Integrated transcriptional analysis reveals macrophage heterogeneity and tumor-macrophage interactions in the progression of pancreatic ductal adenocarcinoma

Kaidi Yang

Southwest Hospital

Xiang Zhao

Southwest Hospital

Jian Gao (✉ gaojian_genomics@163.com)

Yangtze Normal University <https://orcid.org/0000-0001-7636-911X>

Research

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Abstract

Background

Pancreatic ductal adenocarcinoma (PDAC) is a devastating metastatic disease for which better therapies are urgently needed. It highlights the need for an improved understanding of the biological features underlying the progression of PDACs. Macrophages significantly enhance tumorigenesis and development of pancreatic cancer. However, cellular reprogramming and phenotypic changes of macrophages during PDAC progression remain poorly understood, as well as the molecular mechanism underlying tumor-macrophage interactions.

Methods

Herein, we performed the computational analysis by combining scRNA-seq and TCGA RNA-seq datasets, through which we identified myeloid lineage and tumor cells as crucial mediators of intercellular communication networks and crosstalk in the tumor microenvironment.

Results

scRNA-seq analyses highlight the heterogeneity of macrophage with diverse phenotype and functionality. The SCENIC analysis indicated different cell states across the sub-clusters of macrophages through a reconstruction of regulatory networks, and cell trajectory analysis identified the tissue-resident macrophage and inflammatory monocyte as potential sources of tumor-associated macrophages. Furthermore, we uncovered several ligand-receptor pairs lining the tumor cells and macrophages. Among them, we found expression level of *HBEGF-CD44*, *HBEGF-EGFR*, *LGALS9-CD44*, *LGALS9-MET*, and *GRN-EGFR* correlated with poor outcome of patients.

Conclusion

Together, our findings deciphered the macrophage heterogeneity and detected a tumor environmental-specific gene expression program in macrophages of PDAC, which uncover a potential mechanism laying the tumor-promoting role of TAM. The ligand-receptor interactions identified would be potential biomarkers for anti-cancer targeted therapy.

Background

Pancreatic cancer, mostly pancreatic ductal adenocarcinoma (PDAC), remains one of the most lethal cancers with a 5-year survival rate of less than 8% [1]. The intractable characteristics of PDAC are the result of comprehensive factors like demographic factors, genetic factors, medical background conditions and environmental factors, which relates to the accumulation of acquired drive mutations (*K-*

Ras, *HER2/neu*, *TP53*), and as well as abnormalities in growth factors and their receptors, resulting in constitutive activation of downstream signaling pathway and the transcription factors [2–4]. As regards to the limited survival benefits of gemcitabine, the first-line regimen for pancreatic cancer, a better understanding of the mechanisms that contribute to uncontrolled proliferation is urgently needed.

Intriguingly, PDACs have a complex morphology environment where cancer cells are surrounded by a cellular milieu containing diverse cell types such as stellate cells, fibroblasts, endothelial cells and myeloid cells [5]. Myeloid compartment, including mostly tumor-associated macrophages (TAMs) and dendritic cells (DCs) as revealed in single-cell analyses of multiple cancers, contribute to all aspects of tumor progression [6–8]. PDACs could elicit a particularly severe T-cell exhaustion signature and immunosuppressive niche with TAMs [9], which are a heterogeneous cell type that can contribute to gemcitabine resistance by reduced apoptosis of cancer cells and promote malignancy by angiogenesis, immunosuppression and extracellular matrix (ECM) remodeling [10–12]. As the mechanism implicated in the communication between TAMs and pancreatic tumor cells during tumorigenesis is complicated, there is need for more knowledge about the spectrum of cell-cell interactions occurring in TME of PDACs and understanding how these interactions influence the outcome.

Single-cell RNA sequencing (scRNA-seq) enables the characterization of cell diversity and heterogeneous phenotypic states of macrophage compartments in more detail, which has been applied recently for primary pancreatic cancers and mouse PDAC models [13–15]. However, the functional heterogeneity of different subpopulations, cellular reprogramming and phenotypic trajectories of macrophages during PDAC progression remain poorly characterized. Developmental trajectory construction in colon cancer has suggested the origin of TAMs from tissue-resident macrophages and recruited monocytes that subsequently polarized into macrophages[7]. Researches have highlighted that tissue-resident macrophages could be originated from different resources with diverse phenotypes or functions [16, 17]. Tissue environment itself performs environmental programming of tissue-specific macrophage, and can regulate the gene expression program regardless of origin[18]. In PDACs, tissue-resident macrophages could expand during tumor progression and contribute to growth of PDACs [19]. While the understandings of its reprogramming trajectory and molecular phenotype are still limited.

