Quantitative, qualitative and spatial analysis of lymphocyte infiltration in periampullary and pancreatic adenocarcinoma

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Immunotherapeutic modalities are currently revolutionizing cancer treatment. In pancreatic cancer, however, early clinical trials have been disappointing. The optimization of immunotherapeutic strategies requires better understanding of the inflammatory tumor microenvironment. Therefore, the aim of our study was to perform a detailed in situ description of lymphocyte infiltration patterns in resected pancreatic and other periampullary cancers. Multiplexed immunofluorescence imaging was applied to tissue microarrays with tumors from a cohort of 175 patients with resected periampullary adenocarcinoma. A panel of immune cell markers including CD4, CD8α, FoxP3, CD20, CD45RO and pan-cytokeratin was applied to allow for simultaneous spatial analysis of multiple lymphocyte populations. The majority of lymphocyte populations were significantly more abundant in intestinal (I-type) compared to pancreatobiliary (PB-type) tumors. Hierarchical cluster analysis revealed several immune cell signatures of potential clinical relevance. Notably, in the stromal compartment of PB-type tumors, high infiltration of B cells, CD8α+CD45RO+ and single-positive CD4+ T cells, but low levels of FoxP3+CD45RO high and single-positive CD8α+ T cells were associated with improved overall survival (OS). The study also defined prognostic relevant topographical patterns of lymphocytic infiltration, in particular proximity of CD8α+ cells to cancer cells. Moreover, the presence of lymphocytes with potential T-helper capacities (CD4+) in the nearest vicinity to CD8α+ cells was associated with a prolonged OS. Our data demonstrate that the composition and clinical impact of immune infiltrates in periampullary adenocarcinoma differ by morphological type as well as localization. Furthermore, spatial in situ analysis identified potential immunological mechanisms of prognostic significance.

Introduction

Periampullary adenocarcinoma, including pancreatic cancer, is a collective term for cancers arising around the ampulla of Vater. The periampullary region is a complex region which is composed of distinct anatomical structures: the head of the pancreas, distal common bile duct, second portion of the duodenum and ampulla of Vater. Periampullary adenocarcinomas are currently classified by their anatomic location of origin according to the seventh edition of the American Joint Committee on Cancer staging system. They can be divided into two subgroups based on morphology, either intestinal type (I-type) or pancreatobiliary type (PB-type), and there is increasing evidence that morphology is a more important determinant of survival than anatomical origin. Survival rates remain dismally low for this group of patients, with 5-year overall survival (OS) rates of around 6%, partly due to late presentation of the disease, but also because both conventional and targeted therapies have shown limited efficacy. Currently, the only curative treatment of pancreatic and periampullary adenocarcinomas is surgery, however, only 15–20% of the patients are eligible for surgery at the time of...
Aims and methods

The aim of our study was therefore to map the spatial distribution and perform a comprehensive phenotyping of important lymphocyte populations in the tumor microenvironment of pancreatic and periampullary adenocarcinomas, with particular reference to tumor morphology and patient outcome.

Methods

Study cohort

The study cohort consists of a retrospectively collected consecutive series with primary tumors from 175 incident patients who underwent pancreaticoduodenectomy for periampullary adenocarcinoma in the University hospitals of Lund and Malmö from January 1, 2001 to December 31, 2011. Approval for the study was obtained from the Ethics committee of Lund University (reference number 445/07), whereby the committee waived no need for consent other than the option to opt-out. Follow-up started at the date of surgery and ended at death or on March 31, 2017, whichever came first. The Swedish National Civil Register was used to obtain information on vital status. Data on adjuvant treatment were obtained from patient charts. All cases underwent strict histopathological re-evaluation by a board-certified pathologist (JE). Sixty-five tumors were classified as intestinal type (I-type), and 110 as pancreatobiliary-type (PB-type), whereof the anatomical origin of the tumors was 14 duodenal, 70 ampullary, 45 distal bile duct and 46 pancreatic. In 63 cases, paired-samples from lymph node metastases were available, 21 from I-type tumors and 42 from PB-type tumors. In 34 cases, paired-samples from benign tissue was available, 9 from I-type tumors and 25 from PB-type tumors. Mismatch repair status was assessed by immunohistochemistry as previously described.

