31 antibody, and imaged in 3D at 0.65X (100% of total volume) and 2X resolution (35% of total volume starting from midpoint). Representative 3D immunofluorescence images show T3 (**top**, quarter) and WTBM MAT (**bottom**, whole) co-injected tumors, with tumour margins marked out in white dotted lines and indicated.

(I) Subcutaneous PDAC tumors co-injected with T3 neutrophils have greater CD31 vessel density within the tumor core. Quantification of CD31 staining intensity at 2X resolution as in **(E)**. CD31 staining was surfaced with a seedpoint of 16.2, and binned in 10% quantiles according to the distance from left or right margins towards the tumour core. To account for differences in tumor sizes, CD31 staining intensity was further normalized over total slice volume for each quantile. Lineplots represent volume-normalized staining intensity, with dots representing the mean and errors bars indicating SEM for T3 (orange) or WTBM MAT (grey) co-injected tumours. $p < 0.05^*$ by Mann-Whitney U test, one-tailed, alternative = “greater”.

(J) Antibody-mediated blockade of T3 neutrophils reduces their ability to promote rapid tumour growth. Schematic of experimental set-up - sorted T3 neutrophils are co-injected with PDAC tumor with anti-dcTRAIL-R1 targeting antibody (n=8) or isotype control (n=8). T3 neutrophils were further injected on D3 and D7 with the corresponding antibody added, and tumour growth was measured at D14 and D21, 7 and 14 days post tumor injection. Boxplots show median tumour volume with whiskers representing the interquartile range. $p < 0.05^*$ by Mann-Whitney U test.

(K) The T3 neutrophil gene signature is associated with poorer patient overall survival (OS) and disease-free survival (DFS) in pancreatic cancer. Kaplan-Meier plots show OS (**top**) and DFS (**bottom**) for patients in the TCGA-PAAD (The Cancer Genomics Atlas, Pancreatic Adenocarcinoma) and PACA-AU (international Cancer Genome Consortium Pancreatic Cancer-Australia). Patients were split into high and low expression of the T3 curated signature. Median OS and DFS survival are represented on the graph when available. Events are represented by vertical lines and were defined from days to death (from initial pathological diagnosis) or days to first event (from initial treatment, for TCGA, and from clinical disease-free diagnosis for PACA-AU)). $p < 0.05^*$ as calculated by log-rank test.

(L) The T3 neutrophil gene signature is associated with poorer patient overall survival in a subset of solid tumor human cancers. Each data set within the curated TCGA Pan Cancer data set was scored for T3, T2 and T1 signatures (see also **table S4**). Forest plots show hazard ratio (HR) scores associated with patient overall survival (OS) that were significant ($p < 0.05$) by Cox proportional hazards test across all signatures. Dots indicate the calculated HR, whiskers indicate 95% confidence intervals.



Supplementary Materials for

Deterministic reprogramming of neutrophils within tumors

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Materials and Methods

Mice

Six- to ten-week-old C57BL/6 mice were bred and maintained under specific pathogen-free (SPF) conditions in the Biological Resource Centre (BRC) of A*STAR, Singapore. CD45.1 (B6.SJL-Ptprca Pepc b/BoyJ) mice were obtained from The Jackson Laboratory. For all experiments, females were used, and animals were age-matched in each experiment as much as possible. All experiments were performed under the approval of the Institutional Animal Care and Use Committee (IACUC), in accordance with the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore.

Cell lines

The luciferase-stable pancreatic ductal adenocarcinoma FC1242L cell line was generated as described in Leong et al. (66), and was derived from the FC1242 cell line (kind gift from Dr Dannielle Engle, Tuveson lab, Cold Spring Harbour Laboratory) derived from Pdx1^{Cre}; Kras^{G12D/+}; Trp53^{R172H/+} (KPC) mice (67). The mammary adenocarcinoma Py8119 and Lewis lung carcinoma (LLC1) cell lines were obtained from the American Type Culture Collection (ATCC), and grown in standard conditions as per ATCC instructions.

Cell culture of FC1242L pancreatic ductal adenocarcinoma lines

FC1242L cells were cultured in complete media (cDMEM) comprising Dulbecco's modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS), 1% Penicillin-Streptomycin (Gibco) and seeded at a density of 2×10^5 cells/mL in T75 cell culture flask. Cells were grown at 37°C in 5% CO₂ incubator until they reached 80% confluency. Upon reaching confluency, cells were trypsinized with pre-warmed 0.25% Trypsin with Ethylenediaminetetraacetic acid (EDTA), phenol red (Gibco) for 5 minutes at 37°C in 5% CO₂ incubator, washed with pre-warmed cDMEM and counted, before seeding at a density of 2×10^5 cells/mL for the next passage. All experiments were performed using cells from the 3rd passage from the initial gift of the FC1241L cell line.

Tumor models

Orthotopic pancreatic tumor model

Mice were administered intrapancreatic injections of FC1242L tumor cells. Mice were anesthetized with ketamine/xylazine, and had their abdomen shaved and swabbed with antiseptic. A 5mm vertical incision was made in the skin and abdominal layer at a point 1cm down from the xiphoid process of the sternum, and 1cm to the right of the midline. The pancreas was exposed, 1×10^5 tumor cells were re-suspended in 1X phosphate buffered saline (PBS) and mixed with Matrigel (BD) in a 1:1 ratio, and were injected as a volume of 50 µL into the body of the pancreas to form a visible bolus using a 30-gauge insulin needle. The pancreas was then returned to the abdominal cavity. The abdominal layer was closed with absorbable 5/0 sutures, while the skin was closed with non-absorbable 5/0 sutures. Superglue was applied over the sutures to ensure that they do not come undone after surgery. Mice were resuscitated with saline and were subcutaneously

administered Buprenorphine (10mg/kg) and Enrofloxacin (Baytril, 1.5mg/kg) for the 2 days following surgery. Mice were euthanized at week 6-7 following surgery and tumor weights were recorded.

Orthotopic breast tumor model

Mice were implanted orthotopically into the thoracic mammary gland with Py8119 tumor cells (ATCC) derived from MMTV-PyMY transgenic C57BL/6 female. Briefly, mice were anesthetized with ketamine/xylazine, and had their left pectoral nipple area shaved and swabbed with antiseptics. A 5mm vertical incision was made in the skin and the thoracic mammary gland was exposed. The tumor cells were re-suspended in 1X PBS at 1×10^5 concentration, and were injected as a volume of 50 μ L into the body of the thoracic mammary gland to form a visible bolus using a 30-gauge insulin needle. The incision at the left pectoral nipple area was closed with non-absorbable 5/0 sutures. Superglue was applied over the sutures to ensure that they do not come undone after surgery. Mice were resuscitated with saline and were subcutaneously administered Buprenorphine (10mg/kg) and Enrofloxacin (Baytril, 1.5mg/kg) for the 2 days following surgery. Mice were euthanized at week 4 following surgery and tumor weights were recorded.

Orthotopic lung cancer model

LLC1 tumor cells (ATCC) were administered intravenously into the lateral tail vein of the mice. Briefly, mice were warmed for 5-10 minutes with an infra-red heating lamp to dilate the veins. Then, mice were transferred to a mouse restrainer to expose the tail. Mice were injected intravenously at either the left or right lateral tail vein with 2×10^5 of tumor cells in 100 μ L PBS. Mice were euthanized at week 3 following surgery and tumor weights were recorded.

Subcutaneous pancreatic tumor model

1×10^5 tumor cells were re-suspended with/without with 1×10^5 neutrophils (specified in text) in 1X PBS. The cells were then mixed in a 1:3 ratio of Matrigel, and injected (200 μ L) subcutaneously into the right flank of mice using a 30-gauge insulin needle. At 3 and 7 days post the initial injection, 1×10^5 neutrophils were resuspended in 30 μ L of 1X PBS, and injected directly into the observable Matrigel/tumor plug. Starting at day 14 after the injection of the tumor, tumors were measured weekly with Vernier callipers and tumor volumes were calculated using the ellipsoid volume formula – $(0.5 \times L \times W^2)$, where L is length and W is width). Mice were euthanized at day 28 following injection, and tumor presence and weights were recorded. Tumors were deemed to be rejected upon the complete absence of tumor formation, or the discovery of a clear Matrigel plug (**Figure S13D**).

In instances where antibodies were co-injected alongside neutrophils, 10ug of antibody was added to 1×10^5 neutrophils, incubated for 10-20 minutes on ice and made up to the appropriate volume with PBS, before the addition of 1×10^5 tumor cells. The cells were similarly mixed in a 1:3 ratio of Matrigel before subcutaneous flank injection. At 3 and 7 days post the initial injection, 1×10^5 neutrophils were similarly mixed with antibody, incubated for 10-20 minutes on ice, before the volume was topped up to 30ul and the injection is carried out as described above. The blocking/neutralizing antibodies used are VEGFa (2G11-2A05, Biolegend), Rat IgG2a, κ isotype control (RTK2758, Biolegend), dcTRAIL-R1 (Clone #270012, R&D systems), Monoclonal Rat IgG2A isotype control (Clone #5447, R&D systems)

Tissue preparation and data analysis for flow cytometry and cell sorting

Blood

Murine blood was collected via cardiac puncture or incision in the submandibular region using a 5mm single-use lancet. 200 μ L was transferred into an Eppendorf tube containing 20 μ L of 0.5M EDTA. Blood samples were then lysed in 1 X red blood cell (RBC) lysis buffer (eBioscience) for 4 min. Samples were quenched with fluorescence-activated cell sorting (FACS) Buffer (1 X PBS, 4mM EDTA, 3% FCS) and spun down at 400g for 5 minutes. RBC lysis was repeated for a second time for another 2 minutes.

Spleen

Spleens were excised from mice and single-cell suspensions were obtained by homogenizing spleen samples through a 70- μ m nylon mesh filter with FACS buffer. Samples were then spun down at 400g for 5 minutes and lysed with 1 X RBC lysis buffer for 3 minutes. Cells were quenched with FACS buffer and filtered through a 70- μ m nylon mesh filter.

Bone Marrow

A 23-gauge needle through the marrow cavity of the femur and the bone marrow was flushed out with 2 ml of FACS buffer using a 1ml syringe. For sorting purposes that required large cell numbers, both femur and tibia bones were used. Cells were then filtered and washed with FACS buffer, cells were spun down at 400g for 5 minutes.

Tumor

Tumors were excised from tumor-bearing mice and weighed before mincing into smaller pieces using surgical scissors. Digestion mix (RPMI, 10% FCS, 500U/mL DNase I, 385U/mL collagenase D) was added to the minced tumor and incubated at 37°C for 30 minutes. Digestion enzymes were quenched with FACS buffer, and minced tumors were transferred into the gentleMACS™ C tubes (Miltenyi Biotec) for mechanical homogenization by the Xiril Dispomix using 2 rounds of Program 5 (20 seconds). Homogenized cells were filtered through 70- μ m nylon mesh filter and washed with 20mL FACS buffer. Samples were then spun down at 400g for 5 minutes before RBC lysis with 1 X RBC lysis buffer for 3 minutes. Cells were washed filtered through a 70- μ m nylon mesh filter.

Flow cytometry and cell sorting

Antibodies were purchased from BD, Biolegend, eBioscience or R&D Systems. For the identification of mouse myeloid cells from the blood, bone marrow and spleen, cells were stained with fluorophore-conjugated anti-mouse antibodies against CD11b (M1/70), CD16/32 (2.4G2), CD45 (30-F11), CD62L (MEL-14), CD101 (Moushi101), CD115 (AFS598), cKit (2B8), CXCR2 (SA044G4), CXCR4 (2B11), CX3CR1 (SA011F11), Gr1 (RB6-8C5), I-A/I-E (M5/114.15.2), CD43 (S7), Ly6C (HK1.4), Ly6G (1A8) and Siglec-F (E50-2440), together with lineage exclusion markers CD90.2 (53-2.1), B220 (RA3-6B2), NK.1.1 (PK136), and CD11c (N418). After exclusion of cell doublets and dead cells with DAPI, neutrophils were identified as (Lin/CD115/Siglec-F)⁻ Gr1⁺CD11b⁺ cells. PreNeus were further identified as CXCR4^{hi}cKit^{int}CD101⁻, immature

neutrophils were identified as CXCR4^{lo}cKit^{lo}CD101⁻ and mature neutrophils were identified as CXCR4⁺cKit⁺Ly6G⁺CD101⁺.

For the identification of mouse myeloid cells from the tumor, cells were stained with fluorophore-conjugated anti-mouse antibodies against CD11b (M1/70), CD16/32 (2.4G2), CD45 (30-F11), CD62L (MEL-14), CD101 (Moushi101), CX3CR1 (SA011F11), CXCR2 (SA044G4), F4/80 (BM8), Gr1 (RB6-8C5), I-A/I-E (M5/114.15.2), CD43 (S7), Ly6C (HK1.4), Ly6G (1A8), dcTRAIL-R1 (270012, R&D Systems or mDcR1-3, Biolegend) and Siglec-F (E50-2440), together with exclusion lineage markers that include CD90.2 (53-2.1), B220 (RA3-6B2), NK.1.1 (PK136), and CD11c (N418). After exclusion of cell doublets and dead cells with DAPI, neutrophils were identified as (Lin/CX3CR1/Siglec-F)⁻Gr1⁺CD11b⁺I-A/I-E⁻F4/80⁻Ly6G⁺ cells. T1 neutrophils were further identified dcTRAIL-R1⁻CD101⁺, T2 neutrophils were identified as dcTRAIL-R1⁻CD101⁺, and T3 neutrophils were identified as dcTRAIL-R1⁺CD101^{+/+}. Ly6C^{hi} Monocytes were identified as (Lin/Siglec-F)⁻Gr1⁻CD11b⁺I-A/I-E⁻F4/80⁻Ly6G⁻Ly6C^{high}, macrophages were identified as (Lin/Siglec-F)⁻Gr1⁻Ly6C⁻I-A/I-E⁺F4/80⁺.

For identification of dcTRAIL-R1⁺ neutrophils in *in vitro* assays, cells were stained with fluorophore-conjugated anti-mouse antibodies against dcTRAIL-R1 (270012), Ly6G (1A8) and Ly6C (HK1.4). After exclusion of cell doublets and dead cells with DAPI, dcTRAIL-R1⁺ neutrophils were identified as dcTRAIL-R1⁺Ly6G⁺Ly6C^{low}.

For sorting of total neutrophil populations, cells were stained with fluorophore-conjugated anti-mouse antibodies against CD11b (M1/70), CD45 (30-F11), CD101 (Moushi101), CD115 (AFS598), cKit (2B8), CX3CR1 (SA011F11), Gr1 (RB6-8C5), Ly6C (HK1.4), Ly6G (1A8) and Siglec-F (E50-2440), together with lineage exclusion markers CD90.2 (53-2.1), B220 (RA3-6B2), NK.1.1 (PK136), CD11c (N418) and I-A/I-E (M5/114.15.2). Total neutrophils were sorted from the bone marrow, spleen, blood, and tumors from pancreatic tumor mice and identified as Lin⁻CD45⁺CD115⁻Ly6C^{low}Siglec-F⁻Gr1⁺CD11b⁺Ly6G⁺.

For intracellular staining of neutrophils from the spleen and tumor, cells were first stained with a fixable vitality dye (LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit; Invitrogen), then washed and stained with fluorophore-conjugated anti-mouse antibodies against CD11b (M1/70), CD101 (Moushi101), CD115 (AFS598), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CX3CR1 (SA011F11), F4/80 (BM8), Gr1 (RB6-8C5), I-A/I-E (M5/114.15.2), Ly6C (HK1.4), Ly6G (1A8), dcTRAIL-R1 (270012) and Siglec-F (E50-2440), together with lineage exclusion markers CD90.2 (53-2.1), B220 (RA3-6B2), NK1.1 (PK136) and CD11c (N418). Stained cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Buffer (BD) before secondary stained with fluorophore-conjugated anti-mouse antibodies against VEGFA (EP1176Y, Abcam).

Flow cytometry acquisition was performed on BD LSR II and BD FACSymphony A5 (BD) analyzers. Cell numbers were quantified with count beads (CountBright; Life Technologies) according to the manufacturer's instructions. Sorting of neutrophil subsets from blood, BM, spleen, and tumor were performed using a BD ARIAII (BD) to achieve >98% purity.

High parameter flow cytometry staining (LEGENDScreen™) and InfinityFlow

Pancreatic tumor single-cell suspensions were pooled from 5 tumor-bearing mice. Cells were then stained with a backbone panel consisting of: Ly6G (1A8), Siglec-F (E50-2440), CD101 (Moushi101), Gr-1 (RB6-8C5), Ly6C (HK1.4), CD11b (M1/70), I-A/I-E (M5/114.15.2), B220 (53-6.7), CD90.2 (53-2.1), NK1.1 (PK136), CD11c (N418), CD45 (30-F11) and CD43 (S7). The

cell suspension was equally distributed across all antibody wells in the LEGENDScreen™ kit (Biolegend), and staining and washing of the samples were performed according to the manufacturer's instructions. For acquisition, 1μM DAPI was added to the pelleted cells, and 1 million events per well was acquired with a 5-laser BD LSR II (BD) using FACSDiva software. Each FCS file corresponding to each unique PE marker was subsequently compensated and analyzed individually with FlowJo software (BD) for quality control. Compensated live, CD45⁺ singlets were exported from each file and analysed in R using the InfinityFlow package (1.4.0) as described (28, 29). Briefly, the InfinityFlow pipeline transforms the input data using logicle transformations, calculates a Z score for all backbone channels, before non-linear multivariate regression models are trained using the XGBoost R package, and used to impute the expression of each input antibody expression intensities across all cells. Isotype-specific background staining correction is also performed. UMAP is then run on the concatenated backbone data matrix, and a concatenated FCS is also generated as an output. For plotting and data visualization, imputed values were scaled and the upper and lower 0.05% quartiles were removed. Clusters were manually annotated and gated in FlowJo (BD), and the imputed values for each cluster were exported. For heatmap plotting and comparison, the geometric mean was calculated for each cluster, scaled and plotted with the ComplexHeatmap package.