Although macrophages divisions based on their polarization states could be into three types: un-activated macrophages (M0 macrophages), the classically activated type 1 (M1 macrophages), and the alternatively activated type 2 [20]. Recent research on the genetically engineered mouse model of PDACs, as well as human primary gastric cancer revealed no such classification of clusters in the single-cell level [14, 21]. The in vitro programming of M0 macrophage to M1 is thought to enhance the cytotoxicity against tumor cells by pro-inflammatory factors. However, by comparing the early stage and late stage of PDAC mouse model, it was found that PDAC progression is accompanied by an increase of inflammatory features in macrophages [14, 22]. It raised questions about M1/M2 classification and suggested pro- and anti-inflammatory features might not be dependently exited in the in-vivo system. As novel dichotomous functional phenotypes of TAMs have been revealed across different cancer types with a difference of

susceptibility to targeted therapy[7, 15, 23], extended functional classification of macrophage was required to be determined in PDACs.

Here, we utilized scRNA-seq and TCGA RNA-seq datasets to perform computational analysis, through which we constructed a novel cell-cell interaction network to define key cell communication involved in regulating malignancy of PDACs. Besides, we found that macrophages in PDACs is highly heterogeneous and featured with different gene expression profile and regulatory networks (regulons). We further identified that the tissue-resident macrophage and inflammatory monocyte are the potential sources of TAMs with changes of a list of novel gene expression during the differentiation trajectories. Our study uncovered cellular reprogramming programs and cell-cell communication features that specific to TAMs and thus might offer insights on inferring therapeutic targets.

Materials And Methods

Generation of the Primary PDAC Datasets

The Cancer Genome Atlas (TCGA) Pancreatic Cancer project was composed of gene expression data and relevant clinical information (n=182) which were downloaded through the UCSC data portal (<https://xenabrowser.net>). The raw count data obtained from the RNA-sequencing was normalized concerning library size via the R Deseq2 package, then it was performed log transformation and changed into same comparison level. After excluding samples that were not a histological type of primary ductal cancer, we reserved a total of 147 cases of primary samples for further survival analysis.

Single-cell Data Processing

The published PDAC single-cell dataset [13] containing 24 PDACs and 11 normal pancreas tissues was deposited in the Genome Sequence Archive with the accession number of PRJCA001063. We retrieved the raw data matrix and transformed it into a SingleCellExperiment object using the Seurat R package. After removing cells with poor quality (<200 genes/cell, <10 cells/ gene and >10% mitochondrial genes), raw matrix was processed using "Sctransform" methods, which contains an encapsulated function of "NomalizeData", "ScaleData", and "FindVariableFeatures" for automatically high-variance gene identification. Then we generated the ElbowPlot and determined the optimal dimensionality -number of principal components (PCs). As high resolution often leads to an increasing number of clusters, the "FindClusters" function for applying modularity optimization was adjusted to 0.15. The UMAP (Uniform Manifold Approximation and Projection), a dimension reduction technique was used to visualize the sub-clusters. To assign cell identity to each cluster, we consulted previously published literature and the CellMarker dataset (<http://biocc.hrbmu.edu.cn/CellMarker/>) for reference. Following characterizing the identity of myeloid cells/macrophages based on the markers *AIF1* and *CD68*, the same workflow was used to identify sub-clusters of macrophage. To perform over-representation (ORA) analysis, we identified differentially expressed features (cluster biomarkers) for each cluster against other cells using the "FindMarkers" function in which parameters of min.pct (minimum percent of cells expressing the gene)

and log2fold-change are set to 0.1 and 0.5, respectively. Pathway enrichment analysis was based on the items of the Reactome pathway using ClusterProfiler R package.

Correlative cell-cell interactions inferred by combined scRNA-seq and TCGA-PDAC datasets

Based on the TCGA RNA-seq profiles, we estimated the relative abundance of each cell subtype by the average expression of the cell type-specific genes defined above. Next, we conducted a Pearson correlation analysis between the expression of each gene and the relative abundance of each cell type to identify genes that may infer the co-occurring cells with these particular cell types. Since the expression levels of specific signature/marker genes in a given cell subtype were obviously in high correlation with the abundance of this specific cell subtype, we identified these genes based on the single-cell dataset according to the criteria of average expression > 1 and cell frequency of expression > 20%. We then filtered these genes and transformed their correlation coefficient to 0. After that, the top 20 highly correlated non-self-expressed genes were selected from the ranked correlation matrix by the correlation coefficient. To identify which cell subtypes could contribute to the highly correlated non-self-expressed genes, we performed a gene set enrichment analysis by calculating the mean value of z-score transformed expression of these genes for a specific cell subtype. At last, the correlated cell types were identified as the z-score transformed enrichment score > 1.28. If the correlated two types were identified mutually, the maximum of the enrichment scores was chosen.