Tissue microarray construction

Tissue microarrays were constructed as previously described, using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminster, MD). Three 1 mm cores were sampled from viable, non-necrotic areas of the primary tumors. Four-micrometer TMA-sections were then used in the subsequent immunofluorescence staining.

Multiplex immunohistochemical staining

Multiplexed immunofluorescence staining was performed using the Opal Multiplex IHC Kit (PerkinElmer, Waltham, MA). A panel of immune markers was developed with antibodies against CD4 (Mouse/4B12, DAKO/M7310, 1:200), CD8a (Mouse/144B, Thermo Fisher Scientific/MA5-13473, 1:500), FoxP3 (Rabbit/D6O8R, Cell Signaling Technology/12653s, 1:100), CD20 (Mouse/
L26, DAKO/GA604, 1:3,000) and CD45RO (Mouse/UCHL1, Thermo Fisher Scientific/MA1-19452, 1:200). For pan-cytokeratin staining, a combination of several antibodies was used: anti-E-cadherin (Mouse/Clone 36, BD Biosciences/610182, 1:5,000), anti-pan Cytokeratin (Mouse/[C-11], Abcam, San Francisco/ab7753, 1:1,000) and anti-pan Cytokeratin Clone, 1:2,000 (Mouse/AE1/AE3, Thermo Fisher Scientific/MA5-13156, 1:500). To visualize cell nuclei, the tissue was stained with 4′,6-diamidino-2-phenylindole (Spectral DAPI, PerkinElmer). The staining procedure was largely performed as previously described.29

**Imaging**

Imaging of the TMAs was performed with the Vectra Polaris system (PerkinElmer). First, a whole slide scan at 10× magnification was acquired for TMA core annotation and selection of regions on which to apply multispectral imaging. Multispectral imaging was then performed at a resolution of 2 pixels per 1 μm. Spectral unmixing was carried out using inForm software (PerkinElmer).

**Image analysis and thresholding**

First, image analysis was performed with the inForm Analysis software (PerkinElmer), wherein each scanned image was visually assessed by a board-certified pathologist (PM) to exclude large areas of nontumor tissue, cores without tumor tissue, necrosis or artifacts from the analytical process. After this, image analysis was performed in two steps; a training session and an image analysis session. In the training session, a set number of cores were categorized into three types of tissue compartment: tumor compartment (or epithelial compartment in nonmalignant samples), stromal compartment, or blank areas. A machine-learning algorithm then carried out tissue segmentation (Supporting Information Fig. S1).

Cell segmentation was carried out as described before.29 In short, DAPI staining was used to segment cells. The perinuclear area was defined as a 3-μm (6 pixels) area surrounding nuclei and was therefore considered to be cell cytoplasm. The total cell area, including nuclear and cytoplasmic areas, was evaluated for cytoplasmic/membrane marker expression. FoxP3 expression was evaluated in nuclear (DAPI positive) areas only. Additionally, coordinates on the y and x-axis were retrieved for each individual segmented cell. Lymphocyte infiltration was normalized against the area of viable tissue in each core.

The inForm built-in function for cell phenotyping was used to define the intensity threshold for the positivity of each marker individually, using a random selection of TMA cores. The defined thresholds were then applied for the raw output data of the complete cohort. Thus, each cell was characterized by positivity–negativity of each marker. The co-expression data was then used to identify immune cell subtypes.

Immune cell infiltration was evaluated as the number of cells per region, in the stromal compartment, tumor compartment or total viable tissue area of the TMA cores, respectively.

Cell spatial localization was assessed in two ways: to evaluate the relation of immune cells to tumor tissue, the distance of each cancer cell (defined as cytokeratin single-positive cells) to its nearest neighboring immune cell (of each subclass separately) was measured. A median distance value was computed for each case. The potential interaction zone between lymphocytes and/or cancer cells used in the interaction analyses was set to a radius of 15 μm.