Single cell RNA sequencing (scRNAseq)

Cell preparation

Sorted total neutrophils from the bone marrow, spleen, blood, and tumor (n = 2) of pancreatic tumor-bearing mice were spun down and incubated with Totalseq-A anti-mouse Hashtag antibodies 1-8 at a concentration of 0.5μg per 100,000 cells for 30 minutes at 4°C. Cells were washed with FACS buffer, spun down, and resuspended in PBS with 1% bovine serum albumin. Cells were then counted and pooled accordingly and were used as input for 10x Genomics 3' (v3) sequencing on NovaSeq (Illumina) following the manufacturer's protocol.

scRNAseq quality control and analysis

The quality of sequencing reads was evaluated using FastQC and MultiQC. Cell Ranger (version 2.2.0) was used to align the sequencing reads (fastq) to the mm10 mouse transcriptome and quantify the expression of transcripts in each cell. This pipeline resulted in a gene expression matrix for each sample, which records the number of UMIs for each gene associated with each cell barcode. The gene expression matrix was subsequently analyzed in R using the Seurat package (4.0.5) (68). Hashtags were demultiplexed using CITE-seq-Count. Doublets and multiplets were filtered out, as well as UMIs which had 2 or more hashtags associated with it, which resulted in 11,682 remaining single cell transcriptomes. Additional QC included removing UMIs with excess mitochondrial reads (>5%), number of features less than 200 (low read counts) and more than 4200 (outliers). Finally, we used the standard Seurat pipeline running NormalizeData(), FindVariableFeatures(selection.method = "vst", nfeatures = 2000), and ScaleData(). ScoreJackStraw() and JackstrawPlot was used to determine the number of dimensions before running RunPCA(), FindNeighbours(dims = 1:35), and FindClusters(resolution = 0.8). RunUMAP(dim=1:35, n.neighbours = 30) was used to first visualize the data, which revealed 13 clusters, of which two small clusters, cluster 11 and 12 were identified to be contaminating B cells and platelets. This was subsequently removed from the Seurat object, which now contains 11 distinct clusters, containing 11,347 cells. Mapping of a maturation score derived from Evrard et

al. (14) and Xie et al. (19) using FindModuleScore() was used in tandem with tissue identity to identify clusters corresponding to each maturation stage (Precursor, Immature and Mature), or belonging exclusively to the tumor. Diffusion mapping was carried out using the destiny package (3.4.0) (24) with default settings using DestinyMap(). Spliced and unspliced count matrices were obtained using Velocyto and exported as loom files for subsequent input into scVelo. Loom files imported as annotated data matrices, and UMIs filtered out during quality control in Seurat was similarly discarded before further analysis. Velocity analysis was performed with scVelo (2.1.0) (26) using the default stochastic model and velocity vectors were projected into the diffusion map embedding. Differentially expressed genes (DEGs) were calculated using FindMarkers() specifying the use of MAST (1.20.0), where genes were considered differentially expressed with average log₂ fold change > 1 and adjusted *P* values < 0.05. Subsequent gene ontology analysis was performed on tumor neutrophil cluster DEGs using topGO (2.46.0). Regulatory network analysis was carried out with PySCENIC (0.10.4) (27) in Python and exported for visualization in R. Regulon specificity scores (RSS) were calculated for each pre-defined neutrophil cluster to identify the top 50 scoring transcription factor regulons. AUCell scores were averaged for each cluster, and scores corresponding to the top 50 scoring regions for T3 neutrophils were visualized using ComplexHeatmap (2.10.0) in R.

Integration and cell label transfer

scRNAseq data were obtained from publicly accessible datasets: GSE127465 (11), GSE137540 (19), GSE163834 (13), GSE155698 (55), OEP003254 (56). For the GSE127465 dataset, InDrop data was imported using the indRop (0.1.0) package. All downloaded data were processed in Seurat, using the quality control cut-offs specified above.

Integration of our dataset with the GSE137540 dataset containing healthy neutrophils was performed using Seurat. Both datasets were first normalized using the SCTransform() feature, after which 2000 integration features were selected (SelectIntegrationFeatures()), and the default workflow for integration was performed with the normalization.method parameter set to “SCT”. After integration, the standard workflow detailed above was used scale the data, and to calculate PCA and UMAP embeddings for subsequent visualization.

Cell label transfer was performed using Seurat FindTransferAnchors() and MapQuery() functions. In order to map the query datasets (mouse: GSE127465, GSE163834, human: GSE155698, OEP003254), a reference UMAP was generated from our dataset by setting return.model = TRUE, and re-running RunUMAP(). For the mouse label transfer datasets, all 11 annotated clusters were utilized, while for the humans, a simplified classification collapsing immature and mature neutrophil into one immature and mature cluster respectively was used instead. Transfer anchors between both datasets were computed and projected onto the reference UMAP via the MapQuery() function.

ATAC (Assay for transposase-accessible chromatin) sequencing

ATAC-seq was performed based on the Omni-ATAC protocol (69). 50,000 CD11b⁺Ly6G⁺CD101⁻ immature and CD11b⁺Ly6G⁺CD101⁺ mature neutrophils were sorted from: (1) bone marrow from wild-type naïve mice (n = 3), and (2) bone marrow, spleen, blood and PDAC tumor from tumor-bearing mice 6 weeks post orthotopic injection (n = 3). Briefly, cells were washed, pelleted, and lysed for 3 minutes in Lysis Buffer containing 0.1% v/v NP-40, 0.1% v/v Tween-20 and 0.01% v/v Digitonin. Nuclei were then pelleted and transposed at 37°C, for 30 minutes on a thermomixer at 1000 rpm, before DNA purification, indexing and library preparation was carried out. Samples

were checked for nucleosomal patterning via Bioanalyzer before pooling at an equimolar ratio for sequencing on a HiSeqX (Illumina) with paired-end 150 reads.

Paired-end sequencing reads of length 2 x 150 bp were trimmed with cutadapt (ver. 2.5) (70) and quality checked using FASTQC before aligning to the mm10 (GRCm38) mouse reference genome using bwa (ver. 0.7.17) (71). Low quality alignments (MAPQ < 30), secondary alignments, unmapped reads and reads with unmapped mate were discarded using samtools. For reads with multiple alignments only five best alignments were retained. PCR duplicates were removed using Picard MarkDuplicates. The remaining clean alignments were analyzed for fragment length distribution using the ATACseqQC (ver. 1.10.4) (72). Read alignments to the positive strand were shifted 4 bp downstream and alignments to the negative strand were shifted 5bp upstream to centre the reads on the transposon binding events. Subsequently, peaks were called using macs2 (ver. 2.1.1.20160309) callpeak command with the parameters “-p 0.01 --shift -150 --extsize 300 --nomodel -B --SPMR --keep-dup all --call-summits”. Fold enrichment and p-value tracks normalized to input were produced using macs2 bdgcmp command (options -m FE and -m ppois respectively). Peaks with a q-value < 0.01 were shortlisted. Peaks in the blacklisted regions specified by ENCODE project (73) were removed. For each of the conditions (cell phenotype and tissue of origin) pooled samples were generated by pooling together reads from all individual replicates of the same condition. These pooled samples were also analyzed in the same manner as above. Peaks called in the pooled samples were compared against peaks called in the individual samples of the same condition using irreproducible discovery rate (IDR) framework (ver 2.0.4.2). High quality reproducible peaks were shortlisted based on IDR < 0.05. Subsequently, the IDR peaks from all conditions were merged using “bedtools merge” to generate a final set of peaks. Counts of reads aligned at each peak interval in each sample was determined using summarizeOverlaps function of the GenomicAlignments R package (ver. 1.22.1) (74). The counts were loaded into edgeR (75) for differential accessibility analysis where a negative binomial generalized linear model (GLM) extended with quasi-likelihood methods was fitted to the counts data, dispersions were estimated and differential peak intensity across sample groups was determined by the quasi-likelihood F-test method (76) using FDR (false discovery rate) < 0.05, and log2 fold change > 1. DeepTools (3.1.0) was used to generate normalized bigWigs from pooled biological replicates, with scaling factors calculated from the reciprocal of normalization factors derived from edgeR calcNormFactors() multiplied by library size (per million). Normalized bigwig files were visualized with Integrative Genomics Viewer (IGV 2.7.2).

To find differentially accessible open chromatin regions (OCRs) in tumor neutrophils, DA peaks were first computed between each pairwise comparison across tissues (i.e. Tumor vs Bone marrow) for each maturation stage (immature or mature cells). DA peaks obtained from each of the pairwise comparisons of mature tumor neutrophils against mature neutrophils from (1) wild-type bone marrow, (2) tumor bearing bone marrow, (3) tumor bearing spleen, (4) tumor bearing blood were intersected to identify OCRs specific to mature tumor neutrophils. This process was also carried out to determine immature tumor neutrophil specific OCRs. OCRs shared between immature and mature tumor neutrophils were then denoted to be tumor-specific OCRs (see also **table S2**). Background peaks were defined to be regions that were not differentially accessible in immature and mature tumor neutrophils (see also **table S2**). T3 OCRs were defined as tumor-specific peak regions that were annotated to T3 DEGs found in our scRNAseq dataset (see also **table S3**). Heatmaps of T3 OCRs were generated with DeepTools (77).

Motif discovery was performed with HOMER (4.11.1) (78) findMotif.pl with the -size *given* and -mask options, on tumor-specific OCRs that were classified as DA peaks in immature and mature

tumor neutrophils against the background peak set denoted above. Enriched known transcription factor motifs with q value < 0.05 for immature and mature tumor neutrophils were correlated with transcription factor regulons enriched in T3 neutrophils to examine whether motif usage corresponded to the transcription factors predicted to be active in T3 neutrophils.

Spatial transcriptomics

Sample preparation

Frozen samples were quartered before embedding in optimal cutting temperature-filled molds and sectioned to a thickness of 10 μ m at -20°C with cryotome (CM3050S, Leica). To ensure capture of tumor-infiltrating neutrophils, cryosections were first screened for Ly6G immunofluorescence before mounting on chilled Visium Tissue Optimization Slides (1000193, 10X Genomics) and Visium Spatial Gene Expression Slides (1000184, 10X Genomics). For tissue optimization, the manufacturer's protocol was followed, establishing 6 minutes as the optimal permeabilization time. For processing, we fixed tissue sections onto the slide in chilled methanol and prepared them for immunostaining according to manufacturer's protocol. Sections were stained with Rabbit anti-wide spectrum cytokeratin (Abcam) and Rat Ly6G (1A8, Biolegend) for 30 min. Subsequently, sections were washed with wash buffer for 5 times before secondary staining with Anti-Rabbit Alexa Fluor 488 (Invitrogen) and Anti-Rat Alexa Fluor 555 (Invitrogen) and DAPI for 30 minutes. Upon completion of immunostaining, slides were scanned with the slide scanner (EVOS Auto FL2, Thermo Scientific). After immunofluorescence imaging, Visium Spatial Gene Expression libraries were prepared according to the manufacturer's protocol. The libraries were sequenced on either HiSeq X or NovaSeq 6000 S4 (Illumina) at PE150 and a read depth of 50,000 read-pairs per tissue-covered spot.

Data analysis

Fastq data was processed with the spaceranger pipeline (version 1.2.2, 10X Genomics) and mapped to the GRCh38 mm10-2020-A genome assembly. Spatial spots were initially pre-filtered to remove DAPI-low/negative necrotic areas with Loupe browser 6 (10X Genomics). This enhances the spatial clustering and downstream analyses. Each sample was then normalized individually using the SCTransform function of the Seurat R package (v3.2.3) with default parameters. Data from 4 tumor sections (3 biological replicates, 1 technical replicate) were merged with the merge function of the Seurat R package (v3.2.3) with default parameters and Spatial joint clustering was performed with BayesSpace (36). Ten clusters were determined and assigned based on the qTune function. Annotation of each cluster was performed with the top 50 genes expressed in each cluster to derive the top gene ontology biological processes using EnrichR (79). For cell type deconvolution, the cell2location package (38) was employed together with a reference single-cell dataset kindly provided by Florent Ginhoux. The dataset comprised of CD45⁺ immune and CD45⁻ non-immune cell types in orthotopic PDAC tumors. From the dataset, we first converted gene names to ENSEMBL IDs and performed filtering of low expressing and mitochondrial genes with the default parameters. The regression model was trained under default parameters, ensuring reconstruction accuracy was ideal for use. Finally, the Cell2location model was created using the following parameters: N_cells_per_location = 30, detection_alpha = 20, max_epochs = 20000. Cell type abundances were obtained and exported back to Seurat. To visualize cell type enriched neutrophil spots, we first filtered spots based on the cell type abundance values having at least higher than the 75th-percentile value for the neutrophil subset of interest, while having lower than

the 25th-percentile value for the other 2 subsets. Subsequently, we selected Ly6G staining spots which were manually annotated. For visualization purposes, we jittered the spots to show overlaying neutrophil-enriched spots. The UCell package (80) was utilized to score spots independently for enrichment in various GO processes. These values were visualized by setting a 50th -percentile cut-off, and the frequency distribution of each neutrophil subset was then calculated.

MACSima Imaging Cyclic Staining (MICS)

Sample preparation

Cryosectioned tumor sections were mounted onto glass slides, fixed in 4% PFA for 10 min, washed in PBS, and permeabilized for 20 min with blocking buffer containing 5% donkey serum with 0.3% Triton X100 and DAPI. Slides were fitted onto MACSwell Sample Carriers, which enables sections to be flooded in antibody mixes and washes for each imaging cycle. To identify tumor neutrophils, sections were stained with fluorophore-conjugated anti-mouse antibodies against CD11b (REA592), CD45 (REA737), dcTRAILR1 (mDcR1-3), Ly6G (REA526), CD101 (Moushi101), CD68 (REA835) and Gr-1 (REA810). For identifying tumor regions, sections were stained with fluorophore-conjugated anti-mouse antibodies against CD73 (REA778), CD105 (REA1058), CD29 (REA1074) and primary antibody GLUT1 (PA5-16793, rabbit polyclonal) with secondary rat anti-rabbit IgG-PE (2A9). Image acquisition and processing is performed as described previously (81). Briefly, images were acquired using the long-working-distance 20 × objective (NA 0.45), using hardware autofocus. Fluorophores were excited using red, green, blue light-emitting diodes with appropriate emission filters, and images captured using monochromatic scientific CMOS (SCMOS) camera with 106 nm/pixel resolution.

Data visualization and analysis

Images were preprocessed using the MACS iQView software pipeline as previously described (81). Stitched and registered TIFF files of individual channels were then imported into Imaris (Bitplane). Each marker channel was first analyzed for staining artefacts, which will greatly affect downstream analyses. Artefacts were masked using the surface/spot tool and the masked channel was exported back to the MACS iQ View Software. Then, DAPI stained nuclei was used to perform image segmentation using the Advanced Tissue Morphology method with the donut algorithm and visual inspection. Identified cells and related features containing marker intensities for all channels were imported into FlowJo (BD) for manual cell type annotation. Seurat V4 was used to visualize and these cell type annotations, and the SPIAT package (82) was utilized for distanced-based and co-localization metrics determining of neutrophil subset localization with the various tumor regions.

Digital multiplexed gene expression analysis with Nanostring nCounter system

Probe sets were designed to target mRNA corresponding to T3, T2 and T1 DEGs, which were manually curated to ensure specificity (see also **table S3**), as well as for the housekeeping genes *Actb*, *Cltc* and *Rplp0*. Probes were synthesized to be compatible with the nCounter Elements TagSets (Nanostring). Target cells were either sorted (T3, T2 and T1 cells *ex vivo*), or cultured, and counted to determine the exact number of cells before lysis. Cells were lysed in 1/3 diluted RLT buffer (Qiagen) in deionized water, at a concentration of 5000 cells per μ L of buffer, and mixed well. Lysates were frozen down at -80°C to allow for accumulation of biological replicates. 15,000 cells (3ul of lysate) were used in the downstream hybridization reaction, and the nCounter

gene expression assay was run following the manufacturer's instructions. Data was imported into the nSolver (4.0) system, where they were normalized against the geometric mean of the positive control count and housekeeping genes. Values were further scaled for heatmap plotting.

Cytospin and Wright-Giemsa staining

Sorted neutrophil subsets (7.5×10^4 cells each) were spun onto glass slides using Cytospin 4 Cytocentrifuge (Thermo Scientific), dried for 5 min, fixed, and stained with the Hema 3 manual staining system (Fisher Scientific) according to manufacturer's protocol. Images were acquired with an Olympus BX43 equipped with a 100X oil immersion objective, and image brightness was adjusted with Affinity Photo (Affinity).

In vitro cell culture

FC1242L PDAC cell lines were maintained in culture as specified, and conditioned media was collected on the 3rd passage after thawing. The tumor-conditioned media (TCM) was spun down at 1000g, at 4°C for 10 minutes to pellet down dead cells and debris. The supernatant was carefully removed and stored at -20°C. Sorted neutrophil subsets (specified in text/figures) were counted, and resuspended at 2.5×10^5 /ml in cDMEM media or TCM as specified. Culture was carried out in normoxia (20% oxygen) or in a hypoxia incubator at 5% O₂ where indicated in the text or legends. After culture for one/three days, dcTRAIL-R1 upregulation was analyzed by flow cytometry, or wells were pooled and counted before cells were lysed in preparation for the nCounter gene expression assay.

BrdU Pulse-chase Assay

Mice were injected intraperitoneally with 2mg 5-bromo-20-deoxyuridine (BrdU; Sigma-Aldrich) at indicated time points. To detect BrdU incorporation into neutrophil subsets, cells were stained with a fixable vitality dye (LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit; Invitrogen), surface-stained, fixed, permeabilized, and subjected to intracellular staining with FITC-conjugated anti-BrdU antibody, according to the manufacturer's protocol (BrdU Flow kit; BD) before analysis by flow cytometry.

In vivo Matrigel plug assay

Anesthetized mice were shaved at the lower back exposing both left and right flank regions. Sorted neutrophil subsets (5×10^4 cells each) were resuspended in 500μL of growth-factor depleted Matrigel (Corning) and injected subcutaneously into the left or right flank region to form plugs. At days 3 and 7 post-implantation, sorted neutrophil subsets (5×10^4 cells each) were resuspended in 50μL of PBS and injected directly into the plug respectively. On day 9 post-implantation, Doppler laser perfusion imaging was performed at the lower back region that contained the Matrigel plugs. Regions of interest (ROI) were defined by the observable Matrigel plug, and the

amount of flux variation in each ROI was quantified. Only ROIs that were not obscured by hair regrowth were used.