Single-cell regulatory network inference and clustering (SCENIC)

For single-cell regulons (i.e., transcription factors and their target genes) inference in the different sub-clusters of macrophage, the SCENIC (<https://aertslab.org/>) vignette was used for reference [24]. The regulon inference was performed in the following three steps. (1). co-expression modules between transcription factors and the potential target were identified based on the gene-expression matrix through GENIE3 (R package) (2). Next, to remove indirect targets, these modules were pruned by cis-regulatory motif discovery (cisTarget), leaving 94 regulons to be analyzed in the next step. (3) the activity score of each regulon at cellular resolution was computed using the AUcell algorithm. Finally, the cellular activity patterns of these regulons were used for nonlinear projection and clustering. The overexpressed activity scores of regulons for each sub-cluster were visualized using a heatmap.

Cell-cell communication inference

To systematically study the interactions between macrophage and tumor cells, we predicted cell communication via CellphoneDB (www.cellphonedb.org), a publicly available repository of ligand-receptor interacting pairs and multi-subunit protein complex [25]. It considered the expression level of ligands and receptors with each cell type, generate a null distribution of each ligand-receptor pair, and determined the likelihood of cell-specificity of a given receptor-ligand complex based on the *P*-value. Those with the least number of significant *P*-values are chosen to be biologically relevant. After filtering in a criterion of *P* < 0.05 and secreted partners, a total of 94 ligand-receptor pairs were identified as shown in Supplementary Table 3.

Trajectory analysis

The monocle2 package in R was used to determine the developmental trajectory of macrophages. Following the monocle protocol, we passed the normalized data of indicated sub-clusters into monocle2 to establish a monocle object and selected top 2,000 most significantly ($q < 0.1$) differentially expressed genes (DEGs) as the set of ordering genes sorted by the q value. For isolating the root-to-branch specific gene expression patterns, the monocle BEAM statistical test was utilized. For visualizing the fate-dependant gene expression patterns, "plot_genes_branched_heatmap" function was used. For visualization of change tendency for a single gene, "plot_genes_branched_pseudotime" function was adopted.

Statistical analysis

In survival analysis, the means of the average expression level of interacting molecule 1 in tumor cell and interacting molecule 2 in macrophage are calculated using the square root: $\text{mean} = (\text{molecule1} * \text{molecule2})^{1/2}$. The Kaplan-Meier survival curve was built to show the differential survival among the two groups, and the log-rank test was performed to use statistical tests for comparing the survival distributions of two groups ($P < 0.05$). All statistical analyses were performed using R software (3.6.2).

Results

Myeloid and tumor cells comprise the core of a predicted cell-cell interaction network in PDAC

To interrogate global cell-cell interactions in PDACs, we performed the computational analysis by combining scRNA-seq and TCGA-PDAC RNA-seq datasets. The scRNA-seq profiles were obtained from published datasets, which were processed to identified different cell clusters and cell type-specific signatures as previously defined [13]. For each cluster, we identified gene signatures that were expressed in greater than 25% of cells as well with a log2 fold-change greater than 0.5 (Supplementary Table 1). In addition to identifying the abundance of a particular cluster in each TCGA sample using its gene signature, we generated non-self highly correlated genes and matched this gene set back to the scRNA-seq dataset, through which co-occurring enriched cell type was identified to build the correlative networks (Supplementary Figure 1a). With the analyses, interactions were mainly identified between malignant ductal cells, fibroblasts, endothelial cells and myeloid cells, reflecting the crosstalk of them as previous findings defined [10, 26, 27]. Among the interactions, Myeloid cells and tumor cells harbor the most number of partners. Besides, they act as the core of the interaction network accompanying the highest enrichment score, indicative of the strong connection between them (Supplementary Figure 1a). In contrast, we observed no such interaction in normal pancreas tissue, suggesting it is a biologically meaningful interaction. Then we examined the relative population contribution in tumor and normal samples. It was observed that clusters of immune cells (Myeloid cells, T-cell, B-cell), fibroblast cell and stellate cell appeared more frequently distributed in tumor samples (Supplementary Figure 1b),

suggesting close relations of them to tumorigenesis of PDAC. Besides, we identified that myeloid infiltration status is associated with worse prognosis of PDAC patients (Supplementary Figure 1c).

