Shift in cellular crosstalk reveals the immunosuppressive environment in cholangiocarcinoma patients.

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Disclosures: The authors declare that no conflicts of interest exist related to the study.

Funding: This study was funded by Deutsche Forschungsgesellschaft (DFG, German Research Foundation) – Project ID 403224013 – SFB 1382 (UN and TL). This research project was supported by the START Program of the Faculty of Medicine of the RWTH Aachen University, Project ID 19/23 (LH).

Author contributions:

LH, JJGV, RK, and UN designed this study. SL, FU, XT, GW, JB, MB, FP, JN, DH and MH contributed to sample collection and processing. LH, SH, KR, SM, CR, and JJGV were responsible for data analysis and interpretation. LH, DJ and HB provided histological expertise. LH, SH, SM, RK, JA, JD, and CR performed statistical analysis and interpretation. LH, SH, SS, FR, JJGV, JA, CR, and KR contributed to the first draft of this manuscript. UN, RK, SS, TL, DJ, ED, JS, HB and JJGV provided the infrastructure and supervised the study. All authors contributed to the data analysis and manuscript writing.

Data availability:

The code used in analyzing this data is available at https://github.com/hayatlab/cholangiocarcinoma_ici. The data will be made available via https://zenodo.org/uploads/10017884.

Abstract

Objective: Cholangiocarcinoma (CCA) is a deadly disease, and treatment options besides surgery are limited. This cancer entity is characterized by abundant stroma and the tumor microenvironment (TME) plays an important role in the poor response rate to therapeutics; however, underlying pathways are unknown.

Design: To fill this gap, we used high-dimensional cytometry to analyze 16 human CCA samples and identified cell cluster crosstalk in seven human CCA samples using single-cell transcriptomics.

Results: We showed an increased immune checkpoint expression on various key T cell subsets in the tumor niche. PD-1 ($p<0.001$), ICOS ($p=0.002$) and TIGIT ($p=0.03$) were elevated on intratumoral residential memory CD8 T cells (TRM) and PD-1 ($p=0.03$) and ICOS ($p=0.02$) were elevated on intratumoral (EM) CD8 cells. We also identified regulatory T cells to be more abundant in the tumor ($p=0.002$), while CD8 EM cells fail to reach the tumor ($p=0.02$). To further explore the immunosuppressive TME, we used snRNA sequencing on 7 pCCA samples. We identified a shift in crosstalk between the stromal and hepatic stellate cells (SCHSC) to a specific cholangiocytes cluster, mainly present in the poor outcome group. In addition, we identified new cellular crosstalk with macrophage-myeloid cells. The effector memory cells are sparse in the cancer tissue and spatial distribution indicates that the peripheral liver tissue can be used for stratification for personalized care.

Conclusions: This study sheds light on the etiology of the immunosuppressive TME in CCA and uncovers pathways that are promising for further examination.

Introduction

Cholangiocarcinoma (CCA) is a deadly disease occurring along the biliary tract and is considered a rare cancer type with a 5-year overall survival (OS) rate of 7–20%(1, 2). CCA is classified as intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma (pCCA), or distal cholangiocarcinoma (dCCA). Although the histological pattern can be very similar, the subtypes have been considered different entities because of differences in their surrounding tissue and molecular tumor heterogeneity(3, 4, 5). CCA typically shows distinctive perineural invasion (PNI), which is defined as the invasion of cancer cells into and around the nerve trunks. Recently, we showed that both iCCA and pCCA may present a high nerve fiber density (NFD) in the tumor microenvironment (TME), which is linked to better survival in a subset of patients(6, 7). Another shared histological feature of CCA subtypes is the presence of a large stromal compartment. The stroma displays heterogeneous cell types, including immune cells and fibroblasts. Interactions between different cell types result in immune-infiltrated or immune-excluded phenotypes(8, 9, 10).

Recent progress in cancer care has included the development of immune checkpoint inhibitors (ICI) and therapeutic agents that lead to host immune cell recruitment to target cancer.

T cells play an important role in the immune response of the host to cancer and are the main factor leading to curative responses to checkpoint inhibitor immunotherapy. In CCA, regulatory T cells (Tregs) dominate the early immune response and remain present in later tumor stages(11, 12), whereas effector T cells are rarely found, and if present, do not show signs of activation(13). The presence of CD4 and CD8 T cells infiltrating the tumor and peritumoral areas is a sign of better outcomes(14), and high numbers of CD8 T cells in the outer border of the tumor are positively correlated with 5-year survival rates in patients with iCCA(15). Furthermore, a subset of CD8 T cells expressing CD161 is known to have higher cytotoxic potential(16, 17), and high levels of CD161 expression are associated with protective functions in many cancer types, including CCA(18). The immune checkpoint PD-1 is known to promote immune evasion, and high PD-1 levels in tumor-infiltrating CD8 T cells in iCCA were previously found to be associated with an unfavorable prognosis(19). Nevertheless, our understanding of the immune cell environment in CCA remains limited, and additional data related to treatment responses are still needed(20).

In addition to T cells, tumor-associated macrophages (TAMs) constitute another major immune population within the TME. Although TAMs can exhibit both pro- and anti-inflammatory functions, they are thought to be mostly immunosuppressive(21). Despite some disagreement between studies, most studies point to worse prognosis in patients with high levels of TAMs tumor infiltration(22, 23, 24). TAMs have been shown to be a major source of PD-1 ligand (PD-L1) in CCA tumors(25). Furthermore, TAMs can secrete cytokines, such as tumor growth factor-β (TGF-β), which suppresses the T cell response by T cell exclusion and prevents the acquisition of the tumor suppressive TH1-effector phenotype(26). Despite its immune-regulatory functions, modulation of the extracellular matrix (ECM) plays a role in recruiting TAMs in tumors(27). CAFs can secrete cytokines and chemokines that promote TAMs invasion and directly inhibit T cell responses(28, 29).

In this study, we aimed to better characterize the lymphocyte compartment in CCA by means of a comprehensive approach using cytometry by time-of-flight (CyTOF) and single nuclei (sn)RNAseq. We focused on patients with primary resected iCCA and pCCA and characterized the differences in immune landscapes of both entities in primary tumors, compared to matched peripheral liver tissue, and peripheral blood samples. To identify the crosstalk between cell clusters responsible for the immunosuppressive environment, we used snRNA sequencing to characterize the cellular heterogeneity of the transcriptome in patients with pCCA. To limit molecular tumor heterogeneity, we included tissue samples of pCCA patients only. We identified signs of exhaustion and an increased abundance of regulatory T cells in the tumor niche, while CD8 effector memory (EM) and tissue-resident effector memory cells (TRMs) failed to reach the tumor and were restricted to the peripheral liver tissue. To further explore the immunosuppressive TME, we revealed a shift in crosstalk between stromal and hepatic stellate cells (SCHSC) and a specific cholangiocytes cell cluster, mainly present in the patients within the poor outcome group. In addition, these cholangiocytes demonstrated elevated cell communication with macrophage-myeloid cells. This study sheds light on the etiology of immunosuppressive TME in CCA.

Results

To elucidate the heterogeneity of the tumor immune environment, we performed high-dimensional immunophenotyping using mass cytometry time-of-flight (cyTOF) on 16 tumor samples. Of these, seven were iCCA patients and nine were pCCA patients with resectable disease. For all patients, we obtained matched tumor-free liver tissue (hereafter referred to as 'peripheral liver tissue'), central tumor tissue, and peripheral blood samples (Figure 1a). All patients underwent surgery at the University Hospital RWTH Aachen (see Supplementary Table 1) to obtain detailed patient characteristics. Mass cytometry was performed using the panel described in Supplementary Table 2, with strong emphasis on the characterization of different subsets of T cells and the expression of known costimulatory molecules and immune regulators of importance in cancer immunotherapy. This was later combined with results from snRNA-seq to gain a more complete understanding of the TME (Figure 1b).

Analysis of the CD4 and CD8 compartments in the CyTOF data using a combination of dimensionality reduction (UMAP) and clustering (Phenograph) resulted in the identification of 27 CD4 T-cell clusters and 31 CD8 T-cell clusters, which were manually curated and annotated into known immune cell subsets (Supplementary Figure S1). Here, we focused on CD4 and CD8 compartments in both tumor entities.