Whole-mount immunostaining and optical clearing

PDAC tumor samples from mice treated with WT or T3 neutrophils were harvested and fixed in 4% paraformaldehyde overnight at 4°C and then washed with PBS. Samples were permeabilized using a series of methanol gradients, following the SHANEL method for improved permeabilization (83). Briefly, they were further treated with (1) 0.5M acetic acid overnight and washed with PBS; (2) 4M guanidine hydrochloride/0.05M sodium acetate/ 2% triton-x100 pH 6 overnight; (3) 10% CHAPS in PBS for 4 hours and washed with PBS. This process would loosen the tumor tissue, allowing better antibody penetration. Next, the samples were blocked overnight at 4°C in blocking buffer consisting of 5% dimethyl sulfoxide, 2.5% donkey serum, 2.5% goat serum, 0.3% Triton X-100, 0.2% bovine serum albumin, and 0.01% sodium azide diluted in PBS. Samples were then incubated with primary antibody (anti-CD31, clone 2H8, Invitrogen) diluted in blocking buffer, and incubated for 4 days at 4°C. Samples were then washed three times at room temperature with wash buffer consisting of 0.3% sodium chloride and 0.3% Triton X-100 diluted in PBS. This process was repeated for the incubation with secondary antibody (goat anti-hamster Alexa Fluor 647, Life Technologies) and the nuclear dye propidium iodide. Samples were optically cleared using solvent-based optical clearing by immersion in a series of methanol gradients and incubation in a mixture of benzyl alcohol and benzyl benzoate. Samples were transferred into ethyl cinnamate for imaging and long-term storage.

Image acquisition and image processing

Images were acquired using a lightsheet LaVision Ultramicroscope II with a Zoom-body MVX-10, with a 2× MVPLAPO objective (numerical aperture: 0.5). Laser lines 561nm and 639nm were used for fluorophore excitation with emission filters 620/60 and 680/30 respectively. Images were captured using the PCO edge 4.2 sCMOS camera. Images were visualized using Imaris 9.5.0 (Bitplane).

Determination of CD31⁺ blood vessel volume in WT and T3 neutrophil co-injected tumors

All tumors were imaged from one lateral edge to core and to the other lateral edge regardless of tumor size. They were acquired at 2.0x magnification zoom. In total, three imaging experimental replicates were used for image analysis.

Surface creations were carried out prior to performing any statistical analyses to ensure no bias during the analysis. Total tumor volume imaged is determined by surfacing propidium iodide-positive signal in the Imaris software. To standardize the tumor volume used for analysis, both WT and T3 neutrophil-injected tumor images were processed to obtain the same percentage volume from the tumour midpoint, which was then used for the downstream analysis. The image thresholding was kept the same and applied across the image analysis for both WT and T3 tumors within the same experiment. The surface function in Imaris was then used to create surfaces based on CD31⁺ blood vessels staining with a standardized seed point value of 16.2. Next, the distribution of CD31⁺ surfaces from tumor edge to core is calculated using the shortest distance function to the tumor core. The total tumor length from core to edge is further divided into equal parts in

percentiles (10%). The calculated CD31⁺ blood vessels surfaced volume was then binned by percentile according to the relative percentage distance from tumor core to edge. The tumor volume within each percentile bin was also quantified, and used for normalization of CD31⁺ surfaces.

Survival analysis

The T3, T2 and T1 gene signatures were curated from the probe sets used in our Nanostring gene expression assays, where they were matched with DEGs obtained for each cluster using FindAllMarkers() in the mapped PDAC dataset from Steele et al. (55). In the instance where the Nanostring genes did not fall into DEGs in the corresponding human dataset, i.e. T1, the DEGs in FindAllMarkers() were selected instead (see **table S4** for finalized list of genes). Gene signatures were checked to score T1-T3 clusters the highest whether in tumor or adjacent normal tissue.

Gene expression data (HT-seq counts) and corresponding clinical information of The Cancer Genome Atlas Pancreatic Cancer (TCGA-PAAD) cohort were downloaded from UCSC Xena database. Count data was normalized against healthy controls using voom in the limma package (3.50.3) and scaled by Z transformation. Genes with Z score >1.96 (~2 standard derivation) were deemed differentially expressed. Patients were divided into two groups based on high (Z score > 1) and low expression (Z score < 1) of the gene signature assessed. Overall survival was determined by the “days_to_death” field in the clinical data which reflect number of days since the initial pathological diagnosis. Disease-free survival was determined by the “days_to_new_tumor_event_after_initial_treatment” field, denoting the number of days since first treatment. Kaplan-Meier survival curves and log-rank test were computed using the survival package (3.3-1) in R.

RSEM normalized counts and corresponding clinical information of Pancreatic Cancer Australia (PACA-AU) cohort were downloaded from UCSC Xena and the ICGC Data Portal respectively. Since there were no healthy controls for normalization in this dataset, gene signatures were scored by Gene Set Variation Analysis using the GVSA (1.4.0) package in R. Patients were divided into two groups based on GVSA score > 1.0 (high signature expression). Overall survival was determined by the “donor_survival_time” field which denotes the number of days from the initial pathological diagnosis. Disease-free survival was determined by the “donor_relapse_interval” field in the clinical data, which calculates the number of days to the first disease relapse or progression after an initial clinical disease-free diagnosis. Kaplan-Meier survival curves and log-rank test were then computed.

For both TCGA and PACA-AU datasets, further statistical testing was carried out to assess whether confounders were present during the scoring of the T1, T2 and T3 signatures. Data corresponding to each confounder was obtained for the accompanying clinical data. The statistical test carried out and the reported p-values are reported in supplementary tables accompanying this manuscript (**table S5, table S6**).

Gene expression data for the TCGA Pan-Cancer (PANCAN), and the TCGA Pan-Cancer Clinical Data Resource (Liu et al., 2018) was downloaded from National Cancer Institute Genomic Data Commons. As only solid tumors were assessed, DLBC (Lymphoid Neoplasm Diffuse Large B-cell Lymphoma), FPPP (FFPE Pilot Phase II), LAML (Acute Myeloid Leukemia) were removed from the analysis. As there were no healthy controls present in each dataset, the GVSA scoring method was utilized, and a GVSA score > 1.0 was determined as high signature expression.

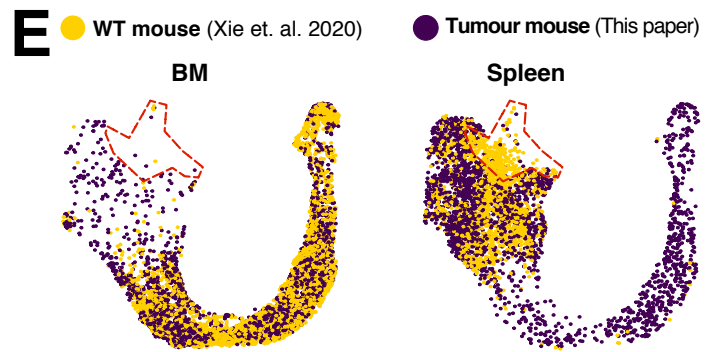
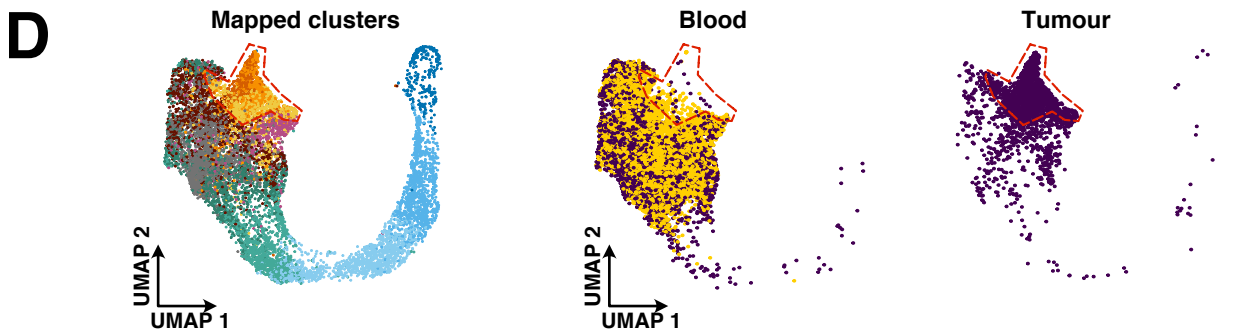
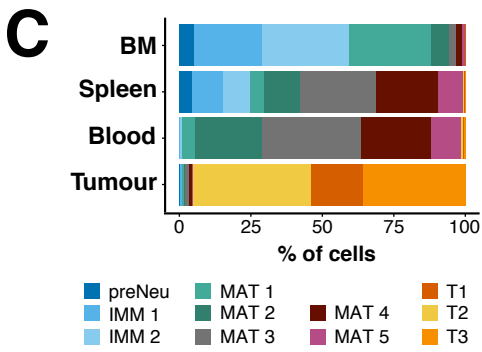
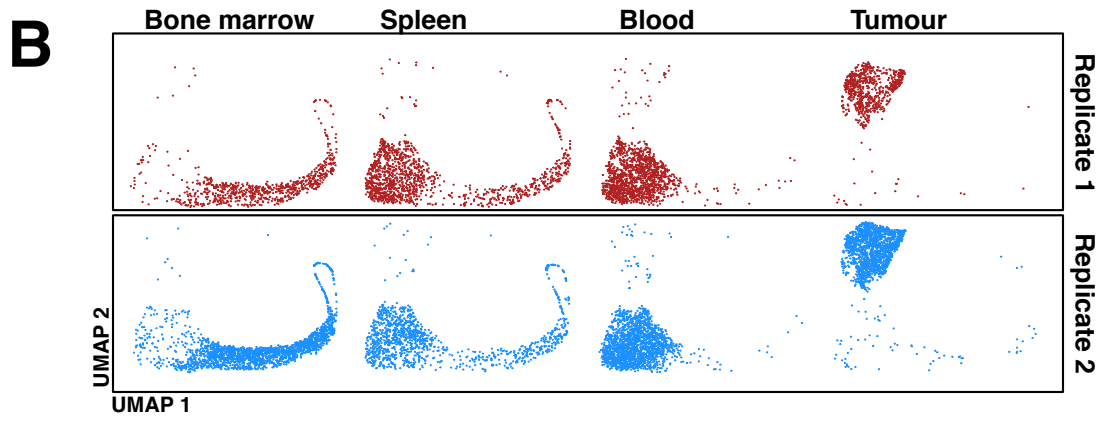
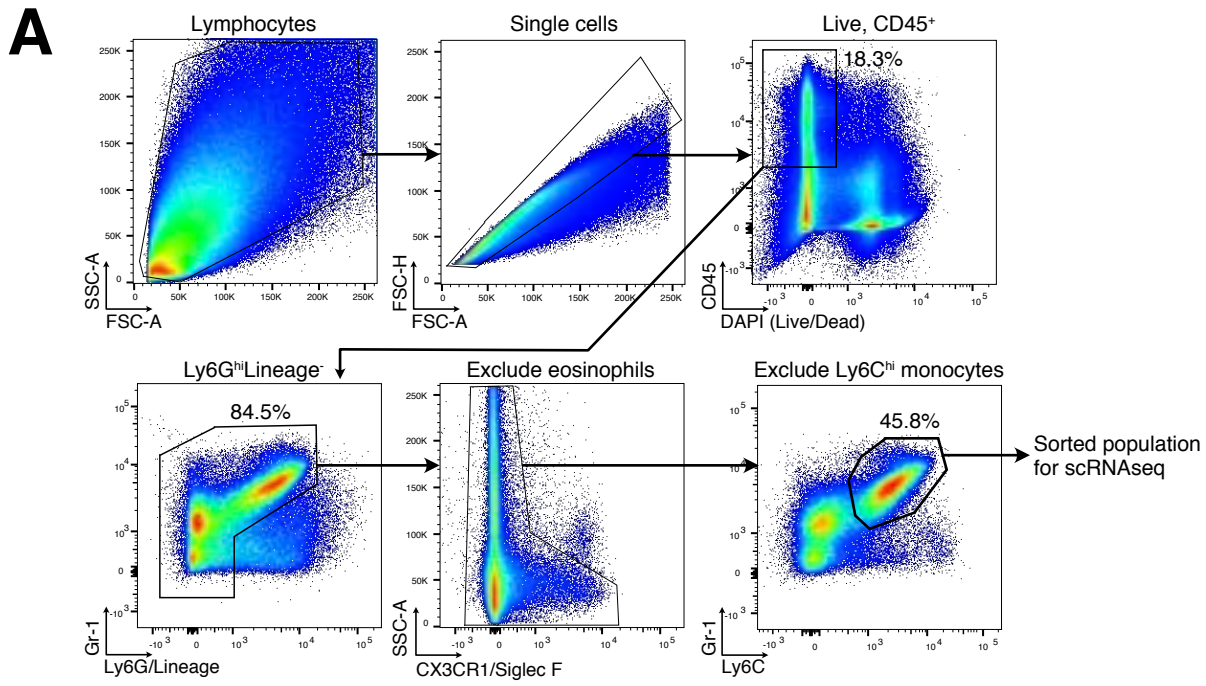
1936 Overall survival was determined by “OS” field in the Clinical Data Resource, which has been
1937 robustly curated and published. Survival analysis, log-rank test, and Cox proportional-hazards
1938 regression was carried out using the survival package (3.3-1) in R. Results are reported in **table**
1939 **S7**.

1940

1941 ***Statistical analyses***

1942 Statistical analyses were performed in R (4.1.3), with the statistical test and number of biological
1943 replicates indicated in the accompanying figure legends.

1944



Supplementary Figure 1. Three distinct neutrophil populations infiltrate the tumor.

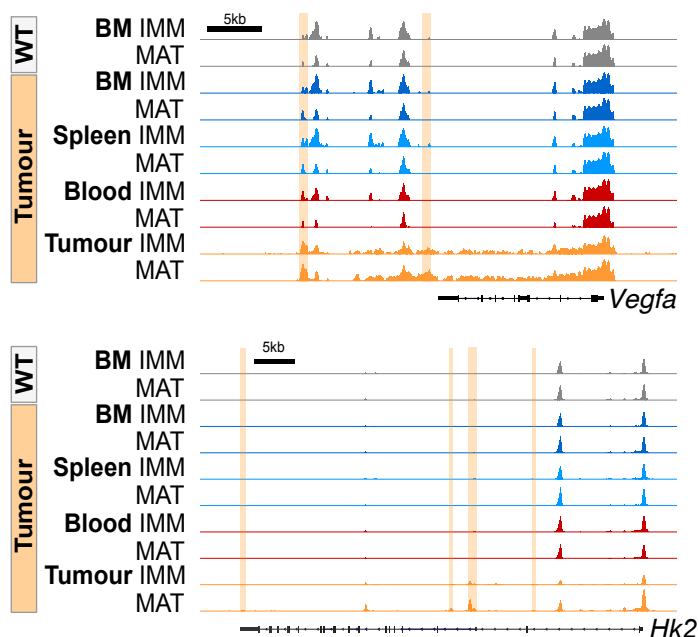
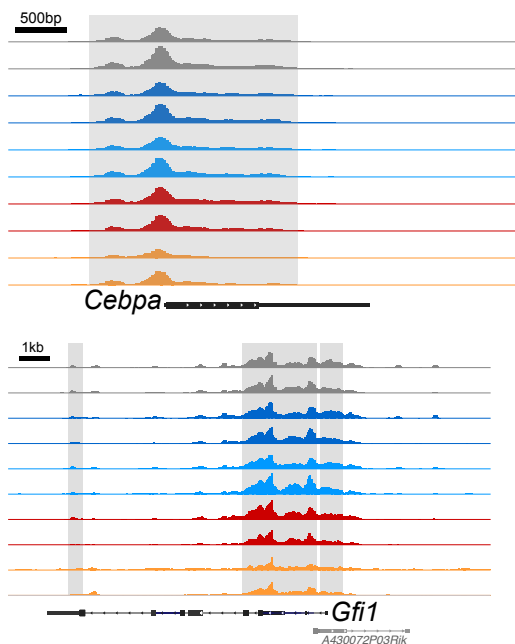
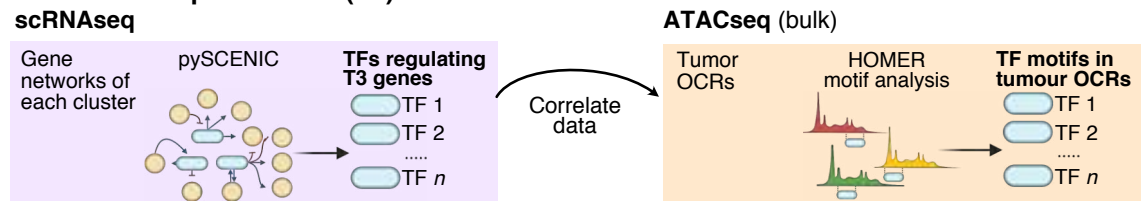
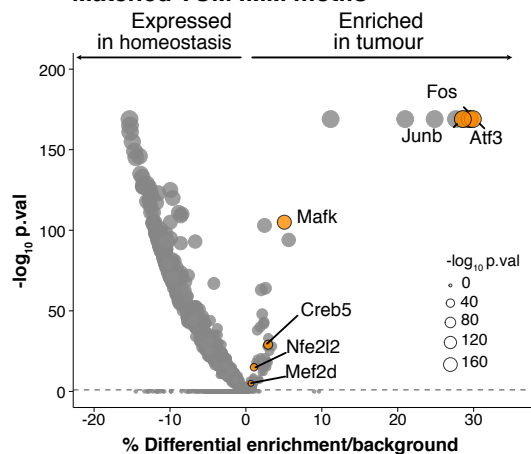
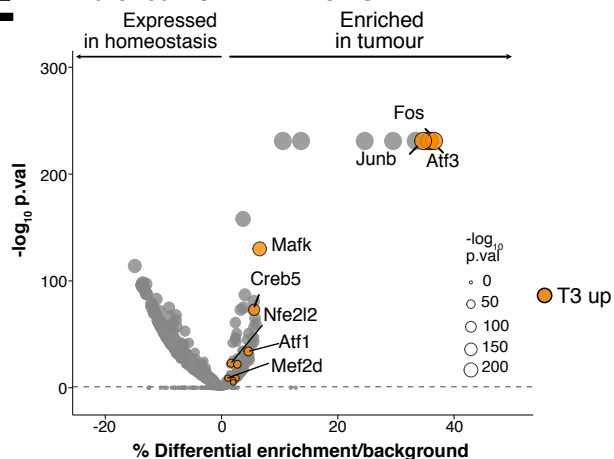
(A) Gating strategy for sorted neutrophils.

(B) UMAP projection of total neutrophils for each biological replicate (n=2) in bone marrow (BM), spleen, blood, and tumor.

(C) Stacked bar plots show frequency of total cells distributed across Seurat clusters by tissue location.