Identification of heterogeneity for myeloid cells

The myeloid cells compartment is comprised of a heterogeneous population of cells [28, 29]. By combining myeloid cells from tumor and normal tissue, the 5408 cells were further clustered into 8 subsets (Fig. 1a). Of these, cluster 5, 6 and 7 belong to myeloid DC lineage as shown by low expression of *CD68*, which was not identified in a previous analysis [13]. Cluster 5 corresponds to plasmacytoid dendritic cell (pDC) as shown by *DERL3*, *CXCR3* and *IGJ*, cluster 6 represents type-2 conventional DC (cDC2) as shown by *CCR7*, *IDO1* and *DAPP1*, and cluster 7 was identified as cDC1 cells with high expressions of *CD1C*, *CD1E* (Fig. 1b, Supplementary Figure 2a). The leakage of these DC clusters into macrophages might be owing to the low resolution (granularity 0.15) of the above main-class clustering. The remaining 5 clusters expressing *CD68* and *CD163* were designated as macrophages, albeit they showed rather a poor separation of clusters (Fig. 1b, Supplementary Figure 2a), suggesting that they represent diverse cell states on a graded scale rather than separate entities. It was noticed that 91.9% of the cells in cluster 2 came from normal tissue, indicating cluster 2 might represent an entitle of tissue-resident macrophage (Fig. 1a, Supplementary Figure 2b). However, we surprisingly found 47.3% of the macrophage population in normal tissue was from cluster 0 and cluster 1 (Supplementary Figure 2b), which suggested the involvement of monocyte-derived macrophages in normal tissues. To further understand the molecular features of the five sub-clusters of macrophages mentioned above, we conducted a DEG analysis and identified cell makers for each cluster (Supplementary Table 2). Cluster 4 expresses *S100A8*, *S100A9*, *VCAN*, and *FCN1* which are associated with inflammatory monocytes [29, 30], we thus defined it as monocyte-like cells; Group 0 highly expresses a lot of MHC class II genes, such as *HLA-DPB1*, *HLA-DPA1*, *HLA-DQB1* et al. Cluster 1 expressed cytokines *CCL2*, *CCL3*, *ADM* et al., apolipoprotein genes *APOC1*, *APOE*, and pro-fibrotic genes *SPP1*, *TIMP1* in control of ECM deposition [31]. Cluster 2, representative of tissue-resident macrophage, expresses previously unknown marker genes, such as *PRSS1*, *CLPS*, *SYCN* et al. We confirmed high expression of *PRSS1* in the normal pancreas tissue as shown by the immunohistochemical images deposited in Human Protein Atlas dataset (Supplementary Figure 3a-c). Cluster 3 highly expresses proliferation-associated genes, such as *TOP2A*, *CDK1* and *MKI67*, et, suggesting it is in a state of rapid proliferation (Supplementary Figure 2c). Intriguingly, different tumor samples consist of a group of macrophages with high heterogeneity, especially the ratio between cluster 1 and cluster 0, two large populations of macrophages among patients (Fig. 1c). It interested us whether the two populations were representative of the different polarized states of macrophage. As there was no recognizable cluster representative of M1 and M2 polarization macrophage, we examined the expression of marker genes representative of M1 (e.g. *CXCL9*, *IL1 β* , *CCL5*) and M2 (e.g. *MRC1*, *CCL18*, *CCL23*, *CD163*) states across the macrophage clusters. However, the expression of M1/M2 markers shows no evident differences among the sub-clusters (Fig. 1d).

t-SNE atlas reveals gene set enrichment state across different sub-clusters of macrophages

To delineate the functional heterogeneity of sub-clusters of macrophages, and to see which cell cluster principally contribute to the malignant progression of tumor cells, we performed gene set enrichment analysis using MsigDB hallmark gene terms and some immune-related terms, which aggregate redundant biological terms and represent well-defined biological states or process. Gene-set variation analysis (GSVA) revealed that cluster 0 displayed upregulation of pathways associated with complement cascade, antigen processing and presentation, PD1 signaling. While cluster 1 was characterized by high expression of cancer-related terms: angiogenesis, inflammatory response, glycolysis, cholesterol homeostasis and epithelial-mesenchymal transition, indicating it is more aligned with the property of TAMs. In correlation with its high proliferating property, cluster 3 expressed the highest level of apoptosis, G2M checkpoint and mitotic spindle (Fig. 2a-c). In addition to GSVA analysis, GRO analysis based on the Reactome pathway database further revealed that pathways related to MHC-II antigen presentation, CD28 family and TCR signaling were predominantly enriched in cluster 0. In contrast, cluster 1 was more activated and enriched with chemokines and extracellular matrix pathways, while cluster 3 was featured with signaling pathways related to cell cycle and mitotic process. Besides, cluster 4, monocyte-like macrophage, was associated with a gene expression pattern towards inflammation and innate immune response (Supplementary Figure 4). Thus, these data suggested the functional heterogeneity of sub-clusters of macrophages and indicated cluster 1 likely represented TAMs.