Abundance of immune cell subsets in CD4 and CD8 memory compartments differ between peripheral and central tumor tissue, but not between tumors entities

Using an unsupervised clustering-based approach on a low-dimension representation, we uncovered differences between the CD4 and CD8 memory compartments (Figure 2a). Notably, the abundance of none of the examined subsets was significantly elevated when comparing the iCCA and pCCA tumor samples (Figure 2b). This suggests a comparable T-cell composition in the iCCA and pCCA subtypes, which enabled a combined analysis of both entities. Looking at cell abundance between the matched tumor and peripheral tissue, we observed a 5-fold increase in CD4 Tregs (p=0.002) and an increase in CD4 tissue-resident memory cells in the tumor tissue (p=0.0003). In the CD8 compartment, CD8 effector memory and tissue-resident effector memory RA cells (TEMRA) were more abundant (p=0.04 and

p=0.02, respectively) in the peripheral tissue, and although CD8 tissue resident memory cell levels were elevated in the tumor tissue, the difference was not significant (Figure 2c).

Exhausted effector cells in the tumor niche

In addition to the sparseness of CD8 effector memory (EM) and TEMRA cells in the tumor, both subtypes showed a decrease in CD161 expression compared to the peripheral tissue, indicating less cytotoxic activity. In addition, many of the identified memory subtypes display higher levels of immune checkpoints in tumors. While PD-1 and ICOS levels were elevated in CD8 EMs, CD8 TRMs, CD4 TRMs, CD8 TRMs, the CD4 TRMs also exhibited higher levels of TIGIT. In addition to the elevated levels of immune checkpoints, we also observed an increase in CD57 expression. CD57 is used as a marker for differentiation/senescence and may promote resistance to ICI in cancer (Figure 3a)(30, 31). Furthermore, the marker expression profile in the small subset of CD4 stem cell memory (SCM) also showed signs of overactivation in tumors with elevated levels of HLA-DR ($p<0.01$), CTLA-4 ($p<0.01$), and ICOS ($p<0.05$).

Regulatory T cells subset is correlated with expression of immune checkpoints in effector cells

Similar to the described effector subsets, CD4 Tregs show elevated levels of the activation markers CD69 and OX40, and the immune checkpoints PD-1, CTLA-4, and TIGIT. The chemokine receptor CXCR3 (p value did not reach significance) and CCR4 showed decreased levels in the tumor (Figure 3a). Both receptors are associated with migration to tumor tissues(32). Within the total Treg population, we identified a distinct cluster of Tregs with lower levels of CCR7 and higher levels of CD69, indicating a more active state (Supplementary Figure S2a). Activated Tregs (aTregs) were almost completely absent in the blood but were more than four times more abundant ($p = 0.0001$) in the tumor than in the normal tissue (Figure 3b). Furthermore, the expression of CD69 by Tregs in the tumor tissue was positively correlated with the mean expression of CTLA-4, TIGIT, ICOS, and PD-1 in CD4 and CD8 effector memory, stem cell memory, and tissue resident memory cells, indicating a connection between immune checkpoint expression in effector cells and the activation status of regulatory T cells (Figure 3c). In addition to the well-studied CD4 Tregs, there was also an increase in CD8+ FoxP3+ CD25+ cells in the TILs population (Figure 3d). Although this population is considerably smaller, it is thought to have regulatory functions similar to those of CD4 Tregs and is more frequent in some types of cancers(33, 34).

Enrichment in a cluster of CD4 EM cells in patients without lymph node metastasis

We also observed a cluster of CD4 EM cells enriched in the peripheral liver tissue of patients without lymph node invasion (Figure 3e). This cluster is characterized by lack of the expression of the examined checkpoints CTLA-4, PD-1 and TIGIT as well as the activation marker CD69 (Supplementary Figure S2b). The presence of lymph node metastasis is indicative of an aggressive cancer biology. There was no correlation with overall survival, most likely due to the small cohort size and high mortality rate within 30 days after surgery of almost 25%.

Multiplexed immunohistochemistry can be used to demonstrate the spatial distribution of CD4 and CD8 residential effector memory cells

To obtain a more spatial context for PD-1 expression in T cells, multiplexed immunohistochemistry (IHC) was performed. CD4 cells and CD103 positive residential memory CD4 cells were mainly present in the tumor and interface areas. The same was true for their PD-1 positive subsets. The regions annotated as peripheral liver tissue showed lower levels of all the examined CD4 subsets (Figure 4a-e). Levels of PD-1 positive CD8 cells and CD8 TRMs were slightly lower than those of their CD4 counterparts, but they were also more abundant in the tumor and interface regions (Figure 4f-j). These findings are in line with the CyTOF data, which showed increased PD-1 expression in CD4 and CD8 TRMs in tumor tissue, but furthermore showed that there is also an increase in CD4 and CD8 TRMs as well as PD-1 positive CD4 and CD8 cells in the interface compared to the tumor region. In our cohort, 24% of the patients (6 of 25 patients) demonstrated either CD4 or CD8 TRM cells being positive for PD-1.

snRNA sequencing reveals heterogeneity in cholangiocarcinoma cell populations

In addition to the previous analysis of the TME using cytometry, we aimed to understand cellular interactions and then performed snRNA sequencing of tumor tissue from seven patients with pCCA, split into two groups based on their clinical outcome. Patients with cancer positive lymph nodes and cancer recurrence within one year after surgery, were identified as having a poor outcome based on an aggressive biology of disease (n=3 with good clinical outcome; n=4 with poor clinical outcome). Detailed patient characteristics are outlined in supplementary Table S3 and available detected genetic variants are outlined in supplementary Table S4.

We identified 10 distinct clusters of cells (Figure 5a), distributed across the two outcome groups (Figure 5b). These clusters were manually annotated into known cell types based on marker gene expression. A large proportion of cells, represented by 4 of the 10 identified clusters, expressed markers associated with cholangiocytes and malignant epithelial cells. Other identified cell clusters included T cells, B cells, and a cluster of macrophages and other myeloid cells as three distinct immune cell populations. Two clusters of hepatocytes and a cluster of stromal and hepatic stellate cells (SCHSC) were identified (Figure 5c). The different clusters were heterogeneously distributed between the patient and outcome groups. Notably, hepatocytes were mainly present in only two patients, and the proportion of cholangiocytes clusters also differed between patients (Supplementary Figure S3). For the total cholangiocytes population, we identified 4167 differentially upregulated and 5578 differentially downregulated genes (FDR<0.05) between outcome groups, including MDM2 and PTPRM (Figure 5d).

Shift in the TME cellular crosstalk between clinical outcome groups

Based on ligand receptor expression, we assessed the cellular crosstalk between different cell populations. For this analysis, we used the CellCall(35) tool and uncovered the cell-cell communication between stromal cells, cholangiocytes, macrophages, and myeloid cells in both groups separately. In contrast, B and T cells were found to be less involved (Figure 6a). Interestingly, we found AXL among the ligand receptor pairs unique for the poor outcome patients. AXL is a member of the three known TAM receptors (Tyro3, MerTK, AXL)

interacting with its ligand GAS6. Specifically, GAS6 – AXL interaction was predicted between GAS6 expressing stromal cells and cholangiocytes and AXL-expressing macrophages (Supplementary Figure S4). TAM receptors are well-known regulators of the immune response in macrophages in various contexts(36). Next, we employed the crosstalker tool to directly compare changes in cell-cell communication between the two outcome groups. We observed a shift in cellular communication towards cholangiocytes cluster 4 in the poor-outcome group. This is mainly driven by communication between cholangiocytes cluster 4 and macrophages and myeloid cells, as well as the SCHSC cluster (Figure 6b). The top ligand in the crosstalk from cholangiocytes cluster 4 to the SCHSC and macrophage myeloid cell clusters was the Versican gene, VCAN. *Versican* encodes an extracellular matrix protein known to be involved in cancer progression and inflammation(37). VCAN was predicted to interact with Toll-like receptor 2 (TLR2) on macrophages and epithelial growth factor receptor (EGFR) in SCHSC. Among the top ligands driving communication from the macrophage myeloid cell cluster was the tumor growth factor β (TGF-β) gene, TGFB1 (Supplementary Table S5). TGF-β can signal trough type I and type II TGF-β receptors and is a well-known regulator of cancer immune evasion as well as other aspects of tumor progression(38).