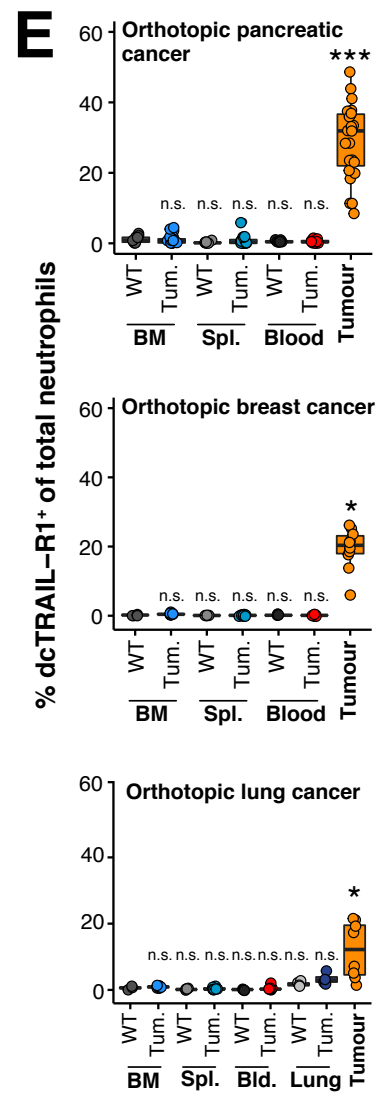
(D) Integration of the pancreatic adenocarcinoma (PDAC) neutrophil scRNAseq dataset (this paper) with neutrophils sequenced from wild type (WT) mice (19). Integrated UMAP embedding showing the identity of clusters defined in the PDAC neutrophil dataset. T1, T2 and T3 populations are outlined in red.

(E) Integrated UMAP shows clustering across the BM, spleen, blood, and tumor, with no separate clusters or branching. Colors correspond to the PDAC (purple) and WT (yellow) scRNAseq datasets. The location of T1, T2 and T3 populations are outlined in red.

A**OCRs at T3 genes****B****Background (non-DE regions)****C****Match transcription factor (TF) to enriched motifs****D****Matched TUM IMM motifs****E****Matched TUM MAT motifs**

Supplementary Figure 2. Epigenetic reprogramming towards the T3 fate is present within both immature and mature tumor-infiltrating neutrophils.

- (I)** Open chromatin regions (OCRs) matched to differentially expressed T3 genes, have increased accessibility only in immature and mature tumor neutrophils. Heatmap shows intensity of fragment mapping across indicated neutrophil subsets, histograms indicate average mapping intensity. T3 genes linked to hypoxia, glycolysis and angiogenesis are annotated. (See also table S2)
- (J)** Chromatin accessibility peaks at *Vegfa* (in log scale) and *Hk2* (in linear scale) loci. Differentially accessible OCRs for tumor neutrophils are colored in light orange.
- (K)** Chromatin accessibility peaks at *Cebpa* and *Gfi1* (both in linear scale) loci. Background peak regions expressed in all neutrophils are colored in light grey.
- (L)** Schematic illustrating the correlation of transcription factor activity across the scRNAseq dataset and the bulk ATACseq dataset by utilizing PySCENIC gene regulatory network analysis and motif analysis.
- (M)** OCRs differentially accessible in tumor immature (IMM, top) and mature (MAT, bottom) neutrophils contain motifs for transcription factor regulons in T3 neutrophils enriched in the same direction. Volcano plots show all motifs enriched in tumor neutrophils. Motifs were colored if they were enriched (orange) in the same direction as T3 neutrophils. (See also table S2).

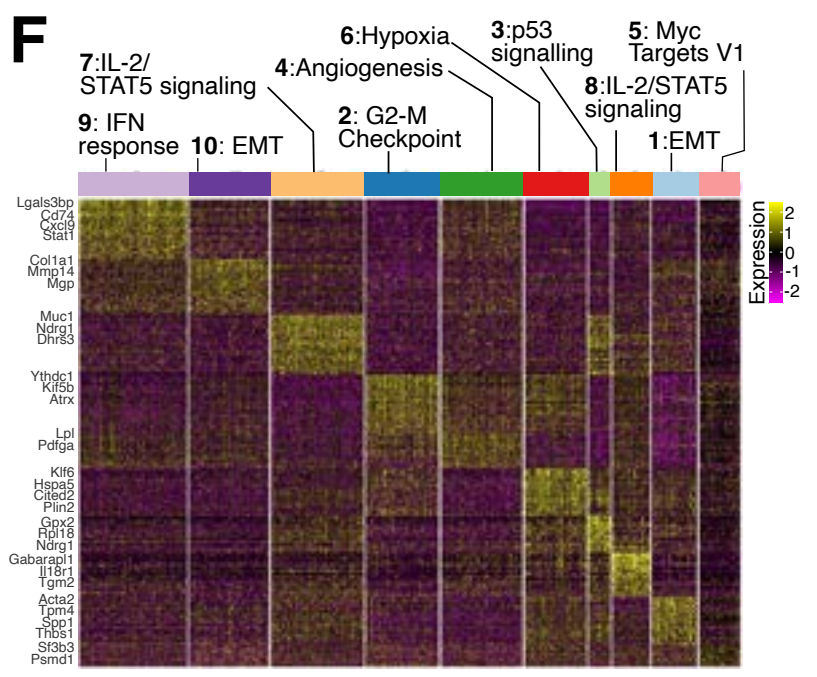
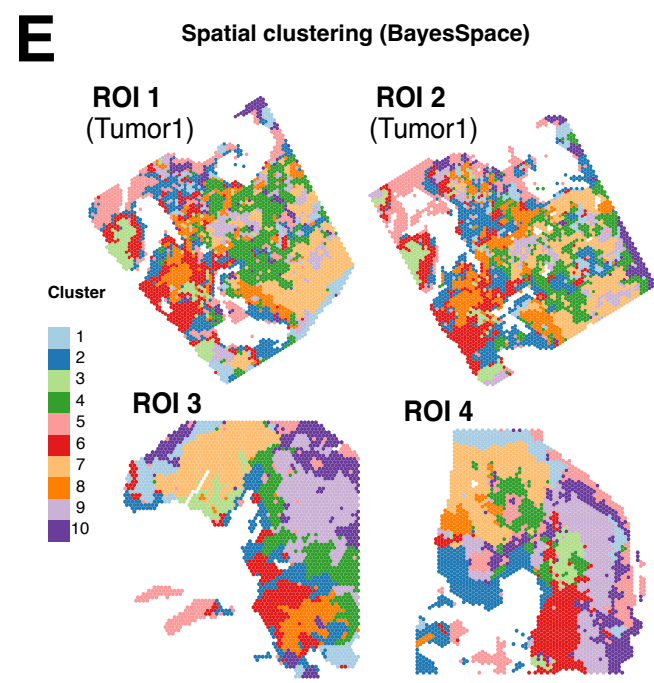
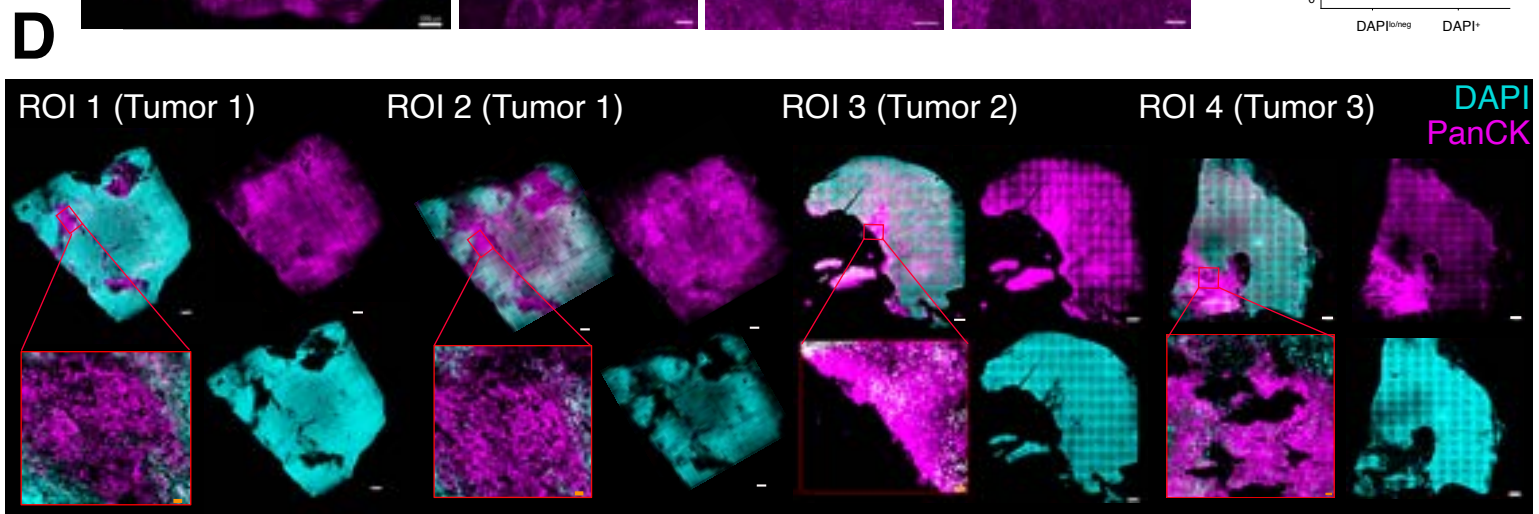
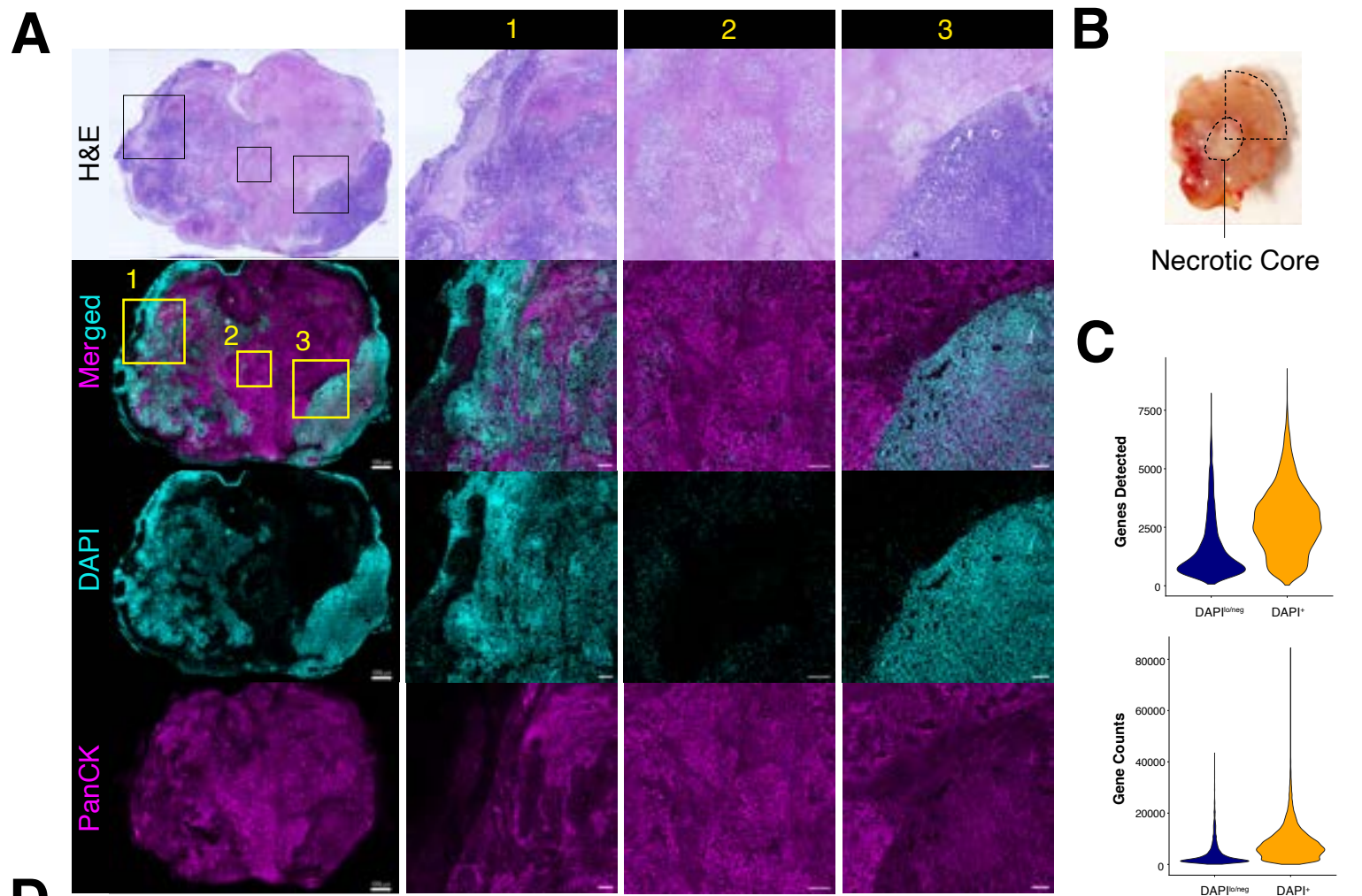


Supplementary Figure 3. The expression of dcTRAIL-R1 on neutrophils is specific to the tumor-bearing state.

- (A) Dot plot shows transcript expression levels of differentially expressed surface markers across T1, T2 and T3 clusters in the scRNAseq dataset. Surface markers were defined based on annotation with the “membrane” classification in the Gene Ontology cell compartmentalization database.
- (B) Heatmap shows scaled, log₂ expression levels of canonical markers in annotated clusters derived from the UMAP projection of all live, CD45⁺ cells in the tumor.
- (C) Heatmap shows scaled, log₂ expression levels of scRNAseq candidate surface in annotated clusters derived from the UMAP projection of all live, CD45⁺ cells in the tumor.
- (D) Representative histograms showing mean fluorescence intensity (MFI) of selected markers from Fig. 4B for bone marrow (BM), spleen, blood neutrophils in the periphery, and macrophages, monocytes and tumor neutrophils as indicated. Dotted lines separate the positive population and were set against the obvious negative populations across the comparisons. Citations indicate published work attributing each surface marker to neutrophils in cancer.
- (E) dcTRAIL-R1 expressing neutrophils are restricted to the tumor across three different cancer models. Boxplots show the frequency of dcTRAIL-R1⁺ neutrophils as gated in Fig. 4C across wild type (WT) and tumor-bearing (Tum.) mice in the BM, spleen, blood and tumor in (top) orthotopic pancreatic ductal adenocarcinoma (FC1242, WT, n=9, Tum., n=23), (center) orthotopic breast cancer (Py8119, WT, n=4, Tum., n=10) and (bottom) orthotopic lung cancer (LLC-1, WT, n=4, Tum., n=8). p<0.05*, p<0.01**, p<0.001***, as determined by Kruskal-Wallis followed by many-to-one Mann-Whitney U test, [setting WT BM neutrophils as the comparison control](#).