**Next-generation DNA sequencing**

Next-generation DNA sequencing was performed as previously described.3 In brief, tissue cores were taken from tumor enriched areas. DNA was extracted using the Qiagen Gen-eRead (Qiagen, Hilden, Germany) kit for formalin-fixed paraffin-embedded tissue according to the instructions from the manufacturer. In total, 102 (58.3%) cases had a sufficient number of tumor cells for analysis. A panel of 70 cancer-associated genes was characterized using Illumina TruSeq custom amplicon assay (Illumina, San Diego, CA) with a MiSeq instrument according to the instructions from the manufacturer. Variant calling and annotation, quality filtering and alignment were performed using the supplier’s analysis pipeline. To exclude single-nucleotide polymorphisms commonly reported in various populations, detected mutations were screened against the COSMIC and ExAC databases.

**Data processing and statistical analyses**

For comparison of two groups of related samples, Wilcoxon signed-rank test with Pratt modification was used. Kruskal–Wallis test was used for comparison of multiple groups. Unsupervised hierarchal clustering was performed on normalized data to identify immune cell signatures. Kaplan–Meier analysis and log-rank test were applied to illustrate any difference in 5-year OS and Cox regression proportional hazard models were used to estimate hazard ratios (HR) for death within 5 years in both univariable and multivariable analyses. Multivariable Cox regression included adjustment for age (continuous), T-stage (T1–T2 vs. T3–T4), N-stage (negative vs. positive nodal status), grade (well-moderate vs. poor), adjuvant chemotherapy (none vs. any), invasion into vascular and lymphatic structures and perineural growth. Morphology (I-type vs. PB-type) was included in the multivariable model in analyses of the entire cohort.

In the entire cohort, three cases were excluded in the survival analyses; one patient with a PB-type tumor who was lost to follow-up due to emigration, and two patients with I-type tumors who died of complications from the initial surgical treatment within the first month.

All statistical calculations were performed with SPSS version 24.0 (SPSS Inc, Chicago, IL) or with R software, version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria) and integrated development environment RStudio, version 1.0.143 (RStudio Team, Boston, MA). All statistical tests were two-sided and p values <0.05 were considered significant.
Data availability
The data that support the findings of our study are available from the corresponding author upon reasonable request.

Results
Expression patterns of immune markers
Immune cell infiltration was possible to determine in 161 (92%) of the cases, whereof 63 were I-type and 98 were PB-type tumors.

A representative example of a multiplex immunofluorescent image is shown in Figure 1a. Individual marker expression levels in each cell were used to identify TIL subtypes: CD4+ single-positive cells (negative for all markers except CD4), CD4+CD45RO+ cells (referred to as activated CD4+ cells; memory CD4+ T cells may be included in this population), CD4+FoxP3+ (referred to as CD4+ Treg cells), single-positive CD8+ cells (negative for all markers except CD8α), CD8α+CD45RO+ (referred to as activated

Figure 1. Heterogeneity of the infiltration of lymphocyte subpopulations in periampullary adenocarcinoma. (a) Upper part: Representative image of a TMA core with heterogeneous infiltration of lymphocytes. Lower part: summarized scheme of the five immune marker combinations, used to define subpopulations of lymphocytes. (b) Immune cell densities in primary tumor and adjacent nonmalignant tissue. (c) Immune cell densities in tumor versus stromal compartment in primary tumor tissue. [Color figure can be viewed at wileyonlinelibrary.com]
cyte in these data indicate a link between tumor histology and lympho-
I-type tumors (Fig. 2). First, we compared lymphocyte infiltration in cancer tissue and adjacent nonmalignant tissue in a pairwise manner. All of the lymphocyte subclases, except single-positive CD4 cells and B cells, were more abundant in malignant tissue (Fig. 1b).

Next, we performed a pairwise comparison of lymphocyte infiltration within the tumor and stromal compartments in samples with cancer tissue (Supporting Information Fig. S1). As illustrated in Figure 1c, densities of B cells and all CD8α+ lymphocyte subclases were significantly higher in the stromal compared to the tumor compartment. CD4+ cells exhibited a different infiltration pattern in that CD4+CD45RO+ T cells were more abundant in the stromal compartment, whereas single-positive CD4 cells and CD4+ Tregs were enriched in the tumor compartment.