VCAN and AXL signaling correlate with M2 macrophage signature

To validate the findings in our pCCA snSeq cohort, we analyzed publicly available microarray data from 182 cases of extrahepatic CCA (eCCA). Although this dataset also contains dCCA, the majority of 130 cases were pCCA patients(39). To assess cell type abundance, we performed cellular deconvolution using CIBERSORTx(40). Using this method, we were able to identify the heterogeneous infiltration of several immune subsets. While some immune cell subsets, such as CD4 naïve cells and M1 macrophages, were predicted to be present only at low levels, M2 macrophages, natural killer (NK) cells, Tregs, and CD8 cells were generally higher and more heterogeneous (Figure 6c). Interestingly, we observed a correlation between VCAN, TLR2, AXL, and the M2 macrophage signature. In addition to the genes involved in cellular crosstalk, we also observed a correlation between the checkpoint genes identified in our CyTOF cohort. Here, PD-1 expression was inferred by the expression of PDCD1, TIGIT, and FOXP3, which correlated with TGFB1 expression (Figure 6d).

In line with our CytOF data, deconvolution predicted high Treg levels. Of all the examined subsets, they were predicted to be the most abundant, and only a small subset of patients had low levels of Tregs (Figure 6c).

Discussion

CCA treatment options remain limited, and even after decades of research, surgery still represents the best chance for a cure. While the role of immunotherapy is increasing in other cancer entities, knowledge about immune responses to cancer cells in CCA remains limited. Furthermore, studies are complicated by CCA heterogeneity, including a wide range of genetic subtypes(41). The immune system can destroy cancer cells, but there is also a complicated immune activation network regulated by immune checkpoints(42). The TME of CCA features a T-cell exclusion mechanism and is dominated by low cytotoxic immune cell infiltration and high levels of Tregs and checkpoint expression, which hinders the immune response against tumors(5). Here, we report the contributions of different immune cells to the immune microenvironment in iCCA and pCCA, with detailed definitions of their components.

Overall, the presence of many CD4 and CD8 cells in the tumor area is a sign of good prognosis, and previous studies have shown higher levels of CD4 and CD8 cells in the tumor margin than in the central tumor area in patients with iCCA(43, 44). Accumulation of Tregs in tumors correlates with poor prognosis(9, 45). Here, we found that both iCCA and pCCA samples were infiltrated with Tregs and exhausted CD4 and CD8 tissue-resident memory cells, while CD8 effector memory cells were mostly excluded. Moreover, we found elevated levels of CD69 in infiltrating Tregs, which correlated with increased checkpoint expression by other subtypes in the tumor. CD69 has been shown to be involved in Treg function and has been reported to be correlated with tumor progression in hepatocellular carcinoma(46, 47).

Immune checkpoints play a role in many cancer types and a subgroup of patients is likely to benefit from immunotherapy. An extensive understanding of immune cell composition and expression of costimulatory and coinhibitory checkpoints is needed to predict whether patients will respond to immunotherapy. PD-1 is a co-inhibitory receptor that can be expressed in many different cell types, including Tregs, activated B cells, and T cells. It suppresses the activity of T cells and downregulates the T cell response when it binds to its ligand PD-L1(19, 48). Our data showed high levels of PD-1 as well as other checkpoint inhibitors such as TIGIT and CTLA-4.

In addition to the suppression of T cells, our snRNA-seq data allowed us to examine the TME beyond the contribution of T cells and identify the crosstalk between cell clusters responsible for the immunosuppressive environment. We identified several immune populations, as well as clusters of stromal cells. The included markers associated with stellate cells, which are believed to be a major source of CAFs(9, 49). In accordance with previous studies, we demonstrated increased expression of genes associated with a worse patient outcome in the cholangiocytes compartment, including MDM2 and PTPRM. MDM2 was previously associated with advanced CCA stages and a worse clinical outcome(50, 51), and PTPRM with a worse outcome in cervical cancer(52). Together with T cells, myeloid cells including macrophages were identified as an integral immune population within the TME and are known to contribute to immunosuppressive cell crosstalk within iCCA(53).

In our pCCA snRNA seq cohort, we identified GAS6, and AXL as unique ligand receptor pairs associated with poor prognosis. *GAS6* codes for the growth arrest-specific 6 protein, which can be recognized by the AXL receptor belonging to the TAM subfamily. TAM receptors play a role in the immune system but are also associated with tumor progression(54). Both GAS6 and AXL were previously associated with worse patient survival in lung cancer(55), and their signaling was associated with the recruitment and polarization of suppressive macrophages in hepatocellular carcinoma(56). In addition to its immunosuppressive role, AXL and TAM receptor signaling are generally associated with PD-L1 expression(57, 58). Furthermore, we observed a shift in the crosstalk between different cell clusters. In our poor outcome group, we identified increased crosstalk between a cholangiocytes subpopulation mostly present in the poor prognosis group, macrophages, and stromal cells. Communication with macrophages was partly driven by the *VCAN* gene encoding for the extracellular matrix (ECM) protein versican and toll-like receptor 2 (TLR2). This interaction was previously shown to promote an immunosuppressive environment(59) and to stimulate metastasis in cancer(60). In addition, versican is predicted to interact with the epithelial growth factor receptor (EGFR) in stromal cells. Versican signaling via EGFR in breast cancer has been shown to promote proliferation(61). EGFR is commonly

overexpressed in CCA, which can be associated with unfavorable patient outcomes(62). We identified TGF-β gene TGFB1 as a major ligand expressed in macrophages. TAM-derived TGF-β is a known factor that promotes various aspects of tumor progression(63) and has been shown to drive the epithelial-mesenchymal transition in hepatocellular carcinomas(64). TGF-β is also a key mediator in the induction of Tregs and can directly inhibit cytotoxic T cell function(26, 65), making it a potent promoter for the suppression of T cells.

In summary, we showed that there is upregulation of checkpoint PD-1 and TIGIT across T cell subsets in iCCA and pCCA. The presence of regulatory T cells, as well as the exclusion of CD8 effector subsets, likely contribute to the lack of anti-cancer immunity and the variable response to immune checkpoint therapy. Furthermore, we examined the crosstalk between different cell populations and identified signaling by GAS6-AXL and VCAN -TLR2 and EGFR-TGF-β as drivers for the suppressive TME beyond T cell immunity. Further examination of these pathways might provide valuable insights into the underlying mechanisms and could potentially give rise to treatment candidates that are tailored to the TME within CCA.

Good biomarkers for predicting a response to immune blockade treatment are still missing. PD-L1 immunohistochemistry is currently used in daily diagnostic care, and its expression in macrophages, immune cells, and cancer cells has been previously reported. Our study demonstrates that in addition to the presence of PD-L1 blocking immune cells in the tumor, the presence of immune cells outside the tumor in the peripheral surrounding liver tissue might be of importance for patient prognosis. A multiplexed panel can be used on peripheral liver biopsies to define the presence of CD4 and CD8 effector memory cells to predict an activated immune phenotype. We hypothesize that further research should investigate peripheral liver tissue for the presence of immune cell clusters and prospectively identify response biomarkers for immunotherapy. Although this is a relatively complex workup, multiplexed imaging is available in most university hospitals and may represent a step towards personalized cancer care in a patient group with sparse therapeutical options.

Materials and Methods

Patient Recruitment

Samples were collected between May 2020 and June 2021 from 16 patients with iCCA (n=7) or pCCA (n=9) with a curative intent for surgery. The patient characteristics are shown in Table S1. All patients consented to participate in this study with local ethical approval under EK106/18 and EK360/19 and the broad consent (EK206/09) of the hospitals centralized biomaterial bank (RWTH cBMB).