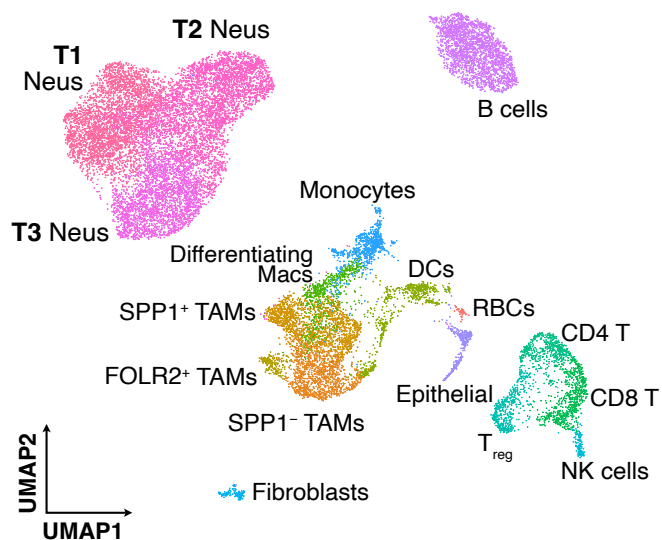
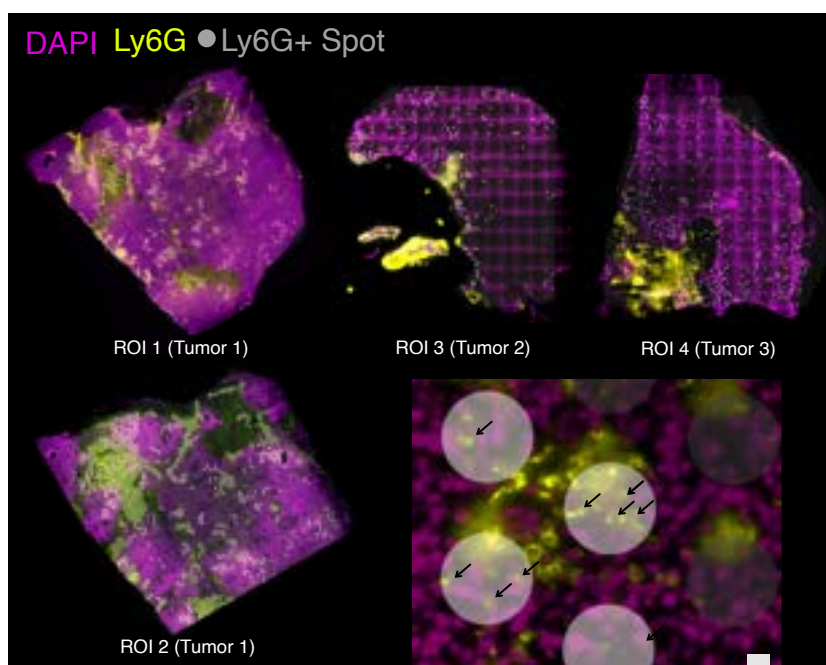
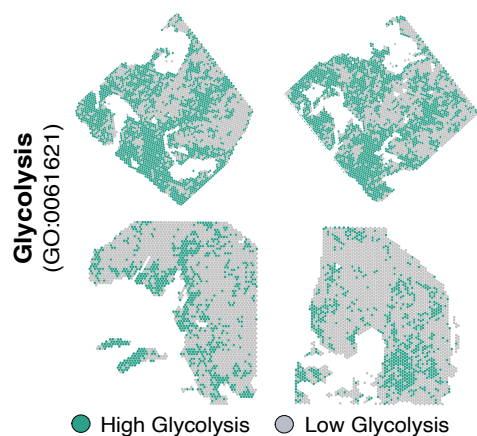
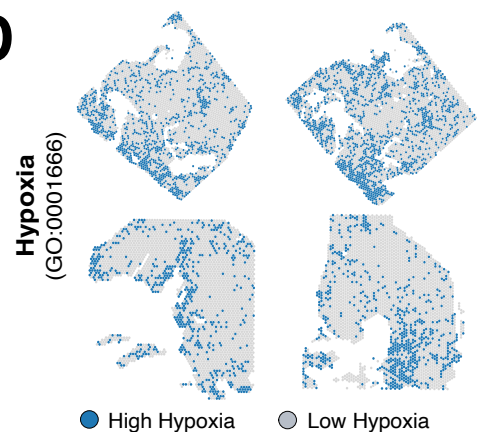
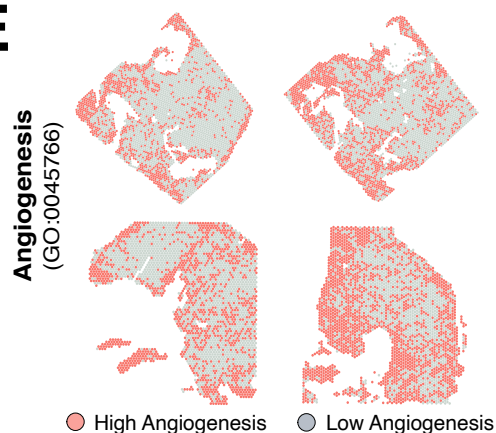
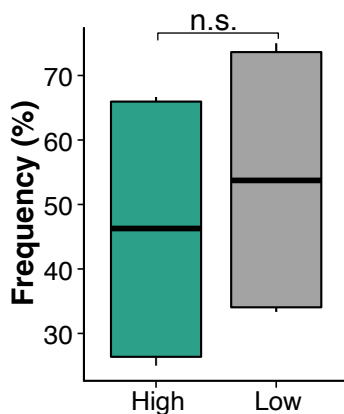
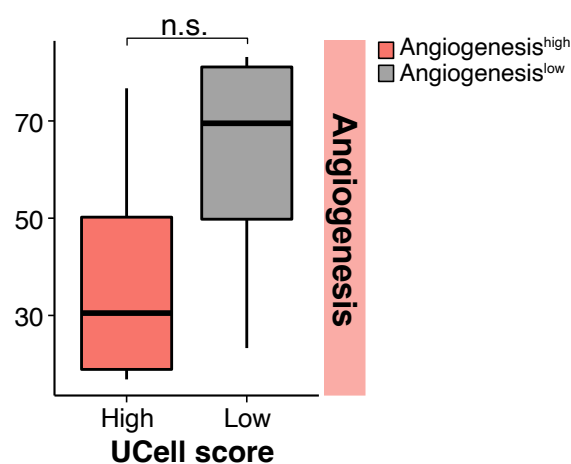
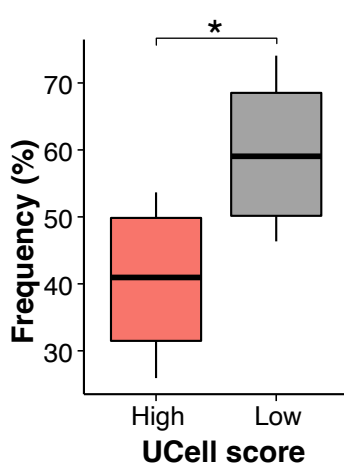
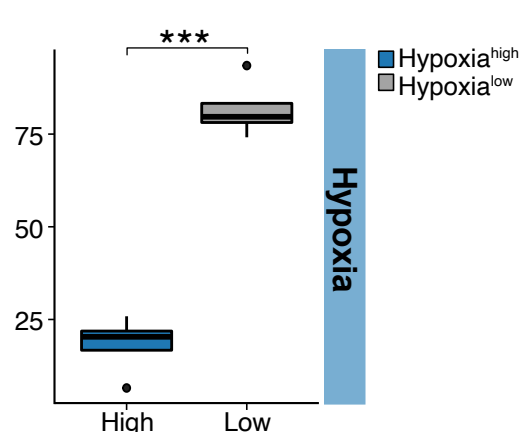
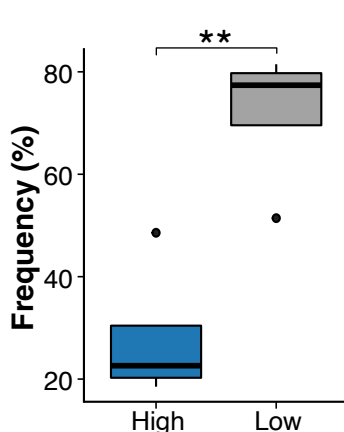
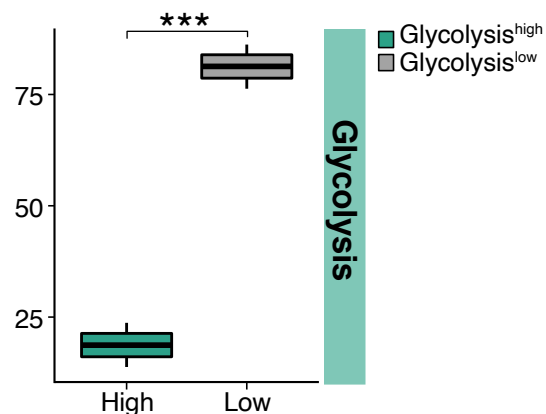
Supplementary Figure 4. Validation of dcTRAIL-R1 and CD101 expression as identification markers for tumor neutrophils

- (A) The T3 population has the highest expression and active transcription of *Tnfrsf23*. UMAP projection of PDAC scRNAseq dataset shows expression density (**top**) and RNA velocity (**bottom**) of *Tnfrsf23*, which encodes for dcTRAIL-R1.
- (B) Definition of T3, T2 and T1 populations by dcTRAIL-R1 and CD101 expression. UMAP shows dcTRAIL-R1 (**top**) and CD101 (**bottom**) expression intensity imputed by InfinityFlow from high (red) to low (blue).
- (C) Cytospin images show the expected nuclear morphology for sorted, putative T1 (immature), T2 (mature) and T3 (mixed) neutrophils. Control pictures show representative morphology from mature and immature neutrophils from the bone marrow of wild type mice. Giemsa-Wright staining was carried out and pictures are representative of four and two biological replicates for tumor and control neutrophils, and at least three different fields of view were considered per replicate.
- (D) Sorted dcTRAIL-R1⁺ neutrophils have the highest expression of the T3 gene signature (n=4 each). Scatter dot plots for T3, T2 and T1 gene signatures, each dot denotes a single gene, lines denote the mean, error bars show standard error mean. $p < 0.001^{***}$, by Wilcoxon signed-rank test for paired genes, comparisons indicated on graph.
- (E) Frequencies of T1-T3 neutrophils within **pancreatic, breast and lung** cancers. Each dot represents one biological replicate, boxplots show media (center-line), interquartile range (box) and whiskers show lowest and highest measurements. $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$ by Kruskal-Wallis followed by Dunn's post-test. (See also table S4).
- (F) Median frequencies from flow cytometry for T3, T2 and T1 neutrophils in the PDAC, **Py8119 breast and LLC-1 lung** tumor models compared against proportions of T3, T2 and T1 defined transcriptionally in the PDAC scRNAseq dataset.



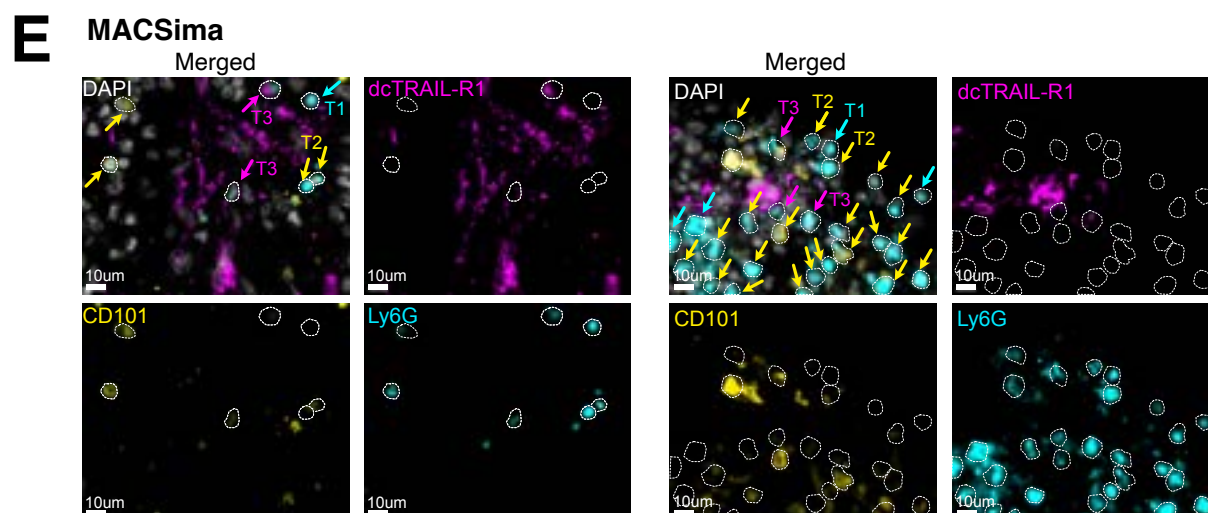
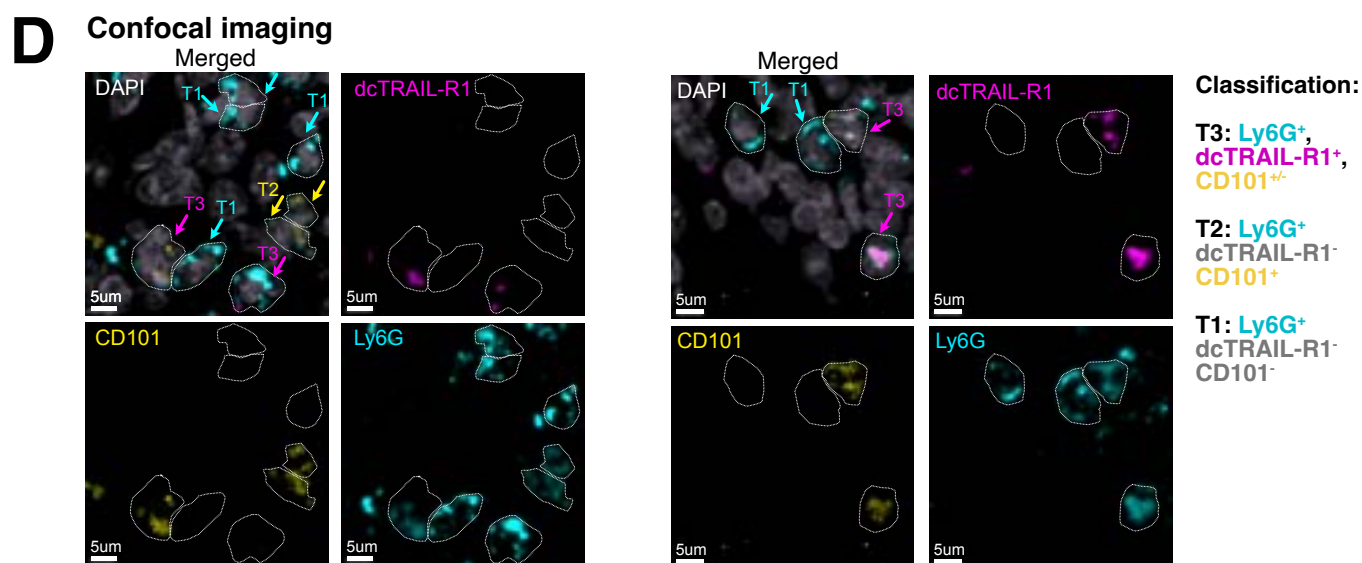
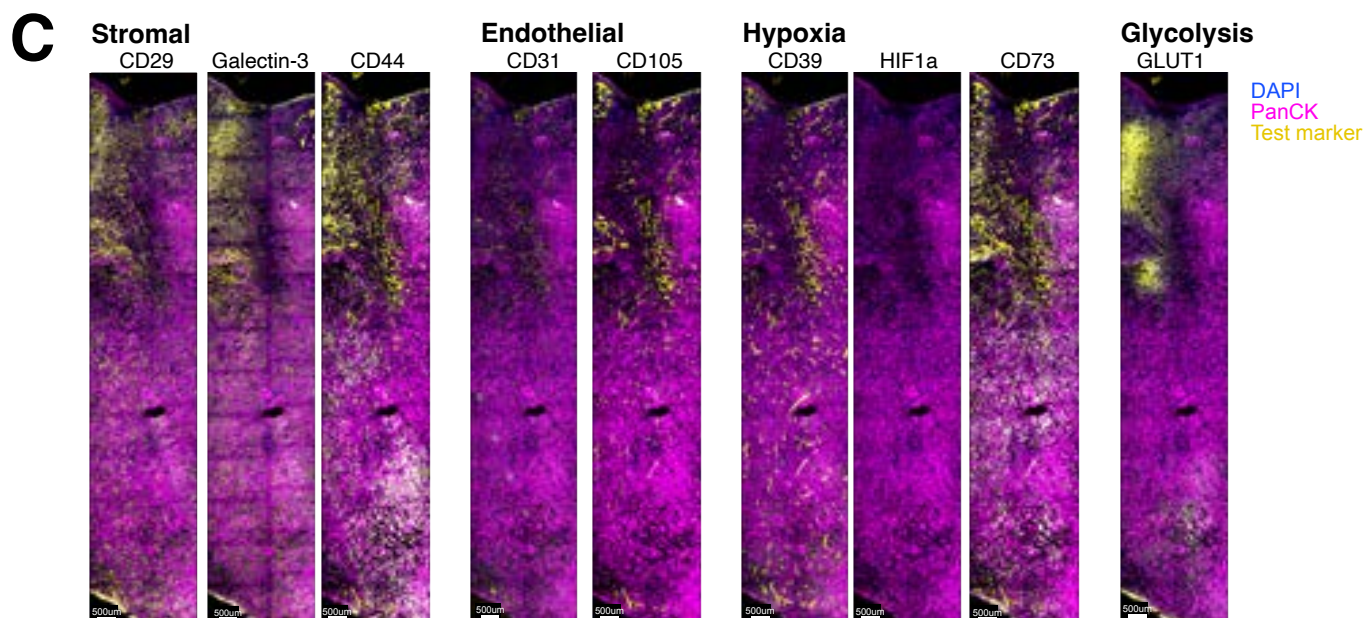
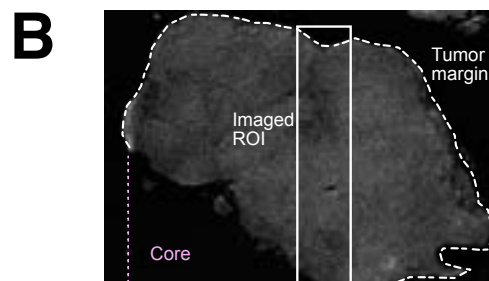
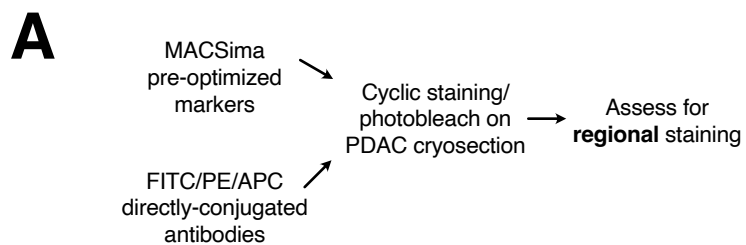
Supplementary Figure 5. Spatial transcriptomics reveals functionally distinct regions in pancreatic tumors.

- (A) Hematoxylin & Eosin-stained section of mouse pancreatic (PDAC) orthotopic tumor and corresponding adjacent section with DAPI (in teal) and pan-cytokeratin (PanCK) (in magenta) immunofluorescence staining, scale bars = 100 μ m. Zoomed Areas 1 (stromal), 2 (core), and 3 (intra-tumoral) represent various regions within the tumors with varying cell densities. Scale bars = 200 μ m.
- (B) Representative pictograph of halved tumors with visible necrotic core. Tumors were then quartered before sectioning, preserving representation of both peripheral and core tumor regions.
- (C) Number of counts and genes detected in DAPI-positive and low quality DAPI-low/negative spots.
- (D) DAPI and PanCK immunofluorescence staining on cryosections taken from quartered PDAC tumors 6 weeks post orthotopic injection (n = 3, ROI=4) and respective zoomed-in images of DAPI-low/negative necrotic regions. White scale bars = 500 μ m, orange scale bars = 50 μ m.
- (E) BayesSpace spatial joint clustering analysis of tumor quarters after DAPI-low/negative spot filtering.
- (F) GO annotated heatmap of spatial clusters of (E) using the top 2000 variable genes. A maximum of 50 DEG markers per cluster is represented and used to derive the GO annotation through EnrichR.

A**B****C****UCell score****D****E****F****T2 localization****G****T1 localization**

Supplementary Figure 6. Spatial mapping of tumor neutrophil subsets in the pancreatic tumor regions

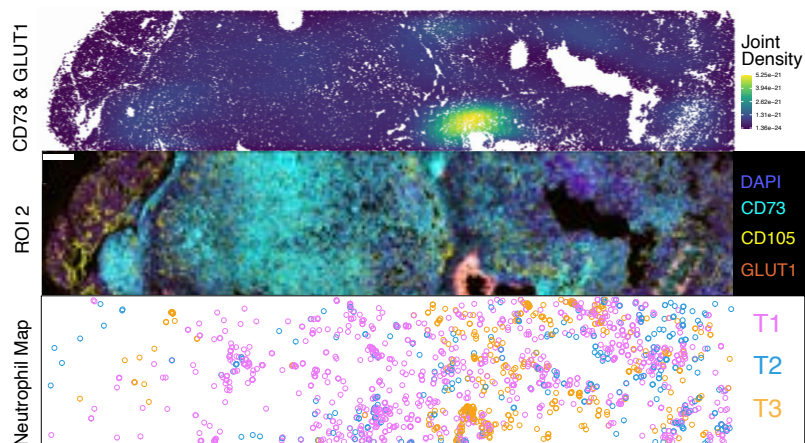
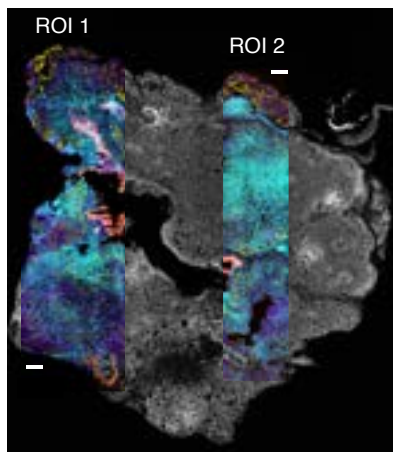
- (A) UMAP projection of pre-annotated single-cell RNA sequencing reference dataset of CD45⁺ and CD45⁻ cells in the PDAC tumor.
- (B) Manual spot filtering of neutrophil-containing spots through Ly6G immunofluorescence staining. Zoomed-in example (bottom-right) of spots containing DAPI⁺Ly6G⁺ cells and non-selected spots (in grey) containing auto-fluorescent emission of Visium spots, requiring manual selection of Ly6G⁺ spots.
- (C-E) Gene signature mapping of (C) hypoxia (GO:001666), (D) glycolysis (GO:0061621) and (E) angiogenesis (GO:0045766) gene ontology (GO) pathways on replicate sections via UCell package. Spots with UCell scores above the 50th percentile are defined as “high” scoring regions and are colored, while low scoring regions are in grey for all plots.
- (F) Frequency of deconvoluted T2 neutrophil-enriched spots in the hypoxic, glycolytic and angiogenic regions. $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, as determined by one-tailed t test.
- (G) Frequency of deconvoluted T1 neutrophil-enriched spots in the hypoxic, glycolytic and angiogenic regions. $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, as determined by one-tailed t test. All boxplots show median (centre line) with standard deviation (whiskers).