The heterogeneity of the immune landscape in tumors and nonmalignant tissue

The relationship between different immune cell populations and pathological characteristics.

In the tumor compartment, we observed nuclear expression of FoxP3 in a fraction of tumor cells. This feature has been reported before, and was not considered in the analyses. Interestingly, in the stromal compartment we also observed FoxP3+ cells that were negative for CD4 or CD8α. These cells were either CD45ROhigh or CD45ROlow and may therefore at least in part be γδ T cells or NKT cells that lack CD4 and CD8α expression (Supporting Information Fig. S2). Since FoxP3+CD45ROlow cells were difficult to distinguish from FoxP3+ cancer cells in the tumor compartment, the analysis of FoxP3+CD45ROlow lymphocytes was restricted to the stromal compartment.

Associations of lymphocyte infiltration with tumor morphological type, anatomical origin and clinicopathological characteristics

The relative proportion of TIL subclasses, stratified by tumor morphology, is shown in Figure 2a. The most abundant cell subclasses in the total tissue area, and the tumor compartment, were single-positive CD4+ and single-positive CD8+ cells. The most abundant TIL subpopulations in the stromal compartment were single-positive CD8+ cells and FoxP3+CD45ROlow cells. No significant differences were observed between I-type and PB-type tumors with regard to total lymphocyte count. However, when the tumor compartment was analyzed separately, levels of CD4+CD45RO+ T cells (p = 0.027) were significantly higher in PB-type tumors, while levels of CD8+CD45RO+ T cells (p < 0.001), CD8+ Tregs (p = 0.001), FoxP3+CD45RO+ cells (p = 0.004) and B cells (p = 0.011) were significantly higher in I-type tumors (Fig. 2a). In the stromal compartment, levels of FoxP3+CD45RO+ cells (p = 0.004) and FoxP3+CD45RO+ cells (p = 0.026) were significantly higher in I-type tumors (Fig. 2a).

These data indicate a link between tumor histology and lymphocyte infiltration patterns. This was further investigated by analyzing lymphocyte densities in strata according to anatomical localization (Fig. 2b). Notably, tumors with pancreatic origin displayed lower densities of all analyzed lymphocyte subclasses, including those with potential immune suppressive activity, in both the tumor and stromal compartments, while duodenal cancers had the all-over highest lymphocyte infiltration (Fig. 2b).

Finally, we compared lymphocyte infiltration with clinicopathological characteristics. Lower densities of most analyzed lymphocyte subclasses were observed in tumors with positive resection margins, presence of perineural growth, invasion in lymphatic and blood vessels and growth in peripancreatic fat (Fig. 2c). Altogether, these data suggest that lower levels of infiltrating lymphocytes are associated with adverse clinicopathological characteristics.

Lymphocyte infiltration in relation to the mutational landscape and mismatch repair status

The relationship between different immune cell populations and the most common mutations (≥10% prevalence) are shown in Supporting Information Figure S3. Notably, the infiltration of several CD8+ cell populations, both in the tumor and stromal compartments, was significantly lower in KRAS mutated compared to KRAS wild-type (wt) tumors. The infiltration of different immune cells subsets did also differ significantly according to mutational status of SMAD4, CDK2NA, APC, but not of TP53, RNF43, SMARCA4, ERBB3 or NF1.

As shown in Supporting Information Figure S4, the infiltration of several immune cell populations was significantly higher in I-type tumors with deficient mismatch repair (dMMR) status. In PB-type tumors there was no association between dMMR status and lymphocyte infiltration, however, the infiltration of FoxP3+CD45ROlow lymphocytes was higher in tumors with proficient mismatch repair (pMMR).

Associations of lymphocyte infiltration with survival

For survival analysis, tumors were dichotomized into two groups with high or low lymphocyte infiltration using the median values as cut-off.