Identification of master regulatory networks (regulons) across different sub-clusters of macrophages

The transcriptional state of each cluster emerges from an underlying gene regulatory networks. We thus applied SCENIC to assess which regulatory networks determine the cell state for each sub-cluster of macrophage. By projecting the cell identities on the t-SNE clustering atlas based on the regulons' activities, we found each cluster of macrophages located separately from other clusters, showing differences of transcriptional states (Fig. 3b). A distinct set of regulatory genes, including EZH2, SOX9, HES4, ATF4, IRF1, IRF2 et al., were revealed to defining the various macrophage populations (Fig. 3a). Consistent with their inflammatory phenotype and functions, SCENIC revealed that genes regulated by inflammatory transcription factors, IRF1, CEBPB, and STAT3, were upregulated in a subset of monocyte-like cells. We also identified EZH2, best known for its function in macrophage activation [32, 33], as a candidate transcription factor driving the proliferating status of cluster 3 macrophages. Besides, we identified STAT1 specifically in cluster 1 macrophages, and upregulation of SOX9 and HES4 seemed responsible for the transcriptional phenotype of tissue-resident macrophages (Fig. 3c).

Pseudotime trajectory reconstruction of macrophage differentiation course

To delineate monocyte-to-macrophage differentiation, the macrophage subpopulation of PDAC tissue was arranged to build single-cell pseudotime ordering. According to a developmental course, the trajectory constitutes one decision point and two termini corresponding to two distinct cell fates (Fig. 4a and 4b). By projecting the cell identity on the trajectory plot, we found cluster 4 was mainly distributed in the root state, verifying its monocyte-like phenotype (Fig. 4c). Besides, cluster 2 was mainly in the location of the root, which might suggest the differentiation of a subset of tissue-resident macrophages

into TAMs. Besides, the cluster 1 randomly appeared in the whole developing course of the pseudotime path, while cluster 3 and cluster 2 cells populated mainly on the two termini of the course constituting the pool of mature differentiated macrophage (Fig. 4c), suggesting they are in a polarized position. Next, we assessed the expression pattern of genes regulated during differentiation in cells at fates 1 and 2 of the trajectory. Profiling of gene expression dynamics along the trajectory revealed a reduction of known monocyte markers (*S100A9*, *S100A8*) and upregulation of *MMP9*, *CCL2*, *CTSL* et al. (Fig. 4d). Pseudotime kinetics of *SPP1* expression showed gradually upregulated from the root to both fates, while *STMN1* was increasingly elevated from the root to fate 2. Besides, *CTSS* expression was upregulated in the late stage of fate 2 differentiation and downregulated early in early fate 1 differentiation. On the contrary, *S100A9* expression slightly decreased at cells from the root to fate 1, but markedly increased in the late phase of pseudotime branch via fate 2 (Fig. 4e).

Inference of tumor-macrophage interactions via CellphoneDB

Having defined the subpopulations of macrophages, we performed an unbiased ligand-receptor interaction analysis between these subpopulations and malignant ductal cells according to the expression level and specificity of each ligand-receptor pair. Among the sub-clusters of macrophages, cluster 3 and cluster 1 are in close physical contact with tumor cells as defined by the total number of interaction pairs (Supplementary Figure 5b). Cluster 1 expresses higher levels of cytokines, such as *CCL2*, *CCL3*, *CXCL3*, *CCL4*, *ADM*, *CCL18* et al. to perform intercellular communication with tumor cells (Supplementary Figure 5c). By contrast, we found cluster 3 express high levels of chemokine receptor *CX3CR1*, the receptor for *CX3CL1*, which might account for the infiltration and recruitment of macrophage with the stimulation of tumor cells [34].

We subsequently sought to assess the degree of interactions for each cluster-cluster pair in the TME. As a result, the presented correlation matrix revealed active macrophages-tumor cell interactions (Supplementary Figure 5a). After filtering with a stringent standard, the master ligand-receptor pairs embedded in the communication between tumor cells and macrophages were illustrated in Figure 5A-B. Previous work has defined the secretion of CSF1 by tumor cells and its interaction with the CSF1R on the macrophages [35]. Besides, Osteopontin (SPP1) - CD44 signaling, which presented in the list with remarkable significance, has been shown to lay an important role in cell migration, tumor growth and progression [36]. This result also raised the previous unknown pairs, such as *CCL3L1-DPP4*, *SPP1-PTG*, *LGALS9-MET*, *TNF-DAG1* et al, which might serve critical functions. (Fig. 5a).