Sample Collection and Tissue Digestion

Blood and tissue samples were collected from each patient. Blood was collected before the operation in 20 ml EDTA tubes. Blood samples were processed within 4h of blood withdrawal. PBMCs were isolated using Ficoll after the addition of 20 mL of 2% FBS/PBS. After centrifuging for 20 minutes at 1300 g without break, we removed the PBMC ring using a pipette. This was then washed and centrifuged again at 300 g and if red blood cells were lysed with ACK lysing buffer and the cells were washed again.

Tissue samples were collected postoperatively from the pathology department of our hospital. Tissue blocks of normal tissue and tumor tissue with a size of 10 × 10 × 5 mm were placed in RPMI medium (Gibco), which was cooled on ice. Our digestion protocol was used as in our previous study(66). The tissue was minced with a scalpel, and enzymes were added for digestion. This solution, along with the tissue pieces, was placed in a shaker for 40 min at 37 degrees Celsius (°C). Then, the medium was refreshed, new enzymes were added, and again placed into the shaker for an additional 30 min. The remaining solid tissue components were pushed through using a cell strainer, providing the greatest possible yield. The tube was centrifuged at 300 g for 10 min, and the pellet was collected and cryopreserved in a medium containing DMSO at -80°C until further analysis.

CyTOF sample preparation

The metal-isotope labeled antibodies used in this study were conjugated using the MaxPar X8 Antibody labeling kit according to the manufacturer's instructions (Fluidigm) or were purchased from Fluidigm pre-conjugated. Each conjugated antibody was quality checked and titrated to the optimal staining concentration using a combination of primary human cells (peripheral blood mononuclear cells, single cell suspensions from tonsils, and single cell suspension from cholangiocarcinoma). The samples were thawed for further CyTOF staining by gently diluting DMSO in warm RPMI and washing 2 times in RPMI. Single-cell suspensions of cholangiocarcinoma tumor tissue, surrounding normal liver tissue, and peripheral blood mononuclear cells were thawed and washed with warm culture media (RPMI + 10%FCS Pen/strep/glut). The cells were incubated with culture medium + 15 U/ml DNase for 10 min at 37°C. Cells were washed in PBS and incubated with 0.5 µM cisplatin (Fluidigm) in phosphate-buffered saline (PBS) for 5 min at room temperature (RT) to label non-viable cells. The cells were resuspended in Truestain FC blocker (BioLegend) for 10 min at RT. After incubation, an antibody mix of targets not capable of fixation (CCR5, CXCR3, CCR, and IL-7RA) was added to the cells for 30 min at 4°C. The cells were fixed with 1.6% PFA in PBS for 10 min at RT. Cells were washed with CSM and fixed with 1.6% PFA in PBS for 10 min at room temperature (RT). Fixed cells were palladium barcoded (Fluidigm) according to established protocols. All surface staining was performed in CSM for 30 min at

RT (antibodies listed in Table S1). Cells were washed in CSM and permeabilized using the FoxP3 staining kit, according to the manufacturer's instructions (eBioscience™ FoxP3/Transcription Factor Staining Buffer Set, Invitrogen. After intracellular staining, cells were fixed with 1.6% PFA in PBS for 10 min at RT. Before acquisition, samples were washed in CSM and resuspended in intercalation solution (1.6% PFA in PBS, 0.02% saponin (Sigma) and 0.5 µM iridium diluted in Fix/Perm solution (Fluidigm)) for 1 h at RT or overnight at 4°C or frozen in aliquots to label DNA. Before acquisition, the cells were washed once with RPMI+30% FCS. The samples were washed twice with CSB and once with larger volumes of CAS. All samples were filtered through a 35 µM nylon mesh cell strainer, resuspended at 0,5 × 106 cells/mL in CAS supplemented with 1/10 EQ four-element calibration beads (Fluidigm), and acquired on a CyTOF2 mass cytometer (Fluidigm) using the Super Sampler injection system. Prior to data acquisition, the instrument was demonstrated to have Tb159 dual counts greater than 1,000,000 and oxidation less than 3%; if the instrument failed these criteria, it was cleaned, tuned, or repaired as necessary.

CyTOF data acquisition and pre-analysis

Immediately prior to acquisition, samples were washed as described above and then resuspended at a concentration of 0.5 million cells/mL in cell acquisition solution containing a 1/10 dilution of EQ 4 Element Beads (Fluidigm). Samples were acquired on a CyTOF Helios mass cytometer at an event rate of <150 events/s. After acquisition, the data were normalized using bead-based normalization in CyTOF software. The data were exported as FCS files for downstream analyses. The data were gated to exclude residual normalization beads, debris, dead cells, and doublets, leaving DNA+ live single events for subsequent clustering and high-dimensional analysis.

Batch effect correction

Data were acquired in five different batches, each containing two technical replicates, one single-cell suspension of tonsil cells, and one PBMCs sample. We used CytoNorm(67) to model the differences in signal intensities between batches. We determined which of the two replicate sets had a larger dynamic range per marker. TIGIT, CD69, CTLA-4, ICOS, PD-1, CXCR3, LAG-3, OX40, CD7, CD95, CD45RA, CD45, CD127, and HLA-DR were modeled and normalized using tonsil replicates. All other markers were modeled using PBMC replicates. The data were arcsinh-transformed (factor 5) and clustered into B cells, T cells, NK cells, and myeloid lineages using FlowSOM. Subsequent settings for CytoNorm were as follows: 21 quantiles from the 0th to 99th percentile. The limits were (0 and 7.4). All FCS files were normalized using both normalization models.

CyTOF data analysis

After batch correction was performed, the data were further analyzed using R (v. 4.2.2). Subsequently, cells were clustered using the FlowSOM(68) algorithm (CATLYST package v. 1.22.0) based on CD2, CD3, CD4, CD8, CD14, CD45, and HLA-DR on a 10 by 10 grid and with elbow metaclustering. The algorithm resulted in eight metaclusters that could be manually assigned to CD4 T cells (FlowSOM metaclusters 1 and 4), CD8 T cells (FlowSOM metaclusters 2 and 3), and miscellaneous cells, included NK cells, CD14+ cells, and others (FlowSOM metaclusters 5-8).

Cells were downsampled to less than 50000 CD4 and CD8 cells per sample, resulting in 918.019 CD4 T cells and 732.047 CD8 T cells. OptSNE dimensionality reduction from the MulticoreTSNE package (v.0.1) using Python (v.2.7.18) with a perplexity of 30, theta of 0.5, maximum of 1000 iterations, and stop of 5000 was performed using CD45RA, CD45RO, CCR7, CD27, CD28, CD69, CD103, CD95, CD57, CD161, CD44, CD25, CD127, FOXP3, CXCR3, CCR5, CCR4, CD11a, and CD49d. Subsequently, Phenograph(69) (Rphenograph package v. 0.99.1) was applied using the same markers with K-nearest neighbors set to 20 and Euclidean as the distance metric. The resulting clusters (25 for CD4 + T cells and 31 for CD8 + T cells) were annotated according to their expression profiles into known immune cell subset categories for data interpretation.

Statistical comparisons involved Friedman's test to compare mean frequencies and marker expression across tissues in either iCCA or pCCA (matched blood, normal tissue, and tumor tissue samples), Mann-Whitney test to compare mean frequencies and marker expression between iCCA and pCCA tumors, and Wilcoxon test to compare mean frequencies and marker expression between normal and tumor tissues. Correlation analysis between cluster frequencies in blood versus tumor tissues and mean marker expression was performed using Spearman's test.

Histology and multiplexed immunohistochemistry

The histology report of each patient was checked for quality control of the right tumor. The pathologist (LH) collected the H&E-stained slides and selected the tumor block. Sections were cut using a Leica RM2235 microscope at a thickness of approximately 4 µm. Antibodies against CK19, CD4, CD8, CD103, and PD-1 were analyzed, and their co-expression was quantified using the TissueFAXS PLUS scanning system with StrataQuest Analysis Software from TissueGnostics(70), Vienna, Austria.