As shown in Supporting Information Figure S5, the total density of all lymphocyte subpopulations, except for single-positive CD8 cells, CD8+ Tregs and FoxP3+CD45RO+ cells, inferred a prognostic value, positive or negative, in univariable Cox regression analysis of the entire cohort. In multivariable analysis, single-positive CD4 cells (HR = 0.53, 95% CI 0.36–0.79), CD8+CD45RO+ T cells (HR = 0.58, 95% CI 0.40–0.87) and B cells (HR = 0.54, 95% CI 0.37–0.80) remained significantly associated with a prolonged OS. In I-type tumors, none of the lymphocyte subpopulations conferred an independent prognostic value, whereas, in PB-type tumors, a high density of CD8+CD45RO+ T cells was an independent predictor of a prolonged OS (HR = 0.54, 95% CI 0.34–0.88).

As further shown in Supporting Information Figure S5, survival analyses restricted to the tumor compartment revealed that only single-positive CD4 cells were significantly
Heterogeneity of lymphocyte infiltration patterns associated with tumor morphological type, anatomical origin and clinicopathological characteristics. (a) Radar plots of relative densities of immune cell subclasses in PB-type tumors (purple) and in I-type tumors (blue), shown separately for total tissue, tumor compartment and stromal compartment. (b) Heat map of the densities of immune cell subclasses in tumor and stromal compartments according to the anatomical origin. (c) Heat maps illustrating the associations between the densities of immune cell subclasses and clinicopathological characteristics. [Color figure can be viewed at wileyonlinelibrary.com]

associated with OS in multivariable analysis (HR = 0.61, 95% CI 0.41–0.91) in the entire cohort. Survival analyses restricted to the stromal compartment revealed that single-positive CD4 cells and B cells were independent predictors of a prolonged OS (HR = 0.62, 95% CI 0.42–0.92 and HR = 0.57, 95% CI 0.39–0.84) in the entire cohort. No significant associations were found between single lymphocyte populations and survival when stratifying for morphology.

Tumor to stroma ratios of infiltrating lymphocytes and their associations with survival
In the next set of analyses, we aimed to distinguish “inflamed” and “immune excluded” tumors. As illustrated in Figure 3a, tumors with an active anticancer immune response (inflamed) are expected to have an enrichment of lymphocyte infiltration in the tumor nest, while “immune excluded” tumors are characterized by an accentuation of lymphocyte infiltration in the
stromal compartment. A “Tumor to Stroma Ratio” metric was generated by dividing the density of lymphocyte infiltration in the tumor compartment with the density of lymphocyte infiltration in the stromal compartment. The tumors were subsequently dichotomized into two groups with high or low Tumor to Stroma ratio using the median values as cut-off. As shown in Figure 3b, high Tumor to Stroma Ratios of CD4⁺CD45RO⁺ T cells, single-positive CD8 cells and CD8⁺CD45RO⁺ T cells were associated with a prolonged OS. These associations were confirmed in univariable analysis (HR = 0.48, 95% CI 0.25–0.90, HR = 0.62, 95% CI 0.42–0.92 and HR = 0.44, 95% CI 0.25–0.76, respectively). However, in multivariable analysis, only a high Tumor to Stroma Ratio of single-positive CD8 cells remained significantly associated with a prolonged OS (HR = 0.64, 95% CI 0.25–0.99). No significant associations with survival were found in univariable analysis when stratifying for morphology. In PB-type tumors, however, the Tumor to Stroma Ratio of single-positive CD8 cells was significantly associated with a prolonged OS in multivariable analysis (HR = 0.50, 95% CI 0.29–0.86).

Lymphocyte infiltration patterns and their associations with survival

When cases were clustered according to total count of TIL density, seven distinct immune cell signatures were defined (Supporting Information Fig. S6). Kaplan–Meier analysis revealed that immune signature 1, characterized by high levels of CD4⁺CD45RO⁺ T cells, CD8⁺CD45RO⁺ T cells, FoxP3⁺CD45ROhigh cells, but low levels of single-positive CD4 and CD8 cells, was significantly associated with a prolonged OS compared to immune signatures 3 and 6 (p = 0.027 and p = 0.022, respectively; Supporting Information Fig. S7). These associations were confirmed in unadjusted Cox regression analysis (HR = 3.18, 95% CI 1.00–9.17 and HR = 3.32, 95% CI 1.14–9.66, respectively), but did not remain significant in adjusted analysis. When stratified for morphology, no immune cell signatures were significantly associated with OS.