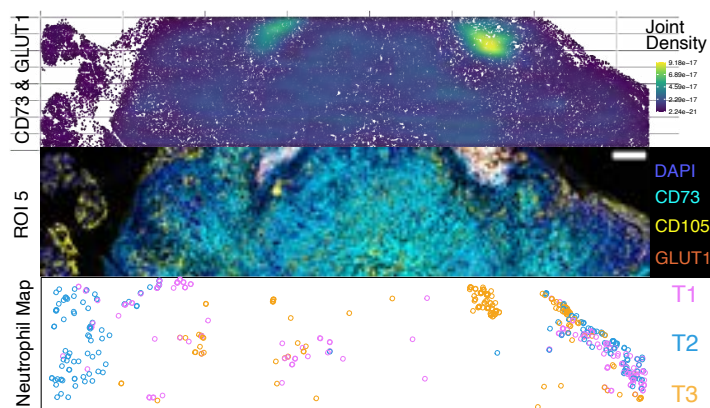
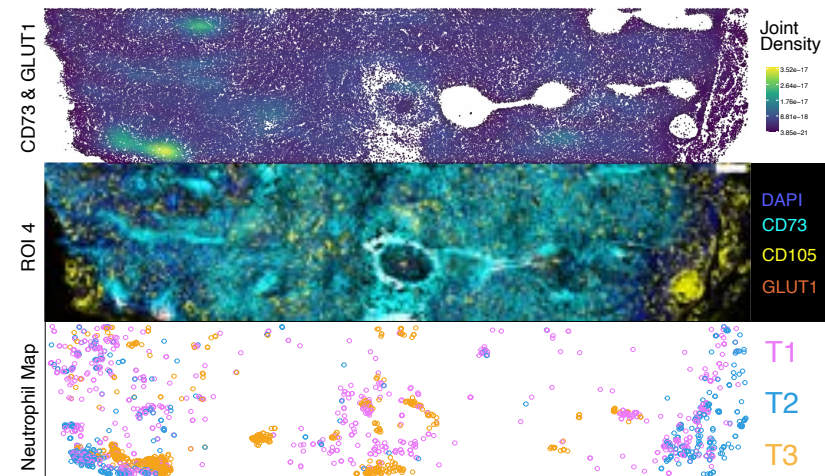
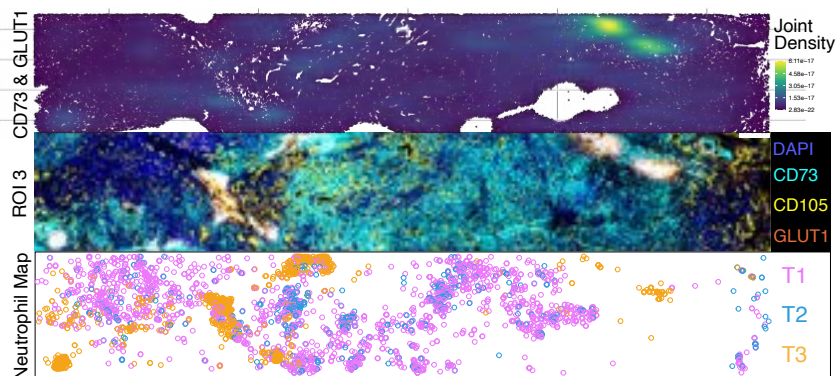
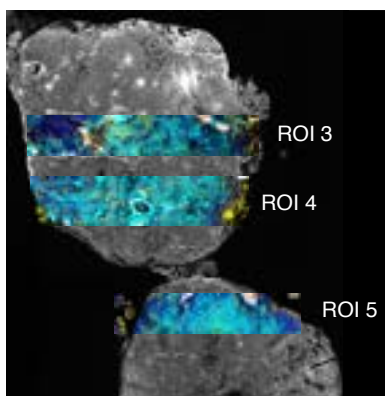
Supplementary Figure 7. Optimization of MICS-based fluorescence staining of tumor neutrophil subsets

- (A)** Workflow for regional marker screening and optimization.
- (B)** ROI selected for imaging screen, covering regions from the periphery to the central core of the tumor section.
- (C)** Marker intensities of tested regional markers (in yellow) against tumor (PanCK) regions. Scale bars = 500 μ m.
- (D)** Representative confocal images of tumor neutrophil subset immunofluorescence staining with Ly6G, dcTRAIL-R1 and CD101. Scale bars = 5 μ m.
- (E)** Representative immunofluorescent images from MACSima multiplexed imaging. Scale bars = 10 μ m.

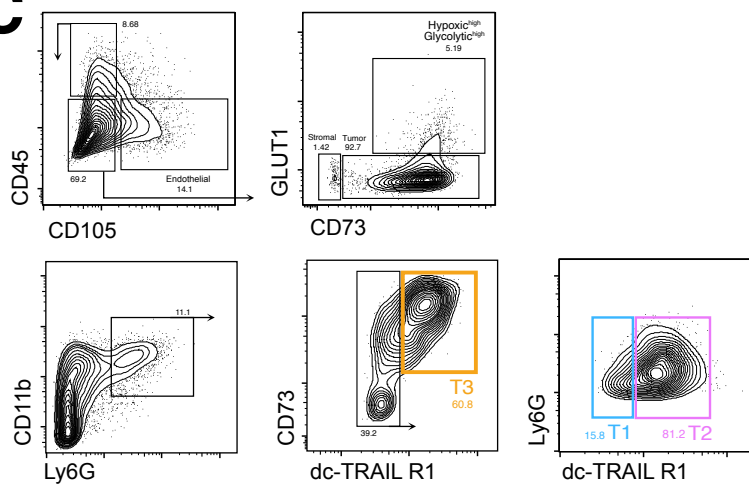
A



B

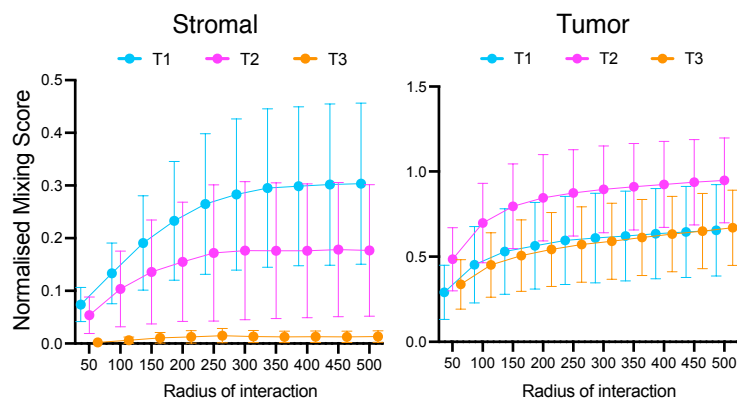


C



D

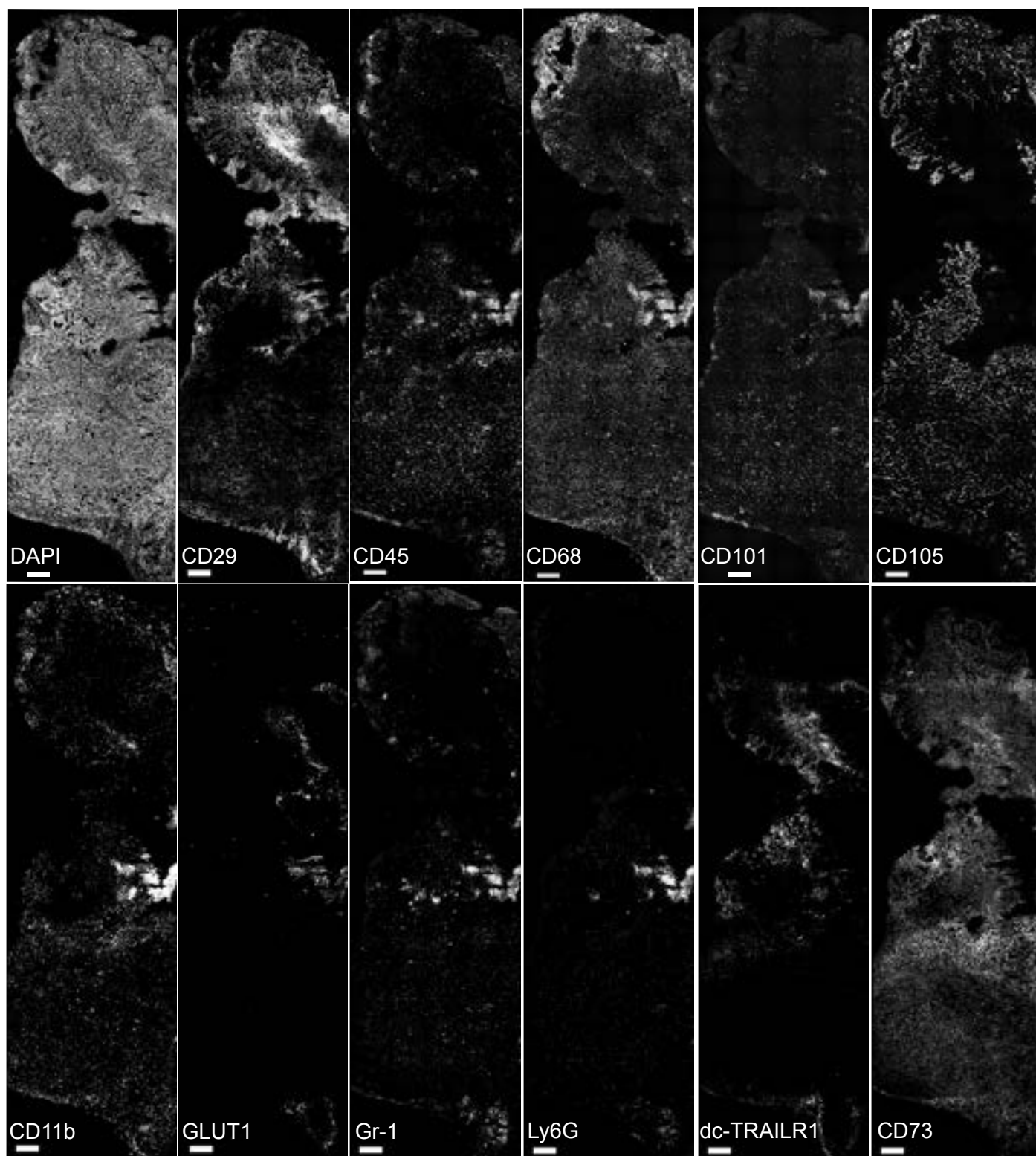
Co-localisation of neutrophil subsets and regions



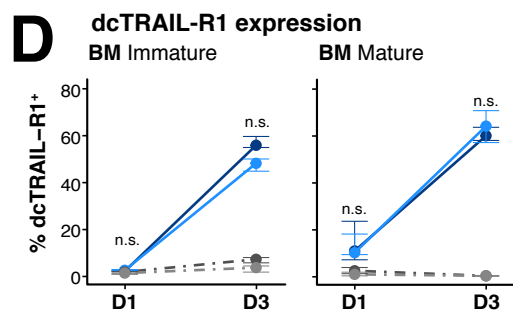
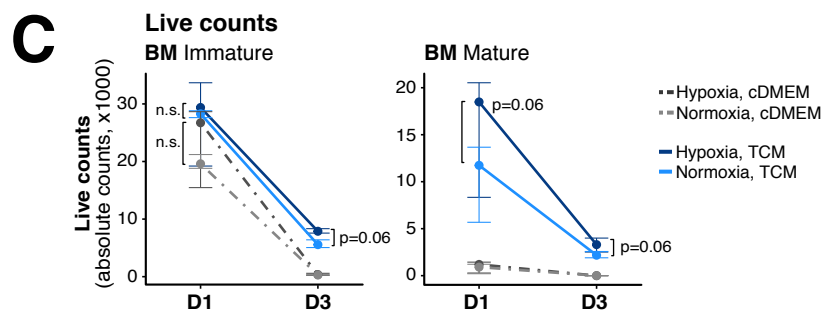
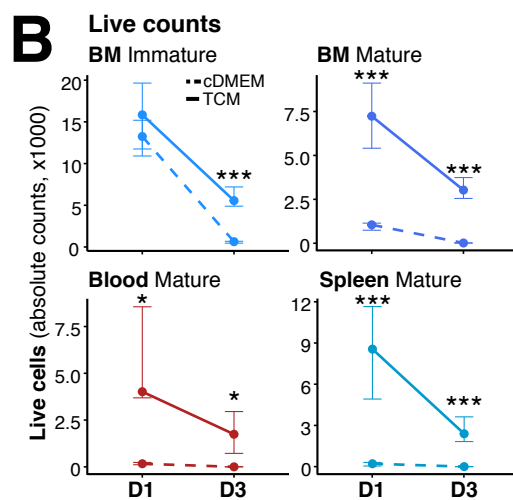
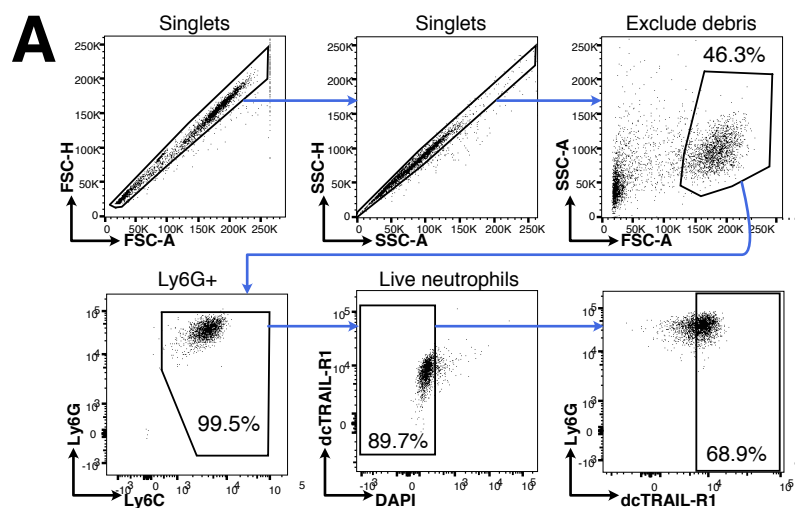
Supplementary Figure 8. Multiplexed spatial imaging of tumor sections validates spatial localization of tumor neutrophils.

- (A) (left) Regions of interests selected in tumor section 1. (right) Immunofluorescence staining of regional markers in ROI2 with corresponding mapping of tumor neutrophils and joint density distribution of CD73 & GLUT1 staining.
- (B) (left) Regions of interests selected in tumor section 2. Immunofluorescence staining of regional markers in ROI3 (right), ROI4 (bottom left) and ROI5 (bottom right) with corresponding mapping of tumor neutrophils and joint density distribution of CD73 & GLUT1 staining.
- (C) Representative gating strategy of segmented cells of populations of interests.
- (D) Co-localization of tumor neutrophil subsets with the indicated tumor regions, represented using a normalized mixing score calculated using SPIAT (82).