Single-nuclei isolation

In addition to cytometric analysis, we also chose seven patients for transcriptomic analysis using single nuclei RNA sequencing (snRNA seq). The patients were divided into two groups based on their clinical outcomes. For the four patients in the poor clinical outcome group, we detected lymph node invasion by the tumor and recurrence within 1 year of surgery. In the three patients in the good outcome group, no lymph node invasion was detected, and they were recurrence-free at the time of the study (at least one year after surgery). NGS data was available in 3 patients, see supplementary table S4. Tumor samples were frozen in cryotubes without fluid as quickly as possible after sample collection. To do so, they were covered in liquid nitrogen and then transferred to a −80 °C freezer for long-term storage. Single-nuclei isolation was performed as described previously(71).

Briefly, tissue samples were cut into pieces <0.5 cm and homogenized using a glass Dounce tissue grinder (Sigma-Aldrich). The tissue was homogenized by subjecting it to 25 strokes with pestle A and 25 strokes with pestle B in 2 ml of ice-cold nuclei EZ lysis buffer. Subsequently, the cells were incubated with an additional 3 ml of cold EZ lysis buffer on ice for 5 min. Nuclei were centrifuged at 500 g for 5 min at 4 °C, washed with 5 ml ice-cold EZ lysis buffer, and incubated on ice for 5 min. After centrifugation, the resulting nuclei pellet was washed with 5 ml nuclei suspension buffer (NSB; consisting of 1× PBS, 0.01% BSA, and

0.1% RNase inhibitor). Isolated nuclei were resuspended in 2 ml NSB, filtered through a 35 µm cell strainer, and counted. Approximately 1,000 nuclei per µL were used for loading on a 10x channel.

snRNAseq preprocessing, clustering and annotation

From the single nuclei using 10X sequencing, we generated raw reads that were further processed by Cellranger version 6.1.2, using GRCh38-2020-A as the human reference transcriptome. This gave us a count matrix that was converted to a fast q file by the default mkfastq function, followed by alignment using the count function, cell calling, and quantification of UMI counts. Only cells that expressed between 1000 and 20000 genes were retained using the filter_cells function. Furthermore, cells with more than 10% of the reads mapped to the mitochondrial genes were removed. To exclude potential doublets, the Scrublet tool was used, with a cutoff of 0.2. For subsequent analysis, we used Seurat (v. 4.3.0). The expression data were log-normalized with a scaling factor of 10000. We applied the Seurat V3 method to select the top 2000 highly variable genes that were subsequently used to calculate the principal components of the data. The harmony package was utilized as a batch-effect correction tool and set on sample IDs as the batch variable. UMAP was applied with Seurat's RunUMAP function using the first 30 principal components generated from the harmony corrected PCA embedding. The cells were clustered using the Louvain algorithm at resolutions ranging from 0.3 1.1. To determine the final resolution, we manually inspected the marker genes and cluster composition. The dataset used for further downstream applications consisted of 20995 cells from 7 human CCA samples. Clusters were annotated using curated marker genes from literature.

Differential expression analyses

We used the MAST algorithm to identify differentially expressed genes in all cell types based on the outcome groups. First, genes with low expression and normalized expression below zero were removed. The hurdle model was set with the good outcome group as the reference condition and the poor outcome as our condition. We extracted the differentially expressed genes, with the logarithm of fold change (logFC) value and adjusted p values from the summary statistics of the output. The Benjamini-Hochberg FDR correction method was used to identify statistically significant DE genes. Gene set enrichment analysis (GSEA) of differentially expressed genes was performed using the clusterProfiler package (v. 3.17) with a minimal gene set size of 3, a maximal gene set size of 500, and a p-value cutoff of 0.05 using Gene Ontology annotations.

Cell-cell communication analyses

To predict ligand receptor (LR) interactions between the previously identified clusters, we used the CrossTalkeR package (v. 1.3.6). The *liana_wrap* function of liana (v. 0.1.12) was used to generate LR prediction. As an input, CrossTalkeR uses cellphoneDB predictions as a database and generates a comparative cell-cell interaction (CCI) network for the conditions. After our unbiased analysis of LR all pairs, we focused on pairs that were among the top 10 pairs with the highest LR scores within the identified cell clusters in the CCI network using the outcome groups as conditions. We also used the CellCall package (v.1.0.7) to identify cell to cell communication trends along different clusters, and internal regulatory signals. The *TransCommuProfile* function in the CellCall package determines the LR score between cell

types. We used default parameters (0.05) for pvalue, Cor Spearson correlation significance and p.adjust value for regulon's GSEA. The cellcall tool uses LR information from the KEGG database to infer the communication and transcription factor activity along with analyzing crucial pathway activity involved in communication between certain cellular connections.

Analysis of public expression data

Transcriptome array data were retrieved from the Gene Expression Omnibus (GEO) dataset GSE132305 for 182 patients with eCCA, in this cohort 130 cases of pCCA were included and processed according to the authors' original methodology(39). For cellular deconvolution, expression data were exported and analyzed using the CIBERSTORx tool(40). For the signature matrix file, we used the LM22 file containing expression data from 22 immune cell types, as described in the original article. For correlation analysis, we imported the results from CIBERSORTx and combined them with the expression data for the genes of interest. Subsequently, the Pearson correlation between the estimated abundance and gene expression for all 182 patients was calculated.