Six distinct tumor nest (TN) immune cell signatures were defined. Kaplan–Meier analysis revealed that in the entire cohort, TN immune signature 3, characterized by high levels of FoxP3⁺CD45ROhigh cells, and CD8⁺CD45RO⁺ T cells (Fig. 4a), was significantly associated with a prolonged OS compared to TN
immune signature 2 ($p = 0.003$; Supporting Information Fig. S7). In unadjusted Cox regression analysis using TN immune signature 3 as reference, TN immune signature 2 (HR = 3.65, 95% CI 1.45–8.61), TN immune signature 5 (HR = 2.47, 95% CI 1.08–5.62) and TN immune signature 6 (HR = 2.64, 95% CI 1.16–6.01) were significantly associated with a reduced

![Diagram of immune cell signatures](image)

**Figure 4.** Identification of immune cell signatures by hierarchical clustering. Heatmaps illustrating hierarchical clustering of cases by normalized densities of lymphocyte infiltration rates in the (a) tumor compartment and (b) stromal compartment. [Color figure can be viewed at wileyonlinelibrary.com]
OS. However, none of these associations remained significant in adjusted analysis. When stratifying for tumor morphology, TN immune cell signature 3 remained significantly associated with a prolonged OS in I-type tumors, however only in relation to TN immune signature 5 (p = 0.045). This association was not confirmed in unadjusted analysis. There were no significant associations between any TN immune cell signatures and OS in PB-type tumors (Supporting Information Fig. S7).

Seven distinct stromal immune cell signatures were defined (Fig. 4b). Kaplan–Meier analysis revealed that in the entire cohort, stromal immune signature 5, characterized by high levels of CD4<sup>+</sup>CD45RO<sup>+</sup> T cells, CD8<sup>+</sup>CD45RO<sup>+</sup> T cells, B cells, FoxP3<sup>+</sup>CD45RO<sup>+</sup> cells and low levels of FoxP3<sup>+</sup>CD45RO<sup>low</sup> cells, was significantly associated with a prolonged OS compared to stromal immune signatures 3, 4 and 6 (p = 0.010, p = 0.011 and p < 0.001, respectively; Supporting Information Fig. S7). In unadjusted Cox regression analysis, using stromal immune signature 5 as a reference, stromal immune signatures 3, 4 and 6 were associated with a reduced OS (HR = 2.38, 95% CI 1.24–4.57; HR = 2.57, 95% CI 1.26–5.23; and HR = 3.09, 95% CI 1.70–5.61, respectively). These associations did not remain significant in adjusted analysis.

When stratifying for morphology, stromal immune signature 5 remained significantly associated with a prolonged OS in relation to
to stromal immune signatures 3 and 6 in I-type tumors ($p = 0.019$ and $p = 0.020$; Supporting Information Fig. S7). Using stromal immune signature 5 as a reference group, stromal immune signatures 3 and 6 were significantly associated with a reduced OS in unadjusted analysis (HR = 3.84, 95% CI 1.28–11.54 and HR = 3.63, 95% CI 1.11–11.82) but not in adjusted analysis. In PB-type tumors, stromal immune signature 1, characterized by high levels of CD8+CD45RO+ T cells, B cells and single-positive CD4 cells and low levels of FoxP3+CD45RO$^{\text{high}}$ cells and single-positive CD8 cells, was significantly associated with a prolonged OS compared to stromal immune signatures 4 and 6 ($p = 0.028$ and $p = 0.014$; Supporting Information Fig. S7). Using stromal immune signature 1 as reference group, stromal immune signatures 4 and 6 were significantly associated with a shorter OS in unadjusted Cox regression analysis (HR = 6.37, 95% CI 1.35–30.14 and HR = 4.87, 95% CI 1.11–21.58). Both signatures remained significantly associated with shorter OS in multivariable analysis (HR = 7.78, 95% CI 1.50–40.26 and HR = 6.19, 95% CI 1.35–28.37, respectively).