A

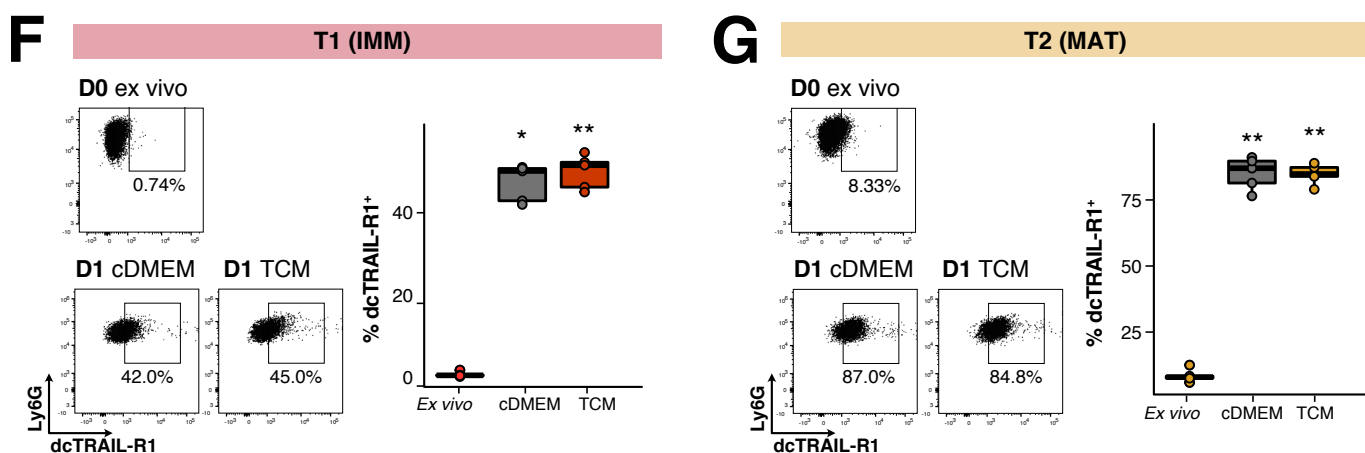
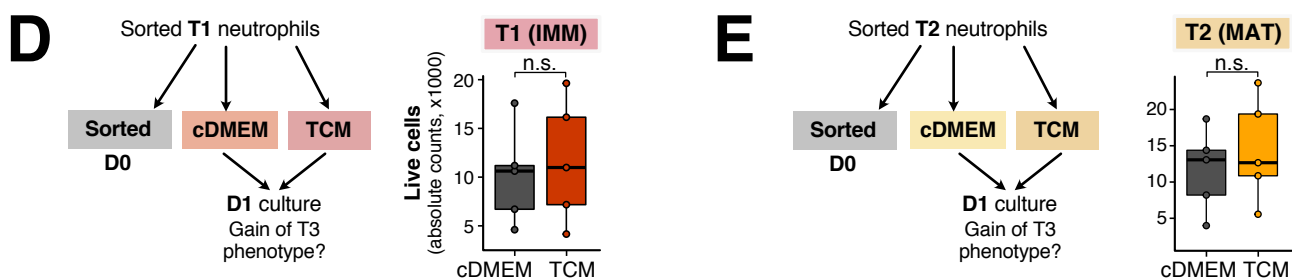
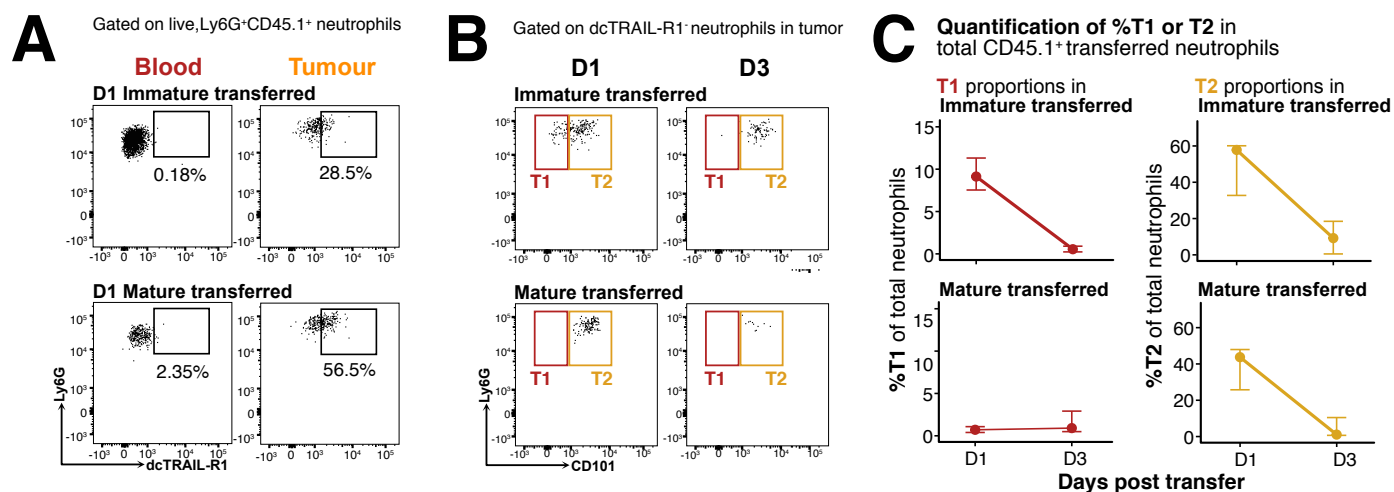


2131 **Supplementary Figure 9. Spatial distribution of marker expressions in multiplexed spatial**
2132 **imaging.**
2133
2134 (A) Expression intensities of each protein marker used in MACSima panel.



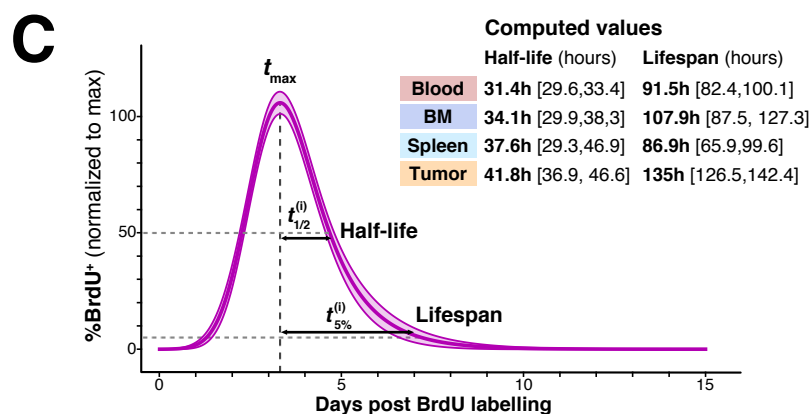
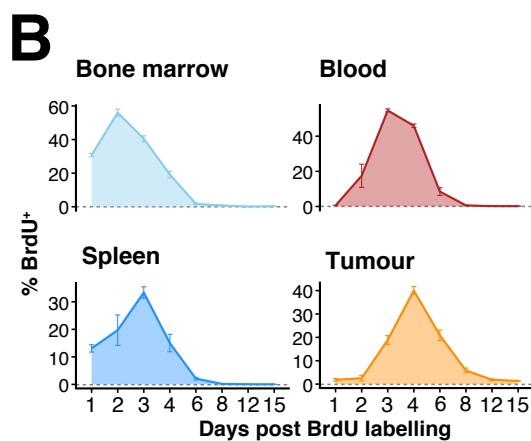
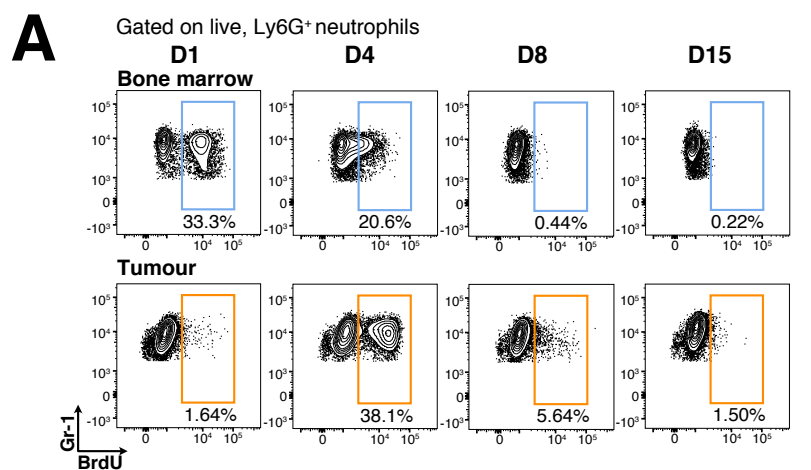
Supplementary Figure 10. Deterministic reprogramming of neutrophils occurs in the presence of tumor-derived factors.

- (A) Gating strategy showing flow cytometry analysis of wild type bone marrow (WTBM) immature neutrophils cultured in tumor-conditioned media (TCM).
- (B) Neutrophils cultured in TCM have increased survival overtime. Line plots show the absolute counts of live neutrophils gated as in (A). Dots indicate the median, error bars indicate Q1 and Q3 intervals for neutrophil subsets cultured in cDMEM (dotted line) and TCM (solid line) over 1 and 3 days. Each group contains the following number of samples – Day 1 cDMEM: BM immature (n=8), BM mature (n=8), Spleen Mature (n=8), Blood Mature (n=3), Day 1 TCM: BM immature (n=8), BM mature (n=8), Spleen Mature (n=8), Blood Mature (n=5), Day 3 cDMEM: BM immature (n=10), BM mature (n=10), Spleen Mature (n=10), Blood Mature (n=4), Day 3 TCM: BM immature (n=10), BM mature (n=10), Spleen Mature (n=10), Blood Mature (n=5). $p < 0.05^*$, $p < 0.001^{***}$, by Mann-Whitney U test.
- (C) Hypoxia does not greatly enhance survival of immature or mature neutrophils cultured in TCM. Line plots show the absolute counts of live neutrophils gated as in (A) when cultured in cDMEM (dotted lines) or TCM (solid lines) either in normoxic (20% oxygen) or hypoxic (5% oxygen) conditions, colors as denoted in plot, n=5 per group. Dots indicate the median, error bars indicate Q1 and Q3 intervals. Comparisons were carried out as denoted, $p = \text{n.s.}$ or stated on graph, by Wilcoxon signed-ranks test.
- (D) Hypoxia does not increase dcTRAIL-R1 expression of immature or mature neutrophils cultured in TCM. Line plots show the dcTRAIL-R1⁺ neutrophils gated as in (A) when cultured in cDMEM (dotted lines) or TCM (solid lines) either in normoxic (20% oxygen) or hypoxic (5% oxygen) conditions, colors as denoted in plot, n=5 per group. Dots indicate the median, error bars indicate Q1 and Q3 intervals. Comparisons were carried out as denoted, $p = \text{n.s.}$, by Wilcoxon signed-ranks test.



Supplementary Figure 11. Transitional tumor neutrophil subsets are committed to T3 differentiation.

- (A) Only neutrophils entering the tumor upregulate dcTRAIL-R1 expression. Representative flow plots show dcTRAIL-R1 expression present on transferred CD45.1⁺ immature and mature neutrophils present in the blood or tumor at 1-day post transfer.
- (B) Transferred immature neutrophils give rise to T1 neutrophils within the tumor. Representative flow plots show gating of T1 and T2 dcTRAIL-R1⁻ neutrophils within the tumor based on CD101 expression for transferred CD45.1⁺ immature and mature cells at day 1 and day 3 post-transfer.
- (C) Line plots quantify percentages of T1 and T2 neutrophils within the tumor as gated in (F) for transferred CD45.1⁺ immature (n=4 at day 1, n=3 at day 3) and mature cells (n=3 at both timepoints). Dots indicate the median, error bars indicate Q1 and Q3 intervals.
- (D) T1 neutrophils do not exhibit differences in viability after one day of culture in complete DMEM (cDMEM) control media or tumor-conditioned media (TCM). Experimental set-up of *in vitro* culture of sorted T1 and T2 neutrophils from PDAC mice in cDMEM or TCM for 1 day (top).
- (E) T2 neutrophils do not exhibit differences in viability after one day of culture in cDMEM or TCM. Experimental set-up of *in vitro* culture of sorted T1 and T2 neutrophils from PDAC mice in cDMEM or TCM for 1 day (top). (E-F) Boxplots show absolute counts of DAPI⁻ Ly6G⁺Ly6C⁺ cells, centre line denotes the median, whisker denote standard deviation.
- (F) T1 neutrophils upregulated dcTRAIL-R1 expression within 1 day of culture in both cDMEM and TCM. Representative flow plots show gating for dcTRAIL-R1 expression *ex vivo* post-sort (D0) and at D1 after culture in cDMEM or TCM. Each group contains the following number of samples – D0 (n=5), D1 cDMEM; hypoxia (n=3), D1 cDMEM; normoxia (n=5), D1 TCM; hypoxia (n=4), D1 TCM; normoxia (n=5).
- (G) T2 neutrophils upregulated dcTRAIL-R1 expression within 1 day of culture in both cDMEM and TCM. Representative flow plots show gating for dcTRAIL-R1 expression *ex vivo* post-sort (D0) and at D1 after culture in cDMEM or TCM. Each group contains the following number of samples – D0 (n=5), D1 cDMEM; hypoxia (n=3), D1 cDMEM; normoxia (n=5), D1 TCM; hypoxia (n=4), D1 TCM; normoxia (n=5). (F-G) All boxplots show median (centre line) with standard deviation (whiskers). p<0.05*, p<0.01** by Kruskal-Wallis test with many-to-one U test against D0.

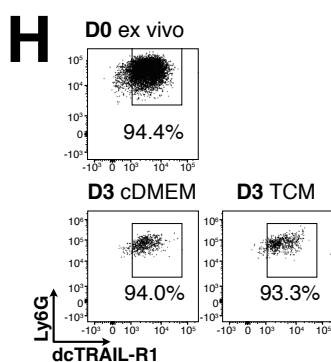
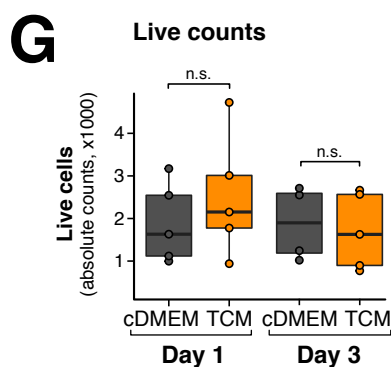
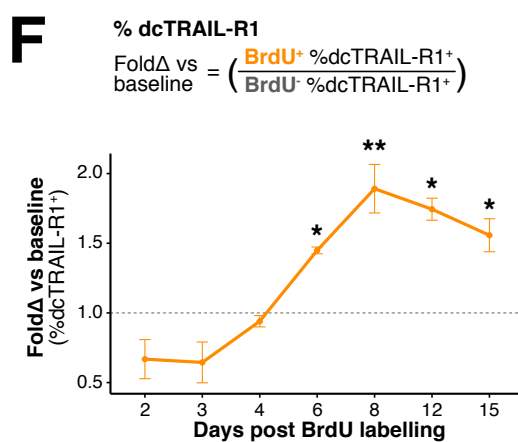
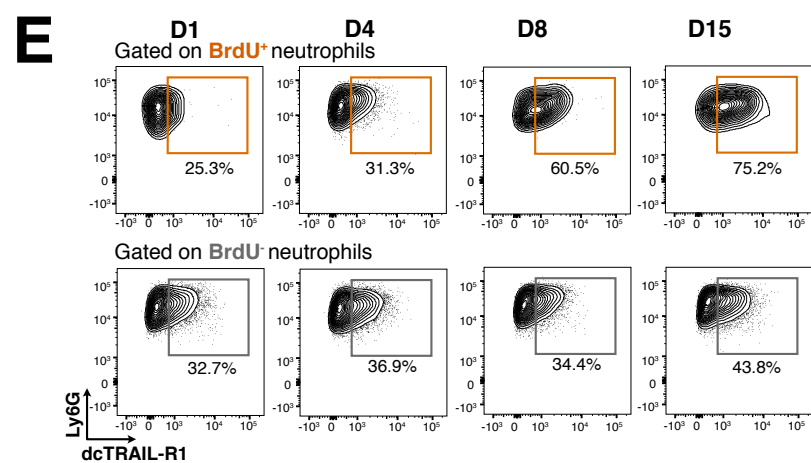


D Neutrophil half-life $t_{1/2}$

p-value	Blood	BM	Spleen	Tumor
Blood	-			
BM	0.26	-		
Spleen	0.18	0.51	-	
Tumor	0.00013***	0.0058**	0.15	-

Neutrophil lifespan $t_{5\%}$

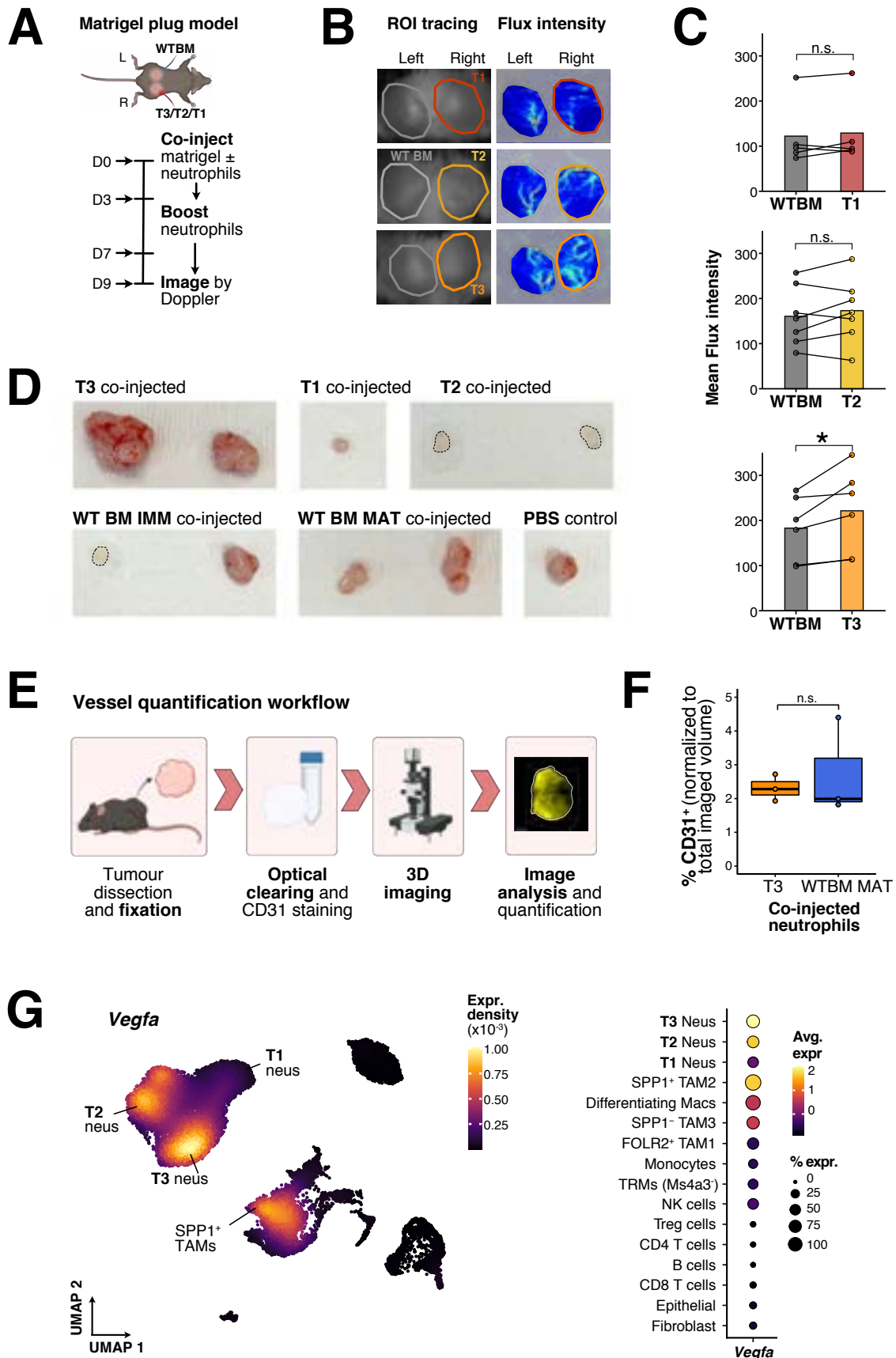
p-value	Blood	BM	Spleen	Tumor
Blood	-			
BM	0.074	-		
Spleen	0.23	0.080	-	
Tumor	0.00014***	0.012*	0.00015***	-



Supplementary Figure 12. Exposure to the tumor microenvironment shapes the longevity and stability of T3 neutrophils.