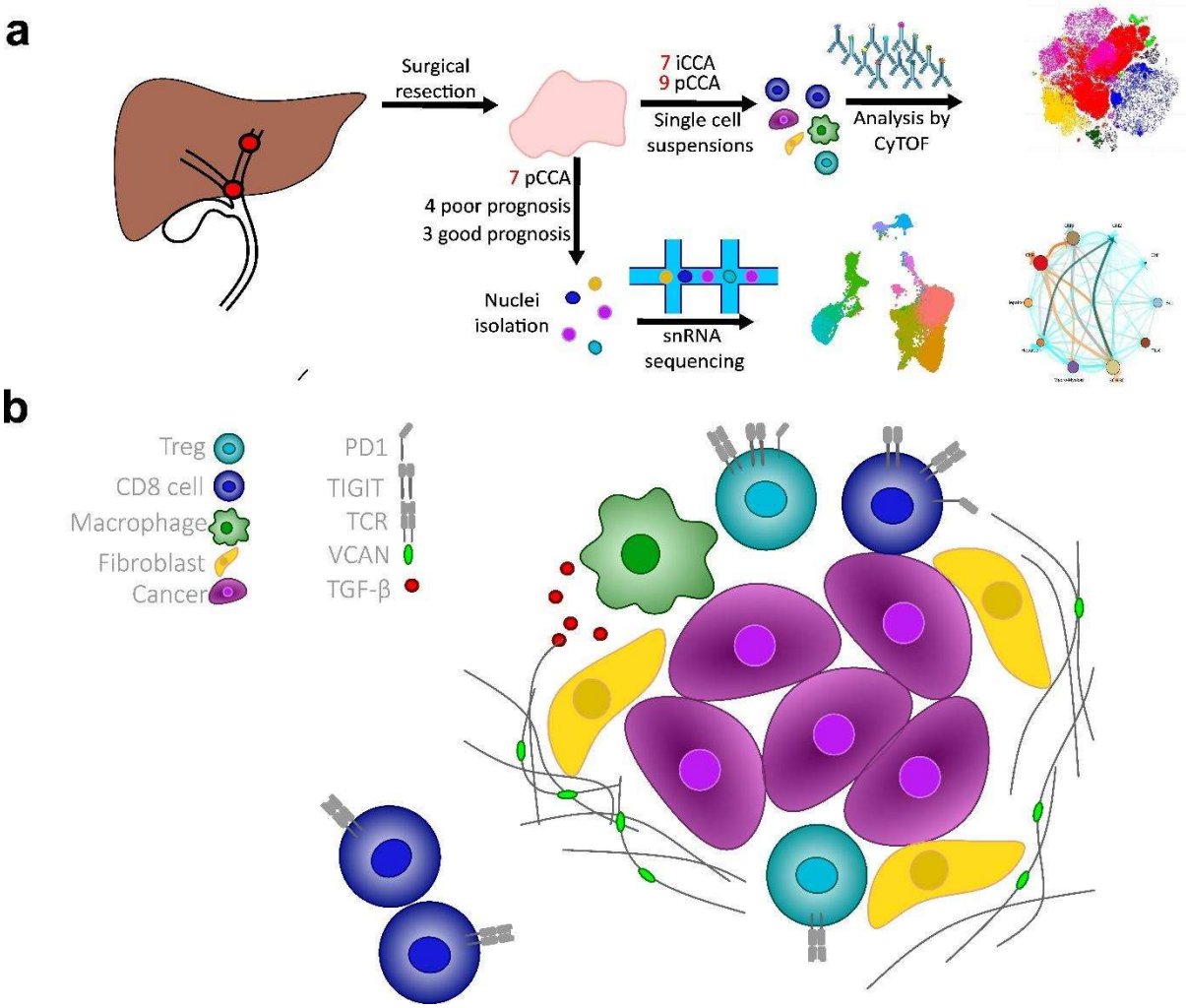

Figure 1 Study workflow

(a) In our study, we included patients with primary intrahepatic cholangiocarcinoma (iCCA) or perihilar cholangiocarcinoma (pCCA) scheduled for resection. For 7 iCCA and 9 pCCA patients we performed Cytometry by time of flight (CyTOF) on cell suspensions derived from tumor tissue and matched peri-tumoral tissue. The used panel was aimed to characterize T cell subpopulations and their functional state. We also select 7 pCCA cases for single nuclei sequencing (snRNA seq). These samples were selected based on clinical data and were grouped into poor prognosis (n = 4) and good prognosis (n = 3) groups. Here we examined cellular crosstalk by looking at ligand-receptor pairs and compared it between the groups. **(b)** Graphical summary of the results. Based on the CyTOF data we observed exclusion of CD8 effector cells and an infiltration of regulatory T cells (Tregs). Tumor infiltrating T cells expressed high levels of the checkpoint molecules PD-1 and TIGIT. Analysis of the Crosstalk using the snRNA data revealed TGF-β secretion by macrophages in poor prognosis patients and high levels of expression of the extracellular matrix protein versican (VCAN).

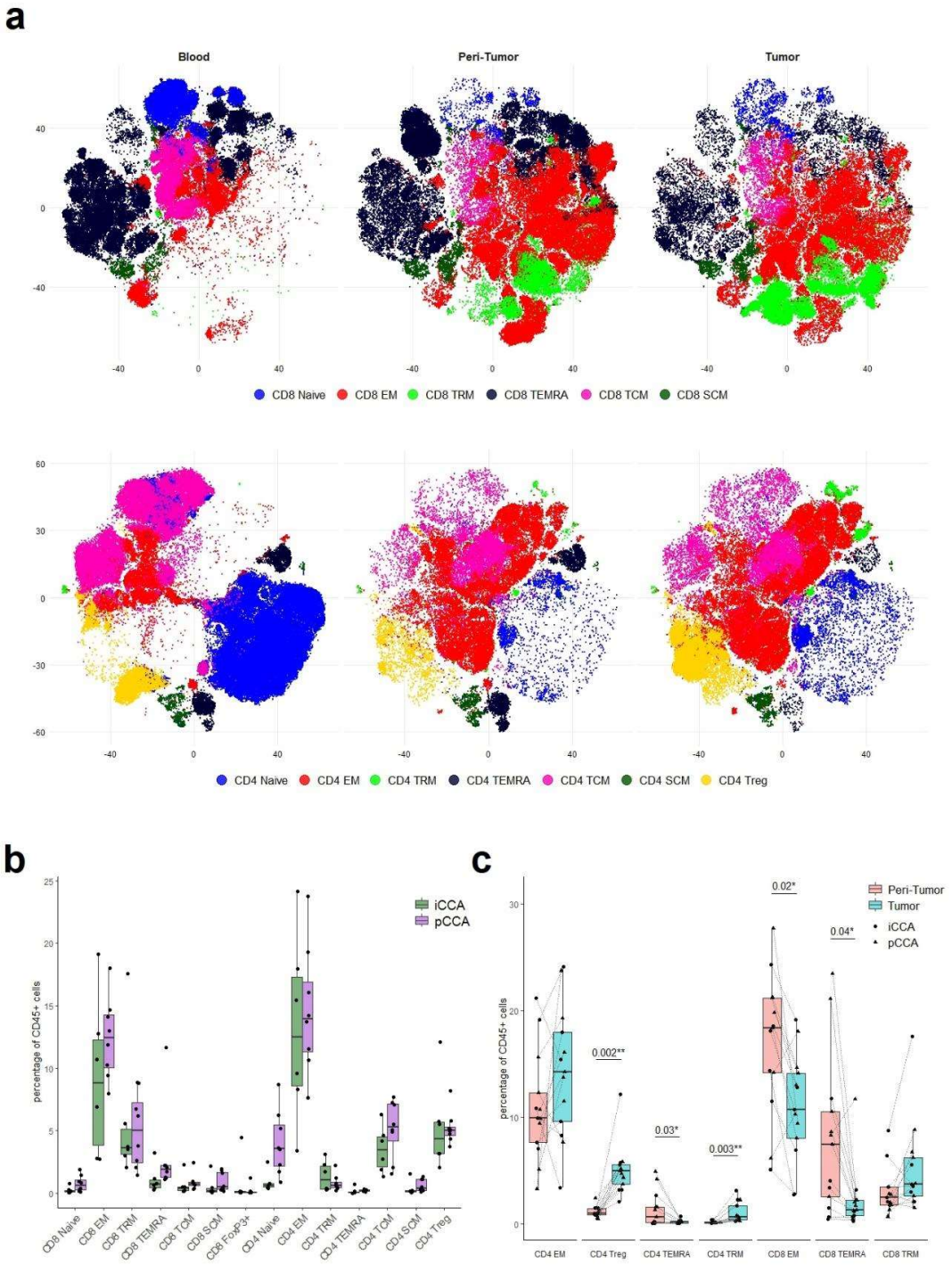

Figure 2. High-dimensional phenotyping in iCCA. (a) Dimensionality reduction plots (optSNE) of CD4 and CD8 T cells from all patients. Annotation was done by manual annotation of clusters after unsupervised clustering using phenograph. **(b)** Mean percentage of total living CD45+ cell types in the tumor sample for each patient split by CCA subtype. **(c)** Mean percentage of total living CD45+ cell types in matched normal and tumor samples. Shown p values were obtained using Wilcoxon test and were adjusted for multiple testing.