**Spatial distribution of immune cells**

In order to investigate potential interactions between TILs and cancer cells, we analyzed the distances from each cancer cell to the nearest lymphocytes (Fig. 5a). The strongest prognostic impact was seen for single-positive CD4 cells and single-positive CD8 cells in proximity to cancer cells (Supporting Information Fig. S8). Similar associations were found in I-type tumors, whereas in PB-type tumors, only the distance to single positive CD8 cells remained prognostic.

To delineate potential interaction mechanisms, we sought to identify direct cell-to-cell interactions of single-positive CD8 cells with other lymphocyte populations. A potential interaction event was denoted when at least one cell of another subclass was found within a 15 μm radius of a reference cell (Fig. 5b). The presence of single-positive CD4 cells in the interaction zone of single-positive CD8 cells was significantly associated with a prolonged survival (Fig. 5c). Moreover, in concordance with the above-described findings regarding average distances, the presence of cytokeratin-positive cells in the interaction zone of single positive CD8 cells was significantly associated with a prolonged survival (Fig. 5c).

**Discussion**

The inflammatory tumor microenvironment is a highly complex, heterocellular system and the concerted interactions within this system determine the anticancer immunity and most likely also the effectiveness of immunotherapy. There is, however, a lack of knowledge regarding the biological basis of the inflammatory tumor microenvironment of periampullary adenocarcinoma and the interactions herein. Our study is, to the best of our knowledge, the first effort to comprehensively map and phenotype the lymphocyte infiltration in the full spectrum of periampullary adenocarcinomas using multiplex immunofluorescence.

The results demonstrate that the infiltration of lymphocytes is dependent upon morphological subtype, with I-type tumors generally harboring higher levels of several lymphocyte subpopulations compared to PB-type tumors. The most striking immune depletion was observed in tumors of pancreatic origin, in particular in the tumor compartment. Additionally, depletion of lymphocyte infiltration was associated with several adverse clinicopathological features such as growth into lymph and blood vessels, and higher T stage. This is in contrast to a previous study using a similar multispectral method, which failed to establish any associations between T-cell infiltration and clinicopathological features in pancreatic cancer. However, our study has a more detailed subclassification of lymphocytes and a different analytical approach, which may explain this disagreement.

In the present study, the infiltration of different immune cells was also evaluated separately in the stromal and tumor compartments, and the results demonstrate significant differences both in absolute values and in their prognostic implications. Such a diverse pattern of lymphocyte infiltration suggests that regulatory mechanisms may differ by tissue compartment and gives a rationale for separate analysis of immune infiltration in the tumor and stromal compartments.

When analyzing paired samples of nonmalignant tissue and primary tumors, we could demonstrate that lymphocyte infiltration was significantly higher in primary tumors, indicating an active immunological response against malignant cells. These results are in concordance with a previous report using a similar method that has demonstrated that CD8+ T-cell infiltration is lower in paired benign tissue compared to tumor tissue in pancreatic cancer. It must, however, be pointed out that FoxP3+ cell subsets (FoxP3+CD45RO$^{\text{high}}$ and FoxP3+CD45RO$^{\text{low}}$ cells), that is, cells with potential immunosuppressive capacities, were among the cells with the highest predominance in tumor versus nontumor tissue. One explanation to this may be that human T cells can induce FoxP3 expression as a result of activation.