Having defined the master ligand-receptor pairs, we investigate the value of these ligand-receptor pairs in predicting patients' survival. We choose the 21 pairs in which ligand was representative of macrophages-origin to observe the tumor-promoting effect of each ligand. Through analyzing RNA sequencing datasets of PDCA from the TCGA portal, we found 5 pairs (*HBEGF-CD44*, *HBEGF-EGFR*, *LGALS9-CD44*, *LGALS9-MET*, *GRN-EGFR*) were correlated with poor outcome of patients (Fig. 5b, Supplementary Figure 5d). Among them, *MET* and *EGFR* were found highly expressed in the malignant ductal cells compared with normal ductal cells by projecting the expression levels of these molecules onto the UMAP clustering.

Correspondingly, *LGALS9*, *HBEGF* and *GRN* were confirmed overexpressed in macrophage cluster (Supplementary Figure 5e). We conclude that over-expressions of *LGALS9*, *HBEGF* and *GRN* in macrophages may help to promote PDAC progression, thus predict adverse clinical outcomes in patients.

Discussion

The 'Bulk' transcriptome sequencing is a commonly used modality to see gene expression changes of specific phenotype and pathway activity. However, it does not resolve problems of observing gene expression changes of specific cells. Consequently, the discrepancy of gene expression profiling between neoplastic cells and stromal cells would obscure the true activity identity for specific molecular signaling. Herein, we employed scRNA-seq methods to provide more insights into phenotypes and gene signatures of macrophage in PDAC.

Our data revealed 8 molecular subsets of myeloid cells that consist of previously ignoring DCs subsets, which perhaps was owing to the different clustering setting for resolution and high expression level of *AIF* in DCs [13]. The other 5 macrophage subsets identified demonstrated distinct phenotypes and functionalities. Among them, cluster 2 was defined as tissue-resident macrophages, which was not identified before [13]. It is generally accepted tissue-resident macrophage plays crucial roles in pancreatic tissue restoration and regeneration [37]. The recent finding indicated that the tissue-resident macrophages become detrimental by promoting pancreatic cancer progression. [19] During tumorigenesis, we found tissue-resident macrophages present significant phenotypic change and cellular reprogramming, as shown by the remarkable reduction of them in the tumor comparing with normal tissue (Figure A2A). Our data revealed one molecular subtype of macrophages conferred with a monocyte-like phenotype for the following reasons. Firstly, this cluster highly expressed *CCR2*, an inflammatory monocyte marker [30]. Besides, through pseudotime trajectory analysis, we found this cluster was present in the starting point of differentiation trajectory. Moreover, cell-cell interaction inference indicated this cluster has the weakest relationship with tumor cells, supporting it is in a condition of immaturity. Besides, the other 3 clusters: cluster 1 expresses numerous chemokine- and inflammation-associated genes, cluster 3 expresses proliferation markers and enriched in the cell cycle pathway, and cluster 0 is rich in MHC-II-associated genes. We dissected the cell communication frequencies for each cluster and found that cluster 3 and 2 were in more close contact with tumor cells. Also, these two clusters showed enrichment of cytokines-related pathways or in rapid proliferation stage, which is consistent with the characteristics of TAMs [38]. According to the pseudotime cell trajectory, we identified both inflammatory monocytes and tissue-resident macrophages as the potential sources of TAMs by trajectory analysis. TAMs compose essential compartment of the cancer-immune microenvironment and play critical roles in the regulation of tumor progression [39, 40]. An in-depth understanding of the ontogeny of TAMs could facilitate therapeutic intervention.

As regards to the polarization states of macrophages, Wang et al. revealed that pancreatic cancer cells activate macrophages to the M2 phenotype in a HIF1/2a-dependent manner, which then promotes the migration, invasion, and EMT of pancreatic cancer cells [41]. While other findings indicated as PDA

progresses, an inflammatory feature with macrophage is substantially increased [14]. In our investigation, the five sub-cluster showed no evident difference between M1 and M2 markers. Consistent with previous studies, neither of these populations are representative of M1 or M2 cells [8, 42]. According to our hypothesis, the M1 is more likely a concept indicating that macrophage performs a pro-inflammatory function in particular settings rather than a specific cell cluster. It was observed that M1-TAMs secrete TNF that induces activation of the NF- κ B signaling pathway to drive acinar-to-ductal metaplasia in the beginning stages of PDAC [43]. While as tumor invasion, the M1 macrophages secrete MMPs, like MMP-9, to degrade the extracellular matrix [44]. In other settings such as proliferation and vascularization, the M2-like phenotype seizes advantage [45]. We hypothesis that the polarization state of each cell frequently changes over the course of tumorigenesis as well as the number of cells presenting a specific polarization state, for which more investigations are required.