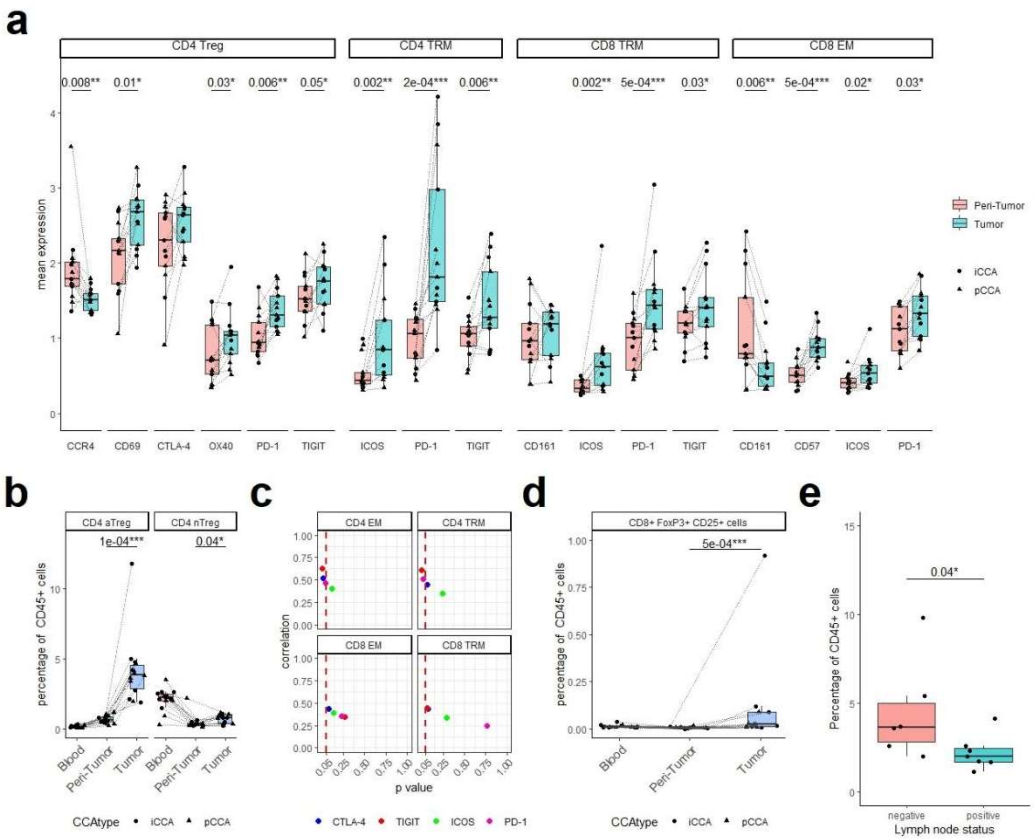

Figure 3. Characterization of marker expression in cell clusters (a) Mean normalized expression in of markers in samples across CD4 Treg, CD8 TRM and CD 8 EM cells. **(b)** Mean percentage of total CD45 positive live cells across tissues and patients. aTreg = activate Treg nTreg = non-activated Treg **(c)** Correlation between mean checkpoint expression in Memory subtypes and mean CD69 expression by total Treg population in the tumor using spearman. Associated p values were calculated using the algorithm AS 89. **(d)** CD25 and FoxP3 positive CD8 cells were identified by sequential gating. Shown is the mean percentage of total CD45 positive live cells per sample. **(e)** Mean percentage of cluster 7 of the CD4 EM cells in peripheral tissue for patients with and with and without cancer cell invasion of adjacent lymph nodes.

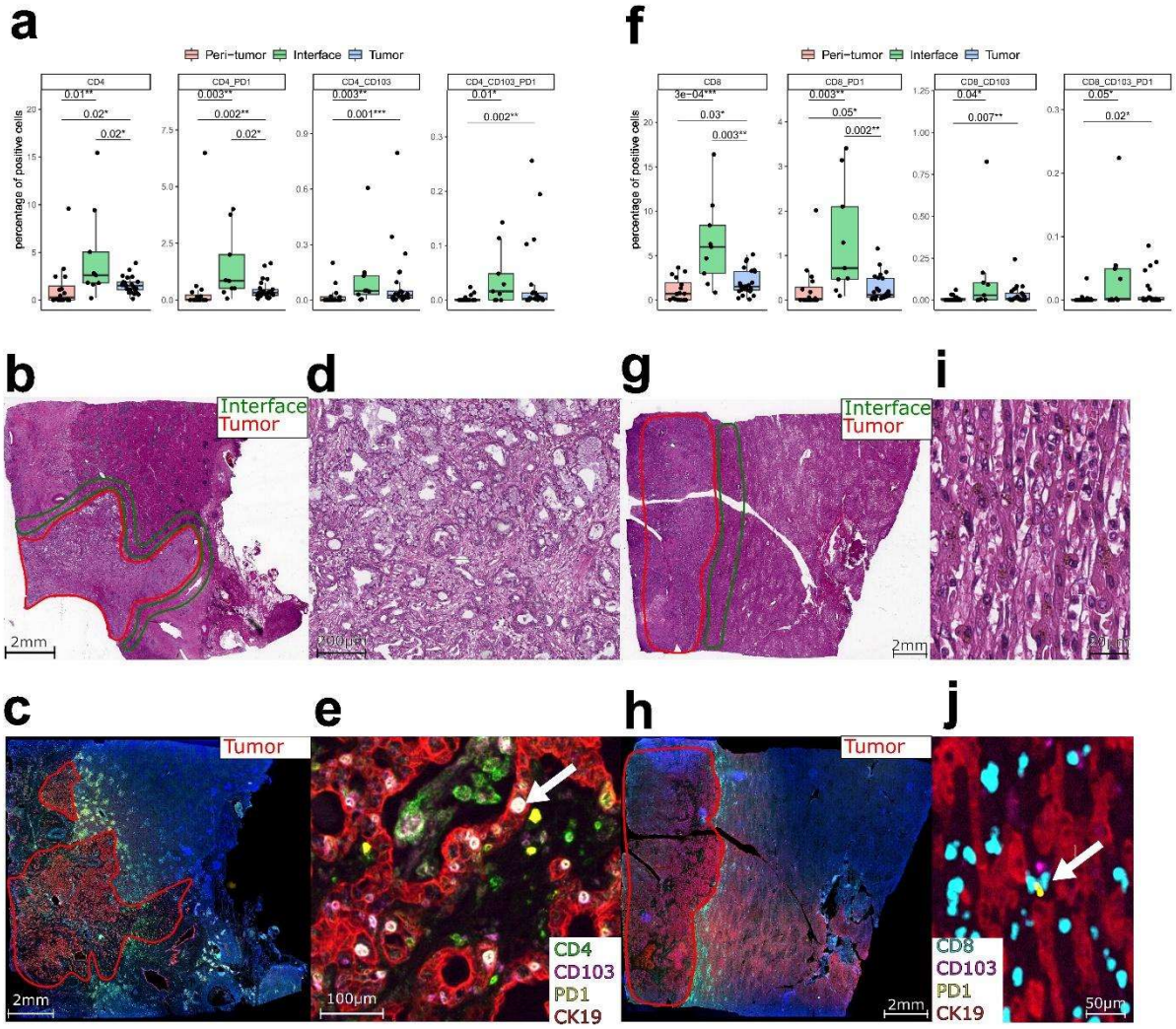

Figure 4. Validation by multiplexed immunohistochemistry. (a) Percentage of cells positive for CD4, CD103 and PD-1 in the different regions of interest (ROIs). PD-1/ CD103 positive cells were mainly found in the interface and tumor area, while positive cells for all markers were rare in the periphery **(b)** Overview of the HE-stained slide of patient 323. **(c)** The corresponding multiplexed image. **(d)** Magnified image of the HE-stained slide of the tumor area of patient 323. **(e)** The corresponding magnified multiplexed image. The highlighted cells with a box are CD4 effector memory cells with expression of PD-1. **(f)** Percentage of cells positive for CD8, CD103 and PD-1 in the different ROIs. Cells positive for the different markers were more abundant in the tumor and interface ROIs. **(g)** Overview of the HE-stained slide of patient 302. **(h)** The corresponding multiplexed image. **(i)** Magnified image of the HE-stained slide of the interface area of patient 302. **(j)** The corresponding magnified multiplexed image. The highlighted cells with a box are CD8 effector memory cells with expression of PD-1.