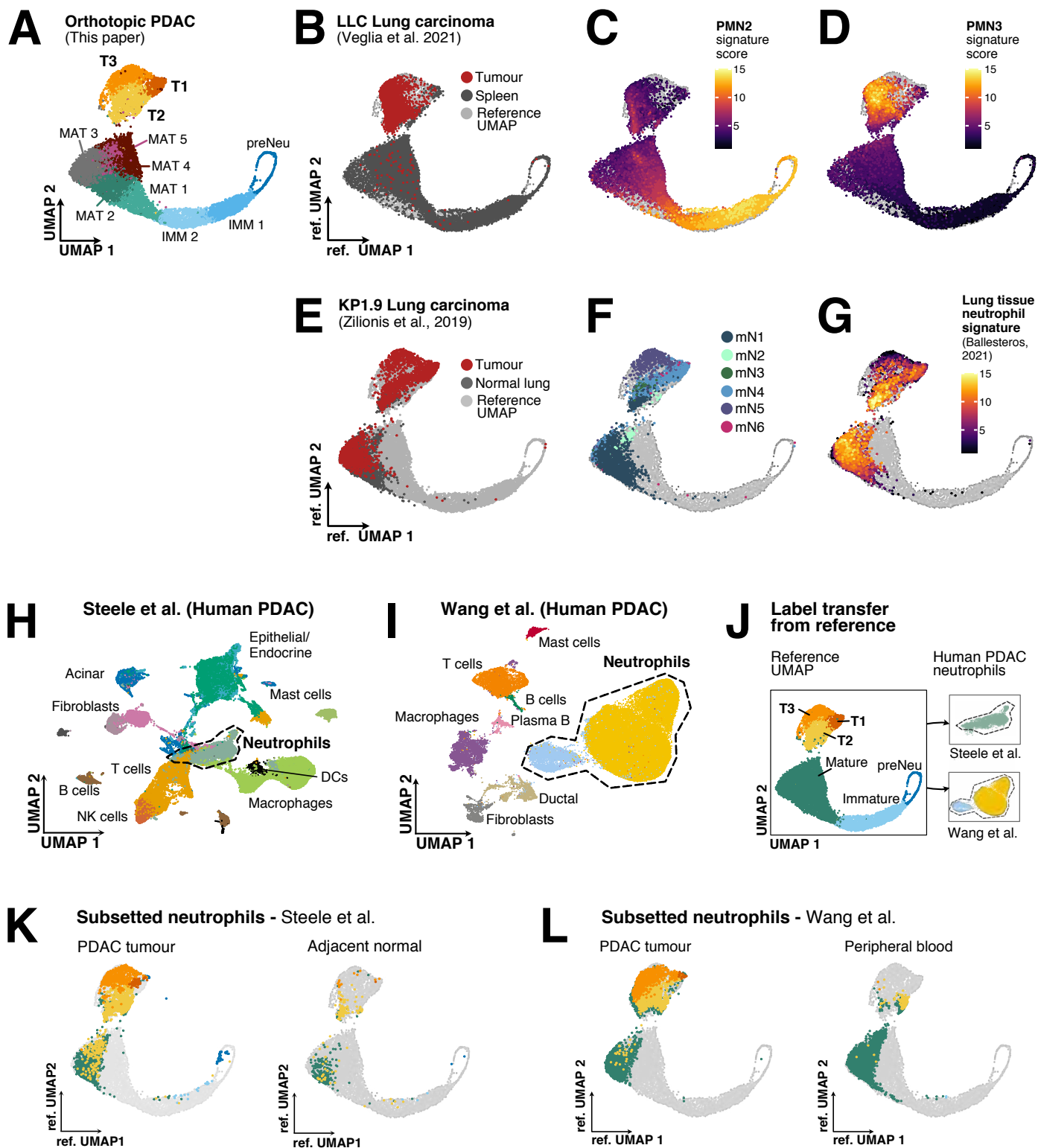
- (A) Representative flow plots show gating of labelled, BrdU⁺ neutrophils within the bone marrow (BM) and tumor at 1-, 4-, 8- and 15-days post labelling. Positive BrdU gates were set on the positive population detected in BM neutrophils at day 1 post labelling and the same gate was used for all tissues and timepoints.
- (B) Quantification of the proportions of BrdU⁺ as in (A). Each dot denotes mean, error bars show SEM.
- (C) Representative line plot showing fitted curve for BrdU percentages normalized to the maximal BrdU⁺ percentage value, which was set to 100% (see Methods for more details). Dots shows mean expression with error bars denoting 95% confidence intervals. A mathematical model capturing the full temporal window was fitted to estimate when the profile achieves its maximum, from which half-life ($t_{1/2}$) and lifespan ($t_{5\%}$) for each organ were calculated after setting 50% and 5% as the reference levels. Computed values half-lives and lifespans with 95% confidence intervals for each tissue are denoted.
- (D) P-values were calculated for each computed half-life and lifespan using boot-strapping. $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, by Mann-Whitney U test.
- (E) Conversion of BrdU⁺ neutrophils into dcTRAIL-R1⁺ neutrophils allow them to persist over time. Representative flow plots show gating of dcTRAIL-R1 expression in BrdU⁺ (top) and BrdU⁻ (bottom) neutrophils within the tumor at 1-, 4-, 8-, and 15-days post labelling.
- (F) Line plots quantify fold increase of dcTRAIL-R1 proportions as gated in (E). Proportions of dcTRAIL-R1⁺BrdU⁺ neutrophils were normalized against proportions of dcTRAIL-R1⁺BrdU⁻ neutrophils, with the BrdU⁻ fraction serving as a measure of baseline T3 proportions within the tumor. Each dot denotes the mean, with error bars showing SEM. $p < 0.05^*$, $p < 0.01^{**}$, by Mann-Whitney U test, one-tailed, alternative = “greater”. *afcl*
- (G) T3 neutrophils do not exhibit differences in viability after one or three days of culture in complete DMEM (cDMEM) control media or tumor-conditioned media (TCM). Boxplots show absolute counts of DAPI-Ly6G⁺Ly6C⁺ cells, centre line denotes the median, whisker denote standard deviation. $p = \text{n.s.}$ by Wilcoxon signed-ranks test.
- (H) T3 maintain dcTRAIL-R1 expression regardless of the culture media or timepoint. Representative flow plots show gating for dcTRAIL-R1 expression *ex vivo* post-sort (D0) and at D1 or D3 after culture in cDMEM or TCM. $p = \text{n.s.}$ according to by Kruskal-Wallis followed by many-to-one U test comparing against the D0 timepoint. Each group contains

2247 the following number of samples – D0 (n=5), D1 cDMEM (n=5), D1 TCM (n=5), D3
2248 cDMEM (n=4), D3 TCM (n=5).
2249



Supplementary Figure 13. T3 neutrophils are pro-angiogenic.

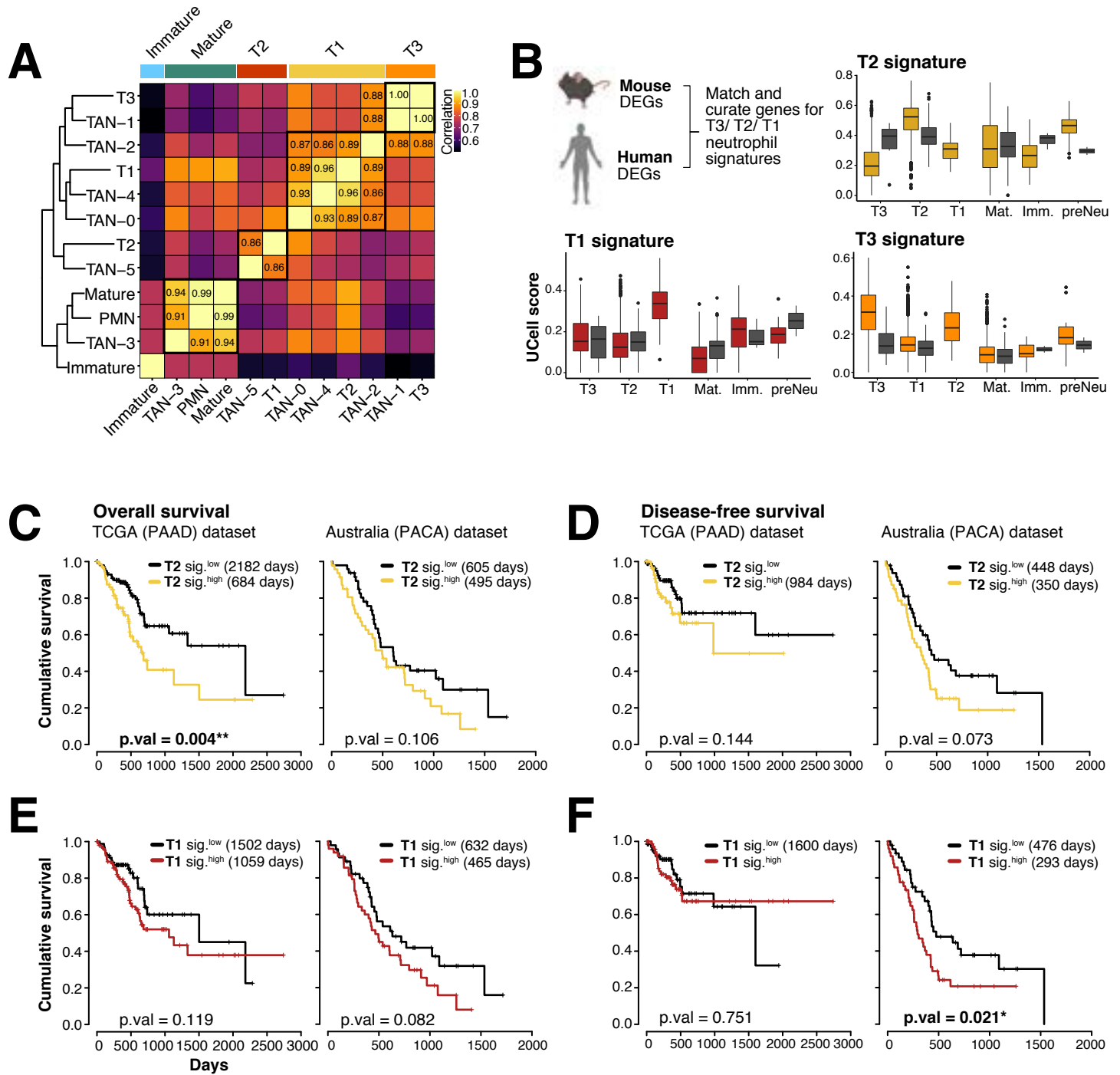
- (A) Schematic of experimental set-up for the Matrigel plug assay to assess tumor neutrophil enhanced angiogenesis *in vivo*. 50,000 sorted tumor neutrophils were mixed in 500ul of Matrigel before subcutaneous injection into the right flank. Neutrophils from the bone marrow of wild type mice (WT BM) were used as controls and injected into the left flank. Additional neutrophils were injected on D3 and D7 by direct injection into the visible Matrigel plug. Imaging of Matrigel plug was carried out by Doppler on D9.
- (B) Representative images showing region of interest (ROI) selection of the visible Matrigel plug in brightfield (**left**) and the flux intensity of blood vessels within the same region with the ROIs marked out (**right**) for plugs injected with T1 (top), T2 (middle) and T3 (bottom). One ROI was drawn for each Matrigel plug injected.
- (C) Quantification of the mean flux intensity of the ROIs selected as in (B) for T1 (n = 7), T2 (n=5), and T3 (n=6) injected plugs with paired WT BM controls. Bar plots show mean, each individual dot represents one biological replicate, and lines indicated paired samples within each mouse. $p < 0.05^*$, by Wilcoxon signed-ranks test.
- (D) Photographs of representative tumors at D28 post injection. Tumors were noted to be rejected at the point of harvest when only the clear Matrigel plug was found (see T1 and WT BM immature samples), or if there was a complete absence of tumor/Matrigel plug.
- (E) Schematic of experimental set-up for optical clearing and 3D imaging of subcutaneous tumors at D28 post-injection.
- (F) Vessel density, as normalized by total imaged volume, is similar in tumors co-injected with either T3 or WT BM mature neutrophils. Boxplots show median (centre line) with standard deviation (whiskers). $p = \text{n.s.}$, by Mann-Whitney U test.
- (G) T3 neutrophils are the highest expressors of *Vegfa* within the pancreatic tumor. UMAP embedding of all cells within the pancreatic tumor shows *Vegfa* expression density (**left**). Dot plot shows the average expression for each cluster identified and the relative percentage expression (**right**).



Supplementary Figure 14. T3 neutrophils are present across cancer datasets in mouse and humans.

- (A) A reference UMAP embedding was created for the PDAC dataset for label transfer and query mapping to other scRNAseq datasets. For all subsequent UMAP plots, the reference UMAP is juxtaposed against the mapped datasets and shown in the background in light grey.
- (B) Label transfer onto a published LLC-1 lung adenocarcinoma scRNAseq dataset (*13*) indicates that T1 – T3 tumor clusters are dominantly enriched in the tumor (red) compared to spleens from WT and tumor-bearing mice (dark grey).
- (C) Module scoring of the PMN2 signature identified in Veglia et al.
- (D) Module scoring of the PMN3 signature identified in Veglia et al.
- (E) Label transfer onto a published KP1.9 lung adenocarcinoma scRNAseq dataset (*11*) indicates that T1 – T3 tumor clusters are dominantly enriched in the tumor-bearing (red) compared to healthy lungs (dark grey).
- (F) Distribution of mN1-6 clusters identified in Zilionis et al. (*11*).
- (G) Module scoring of lung tissue neutrophil signature from Ballesteros et al. (*49*)
- (H) Single cell RNA sequencing (scRNAseq) data of a human pancreatic adenocarcinoma (PDAC) cohort was obtained from Steele et al., (GSE155698) (*55*). UMAP projection of all annotated cell types, from which neutrophils (dotted outline) were subsetted for further analysis.
- (I) scRNAseq data of a human pancreatic adenocarcinoma (PDAC) cohort was obtained from Wang et al., (NODE OEP003254) (*56*). UMAP projection of all annotated cell types, from which neutrophils (dotted outline) were subsetted for further analysis.
- (J) UMAP projection of mouse neutrophil scRNAseq (this manuscript) used for reference mapping and subsequent label transfer to neutrophils subsetted from human scRNAseq dataset from Steele et al., (GSE155698) (*55*) and Wang et al., (NODE OEP003254) (*56*). For all subsequent UMAP plots, the reference UMAP is juxtaposed against the mapped datasets and shown in the background in light grey.
- (K) Mapping of T3, T2 and T1 neutrophils reveal that tumor neutrophil subsets are strongly enriched in the human PDAC tumor and not in adjacent normal tissue. UMAP projection of total neutrophils subsetted from Steele et al., after label transfer showing neutrophil cluster annotations for tumors (**left**) and adjacent normal (**right**) tissue.

2330 **(L)** Mapping of T3, T2 and T1 neutrophils reveal that tumor neutrophil subsets are strongly
2331 enriched in the human PDAC tumor and not in peripheral blood. UMAP projection of total
2332 neutrophils subsetted from Wang et al., after label transfer showing neutrophil cluster
2333 annotations for tumor **(left)** and peripheral blood **(right)** tissue.



Supplementary Figure 15. Mapping of tumor neutrophils from mouse to human datasets to reveal their resultant impact on *in vivo* tumor growth.

- (A) Correlation of mapped tumor clusters with neutrophil cluster identities identified in Wang et. al., (56) reveals that pro-tumoral TAN-1 neutrophils are T3 neutrophils. Heatmap shows Spearman correlation performed on 2000 highly variable genes, R^2 values of selected comparisons are noted on plot. Hierarchical clustering was performed on distance matrix calculated from the correlation matrix.
- (B) Curation of a shared gene signature between mouse and human PDAC neutrophils. Gene signatures were mapped from mouse DEGs obtained in Fig. 3A-C and assessed for the ability to distinguish annotated T3, T2 and T1 neutrophil clusters within the human dataset derived from Steele et al., (55). Clusters originating from the pancreatic tumor are colored in orange, while those mapping to the adjacent normal region are colored in grey. Boxplots show median and interquartile range, with whiskers denoting outliers. Groups with less than two observations, i.e. T1 in adjacent normal, were not plotted.
- (C-F) All Kaplan-Meier plots show patients split into high and low expressions of the curated signatures from (B). Median overall survival and disease-free survival are represented on the graph when available. Events are represented by vertical lines on the graph and were defined from days to death from initial pathological diagnosis (overall survival) or days to first event (disease-free survival) from initial treatment for TCGA (The Cancer Genome Atlas) datasets or from clinical disease-free diagnosis for PACA-AU (Pancreatic Cancer-Australia). $p < 0.05^*$, as calculated by log-rank test.
- (C) Patient overall survival for T2 signatures in TCGA-PAAD (TCGA - Pancreatic Adenocarcinoma, **left**) and PACA-AU (International Cancer Genome Consortium Pancreatic Cancer-Australia, **right**) cohorts.
- (D) Patient disease-free survival for T2 signatures in TCGA-PAAD (**left**) and PACA-AU (**right**) cohorts.
- (E) Patient overall survival for T1 signatures in TCGA-PAAD (**left**) and PACA-AU (**right**) cohorts.
- (F) Patient disease-free survival for T1 signatures in TCGA-PAAD (**left**) and PACA-AU (**right**) cohorts.

2372 **Table S1.**

2373 Gene signature for murine neutrophil maturation score

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Table S2.

Differentially accessible open chromatin regions (OCRs) derived from the intersection of OCRs belonging to tumor neutrophils against all other peripheral neutrophil subsets, for which the log₂ fold changes and FDRs are shown. Peak regions belonging to background peaks used for HOMER analysis, as well as peaks matched to T3 specific genes are also included.

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Table S3.

Gene signatures utilized for digital multiplexed gene expression analysis with Nanostring nCounter system for mouse neutrophils and survival analysis for PDAC cohort datasets.

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Table S4.

Differentially expressed genes for T3, T2 and T1 tumor neutrophil clusters from scRNAseq derived from FindMarkers().

2388 **Table S5.**
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2390 Chi-square testing for potential confounding categorical variables for The Cancer Genome Atlas
2391 pancreatic cancer dataset.

2392 **Table S6.**
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2394 Chi-square testing for potential confounding categorical variables for International Cancer
2395 Genome Consortium Pancreatic Cancer-Australia dataset.

2396 **Table S7.**
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2398 Reporting table for hazard ratios, adjusted p-values and 95% confidence intervals calculated for
2399 T3, T2 and T1 signatures within The Cancer Genome Atlas Pan-Cancer dataset.

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Movie S1.

Representative example of CD31 (yellow) and PI (magenta) staining intensity in optically cleared a subcutaneous tumor co-injected with T3 neutrophils illustrating greater vessel formation within the tumor core.

Movie S2.

Representative example of CD31 (yellow) and PI (magenta) staining intensity in optically cleared a subcutaneous tumor co-injected with mature neutrophils from wild-type bone marrow illustrating lesser vessel formation within the tumor core.

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