Beyond characterizing the cellular composition of PDACs, it is crucial to understand how the different cellular components interact with one another to give rise to the biology of tumorigenesis. Numerous studies have begun to utilize scRNA-seq data to characterize cell-cell interaction [46, 47]. However, limited to the small sample size, inter-tumoral heterogeneity tends to skew the results. Here, we have leveraged the advantages of scRNA-seq and large bulk RNA-seq samples to deduce a cell-cell interaction network, through which we identified myeloid lineage and tumor cells as key mediators of cellular cross-talk in the tumor microenvironment. Investigation of cell-cell interactions via CellphoneDB database suggested a cell-cell interaction profile which may be activated and work in mutual supporting processes between macrophage and tumor cells, therefore contributing to GBM progression. Depend on the CSF1-CSF1R interaction, the crosstalk between tumor and macrophage shifts the balance towards an immunosuppressive environment. Previous work by others demonstrated that CSF1R blockade effectively decreases the number of TAMs, reduced immune suppression and elevated immune response [35]. SPP1, also known as Osteopontin, was identified as secreted factors in our interaction pairs, showing an association with macrophage activation and polarization [48, 49]. It has been substantiated that TAM secreted SPP1 to promote the survival of glioblastoma cell and angiogenesis [50].

Second, we identified a correlation between the ligand-receptor pair and poor prognosis of PDAC patients. Previous work highlights the critical role of galectin-9 in regulating immune response. Galectin-9 enhances iTregs function and stability through CD44, which strengthens the iTreg cell phenotype [51]. However, lack of evidence focused on the function of paracrine galectin-9 on tumor cells. Our data demonstrate that galectin-9-CD44/MET generates an autocrine loop underlying the interactions with a unique value in predicting the prognosis of patients. Besides, Galectin-9 has been reported to been highly expressed in human PDAC comparing with the normal pancreas and is capable of polarizing macrophages toward a pro-tumorigenesis M2 phenotype. It is also available to be detected in the blood serum, thus showed great value in diagnosing PDAC [52, 53]. A study on pancreatic cancer identified expression and cleavage of HB-EGF were required for proteasome inhibitor-induced EGFR activation. Ray et al. demonstrated the macrophage expression of HB-EGF links chronic pancreatitis and K-Ras initiated PDAC [54]. We verified macrophages are the primary source of HB-EGF production and firstly illustrated that HB-EGF and EGFR signaling in pancreatic tumor-stroma interactions may explain the limited efficacy

of therapy and poor prognosis in pancreatic cancer patients. Another ligand-receptor pair for concern in prognostic determination was granulin (GRN)-EGFR. Recent work demonstrated that macrophage-derived granulin facilitates pancreatic cancer metastasis and resistance to αPD-1 therapy[55, 56]. Our data may provide the potential mechanism behind it.

Conclusion

In brief, our study defines a phenotypically diverse subpopulation of macrophage in TME of PADC through computational analysis. Our results improve understandings of the macrophage's related impact on the tumor, and interactome analysis demonstrated the underlying mechanism of intercellular communication between macrophage and cancer cells. Regarding limited immunotherapy options in hand, tumor-macrophage interactome represents potential treatment targets to inhibit cancer proliferation, overcome the immunosuppressive microenvironment and aid in the implementation of immunotherapy approaches in PDAC.

Abbreviations

DCs: Dendritic cells; DEGs: Differentially expressed genes; ECM: Extracellular matrix; GSVA: Gene-set variation analysis; ORA: Over-representation; PCs: Principal components; PDAC: Pancreatic ductal adenocarcinoma; scRNA-seq: Single-cell RNA sequencing; SCENIC: Single-cell regulatory network inference and clustering; SPP1: Osteopontin; TAMs: Tumor-associated macrophages; TCGA: The Cancer Genome Atlas; UMAP: Uniform Manifold Approximation and Projection;

Declarations

Ethics approval and consent to participate

Not applicable

Authors' contributions

Y.K.D. was responsible for the study designing and study supervision; G.J. was responsible for raw data processing and data clearing. Y.K.D. and Z.X. were responsible for the development of methodology, analysis, and interpretation of data. G.J. and Z.X. reviewed, revised and approved of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The single-cell dataset has been deposited to the Genome Sequence Archive with the accession number of PRJCA001063 by the original authors.