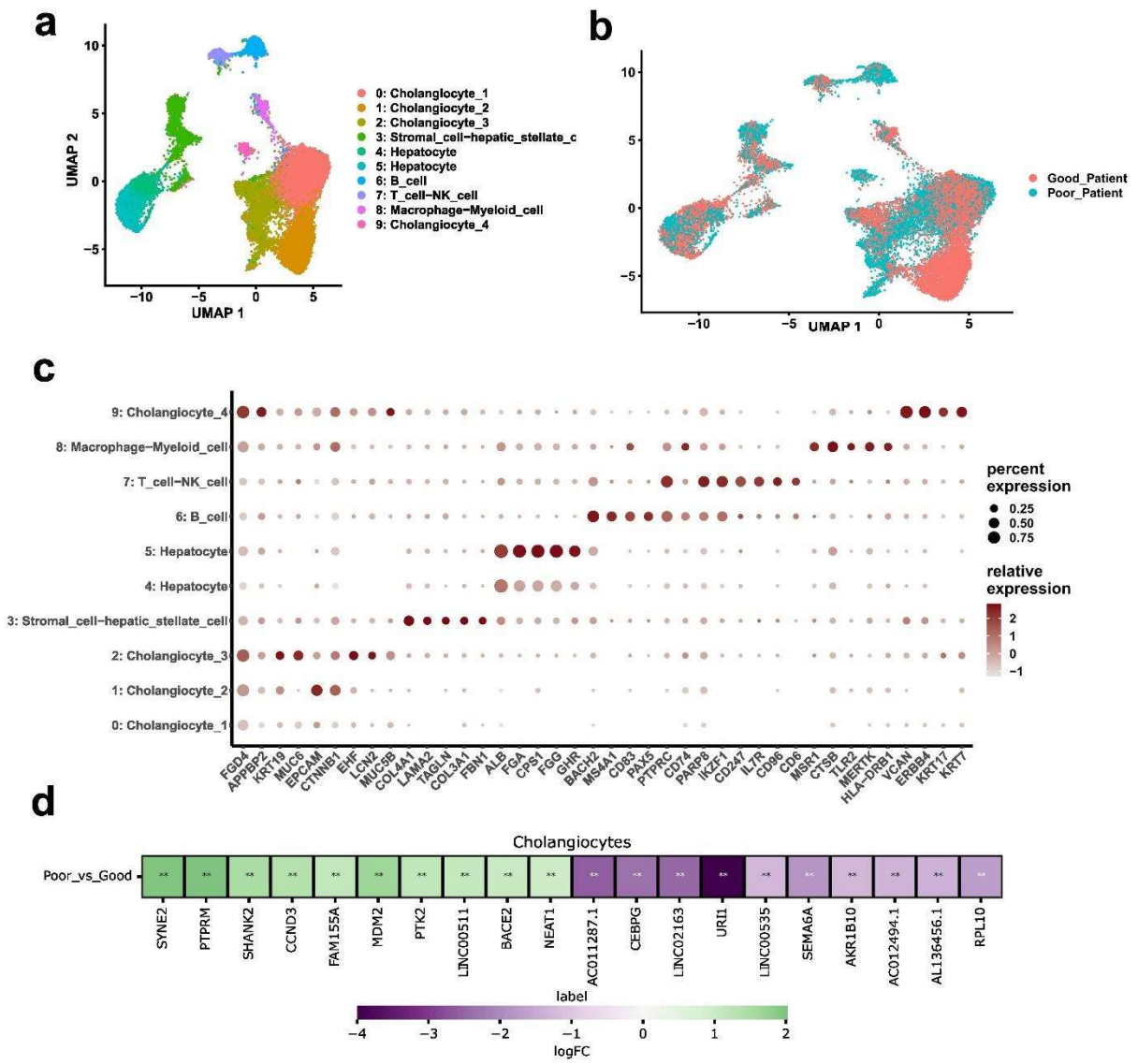

Figure 5 SnRNA reveals heterogeneity in cell populations between clinical groups,

Single-nuclei RNA sequencing (snRNA seq) was performed on seven tumor sections. **(a)** UMAP projection of the 10 identified distinct cell clusters. **(b)** Distribution of cells from outcome groups of patients based on clinical data. **(c)** Marker gene expression was used for annotation across the identified clusters. **(d)** Top differentially expressed genes in good versus poor patients for all identified combined cholangiocyte clusters. Shown at the log Fold changes and significance levels.

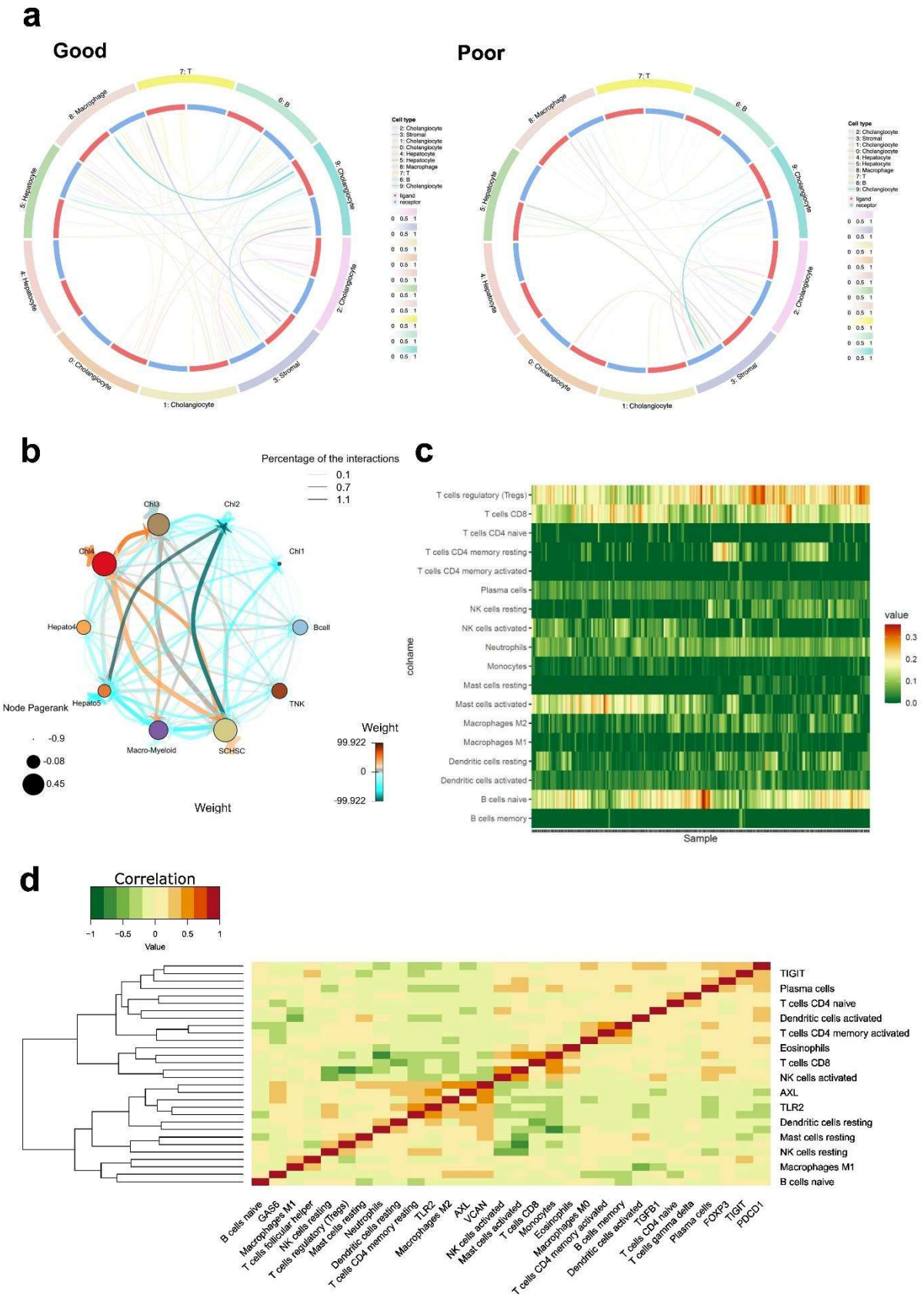

Figure 6 Ligand receptor networks show shift cellular crosstalk between outcome groups,

Cell communication between clusters was inferred based on expression of known Ligand receptor pairs. **(a)** Circus plot showing cell-cell communication between clusters in patients with good (left) and poor (right) prognoses. Thickness indicates the number of interactions between clusters, and **(b)** cellular crosstalk between clusters was inferred from ligand and receptor expression using the CrosstalkeR tool. Differential cell-cell interaction (CCI) plot between the good and poor outcome groups Higher weights indicate interactions with higher weights in the poor outcome group, while negative weights indicate higher weights in the good outcome group (TNK = T cells/NK cells, Macro-Myeloid = Macrophages/myeloid cells, SCHSC = stromal and hepatic stellate cells, Chl = Cholangiocytes, Hepato = Hepatocytes). **(c)** Cellular deconvolution of publicly available microarray data from 182 eCCA samples using CIBERSORTx. Shown are cell enrichment scores per sample. **(d)** Correlation of gene expression related to VCAN, TGF-β, AXL signaling, and genes coding for checkpoints identified in the CyTOF cohort and predicted cell type abundance. The features were sorted by hierarchical clustering, as shown in the dendrogram.

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