Consent for publication

Not applicable

Competing interests

The authors have no conflicts of interest to declare.

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Supplementary Information

Additional file 1. Supplementary Figure 1. Myeloid and tumor cell comprise the core of a predicted cell-cell interaction network in PDAC. Supplementary Figure 2. Differential cell origin and gene expression across the sub-clusters of macrophages. Supplementary Figure 3. Validation of molecular markers of tissue-resident macrophages in the normal pancreatic tissue by Immunohistochemical analysis. Supplementary Figure 4. Identification of characteristics of each sub-clusters of macrophages via over-representation (ORA) analysis. Supplementary Figure 5. Interaction matrix reflecting the degree of cell-cell communication and expression pattern of survival-related molecules in PDAC.

Additional file 2. Supplementary Table 1. Cell markers of each cluster in PDACs. Supplementary Table 2. Gene markers for each sub-cluster of macrophage. Supplementary Table 3a. Significant ligand-receptor pairs between pancreatic tumor cells and macrophages (Macrophage | Tumor cell pair). Supplementary Table 3b. Significant ligand-receptor pairs between pancreatic tumor cells and macrophages (Tumor cell | Macrophage pair).

Figures

Figure 1

a t-SNE projection of 5408 myeloid cells from the normal tissues and PDAC tumors colored according to graph-based clusterings (left panel) or sample origin (right panel). b Expression plots based on t-SNE clustering. Normalized expression levels of the *S100A8*, *CD68*, *PRSS1*, *DERL3*, *CCR7*, *CD1C* representative of cell markers for certain cell types in individual cells. c Hundred-percent bar chart illustrates the percentage of different sub-clusters of macrophages across different PDAC patients. d Heat map

illustrating expression levels of M1 and M2 markers across different sub-clusters of macrophages. pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; t-SNE, t-distributed stochastic neighbor embedding.

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Figure 2

a t-SNE projection of cluster 0-4 macrophages colored according to cell identity. b Heatmap showing the normalized GSVA score of representative MigDB hallmark and immune terms across different sub-clusters of macrophages. c t-SNE projection of normalized GSVA score of representative MigDB hallmark and immune terms across different sub-clusters of macrophages. GSVA, Gene Set Variation Analysis.

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Figure 3

a Heatmap showing the normalized activity score of representative regulons across different sub-clusters of macrophages. b t-SNE clustering plots based on the activity matrix of 94 enriched regulons colored by cell type annotations. c Expression plots based on t-SNE clustering. Activities of IRF1, STAT3, EZH2, KLF5, TCF4, HES4 regulons representative of a specific cluster. In each t-SNE plot of regulon, cells are colored according to the corresponding activity of regulon. *g included in the parenthesis represents the number of genes in the regulons. PC, principal component; AUC, Calculate the Area Under the Curve (indicating the activity score of each regulon).

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Figure 4

a,b Pseudotime trajectory was reconstructed on the identified macrophage cluster, which contains one branch point. States within the pseudotime trajectory are emphasized with different colors (left panel). Cells are highlighted according to the pseudotime ranging from 0 to 20 (right panel). c Each cluster of macrophages was projected onto the trajectory plots with different colors assignment (left panel). The right panel indicates pseudotime trajectory of all macrophages colored by cell cluster identity. d Heatmap

presents differentially expressed genes from a comparison of two branches ($q < 1E-50$), each row represents the expression level of a gene along the branch trajectory. e Pseudotime kinetics of indicated genes from the root of the trajectory to cell fate 1 (solid line) or cell fate 2 (dashed line), with each dot representing a single cell and color-coded by cell cluster identity.

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stratified by the average expression level of specific ligand-receptor pairs. $P < 0.05$ was identified as significant.

Figure 5

a Overview of selected ligand-receptor interactions with significant functional meaning. P values are indicated by circle size; The means of the average expression level of interacting molecule 1 in cluster 1 and its interacting molecule 2 in cluster 2 are indicated by color. The terms identified as ligand

(macrophage)-receptor (tumor cell) pairs rather than receptor (macrophage)-ligand (tumor cell) were labeled with ticks. b Kaplan-Meier curves of the overall survival in different groups of PDAC patients stratified by the average expression level of specific ligand-receptor pairs. $P < 0.05$ was identified as significant.

Supplementary Files

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