Mutations in several genes were found to be associated with altered infiltration rates of lymphocytes. KRAS mutation, a very common event in pancreatic cancer, was found to be associated with lower levels of several effector T cells including single CD8+ cells and CD8+CD45RO+ T cells as well as CD8+ Tregs. Previously, a higher abundance of CD4+ Tregs has been found to be associated with KRAS$^{G12D}$ mutations. Additionally, KRAS mutations were found to be significantly associated with lower levels of B cells, a relationship that has not been described previously. CDKN2A mutations were found to be associated with lower infiltration of CD8+ Tregs, which is in line with previous findings of lower levels of immune cell infiltration in a wide range of solid tumors with CDKN2A mutation, including pancreatic cancer. Mutations in SMAD4 and APC were found to be associated with higher abundance of several CD8+ lymphocyte subsets and in the case of APC also with CD4+ lymphocytes. In I-type tumors, dMMR status was...
associated with higher lymphocyte infiltration. It has previously
been shown, with single stain immunohistochemistry, that
CD8+ T cells are more abundant in dMMR I-type tumors,26
but the present study provides a much more detailed
phenotyping. dMMR leads to hypermutation and increased
neoepitope formation,36 in turn potentially leading to a more
efficient immunological response and increased infiltration of
immune cells into the tumor. This association was not seen in
PB-type tumors, which is in line with previous findings in pan-
creatic cancer.37

Several associations were observed between certain lym-
phocyte subpopulations and survival. Of particular interest
were CD8+ cell subsets, which demonstrated consistent associ-
atons with a prolonged survival. In particular, activated
and/or memory CD8+ T cells (i.e. CD8α−CD45RO+), but not
single-positive CD8 cells, were prognostic when analyzed as
total cell density. One should, however, be cautious when
drawing conclusions from these data. The distinction between
activated and/or memory and single-positive CD8 cells was
made by applying a cut-off for the CD45RO expression, and it
must be pointed out that this protein has a continuous expres-
sion in lymphocytes, potentially reflecting a continuum of the
activation status of CD8+ cells. Identifying a proper methodol-
gy for handling this biological complexity is a matter of fur-
ther studies.

Of note, Tregs (CD4+FoxP3+), commonly suggested to be
immune inhibitory, were not associated with adverse patient
outcome in our study. These data are in line with previous
research in pancreatic cancer25 and lung cancer,32 suggesting
other immune inhibitory mechanisms. Also, it may indicate
that FoxP3 expression in tumors can be induced by activa-
tion per se, without being linked to an immunosuppressive
function.33 This hypothesis is in concordance with a previ-
ous study by Stromnes et al., demonstrating that FoxP3
levels on Tregs from pancreatic tumor tissue were signifi-
cantly higher than on Tregs in adjacent benign tissue,21 pos-
sibly indicating that FoxP3 is elevated due to activation. This
observation may be important for understanding the mecha-
nisms underlying immune evasion in peripancreatic adeno-
carcinomas.

Further, the present study identified several immune cell
infiltration signatures that were significantly associated with survival,
many sharing the common characteristic of having compara-
tively high levels of CD4+CD45RO+ and CD8+CD45RO+ T cells
and FoxP3+CD45ROhigh cells. Interestingly, in the stromal com-
partment, the signatures that were found to be prognostic also
had high levels of B cells. Several differences were also identified
between the two morphologies regarding the prognostic impact
of the defined immune cell signatures. Notably, stromal immune
cell signature 5 (characterized by high levels of CD4+CD45RO+
and CD8+CD45RO+ T cells, FoxP3+CD45ROhigh and B cells, but
low levels of FoxP3+CD45ROlow cells) was significantly associ-
ated with a prolonged survival only for patients with I-type
tumors, and this survival benefit was retained against most of
the other defined stromal immune cell signatures. In mice and
in in vitro studies, FoxP3 has been shown to be expressed in
tumor promoting, immunosuppressive macrophages and den-
drivic cells as well,38,39 whether this may also be the case in
humans is as of today not yet known, but will be interesting to
follow.

As noted above, stromal immune cell signature 5 was defined
by high density of FoxP3+CD45ROhigh cells but low density of
FoxP3+CD45ROlow cells, which could, at least partly, represent a
naïve Treg population,40 indicating a different functionality
between these distinct FoxP3+ cell populations in I-type tumors.
Previously, it has been shown that activated/memory Tregs and
naïve Tregs have the same suppressive capabilities,41 therefore
one could speculate that the functionality does not lie in the
FoxP3+ cell populations themselves, but rather in the interplay
between the FoxP3+ cell populations and other immune cells,
which have been shown to be of prognostic value in previous
studies.32,42,43 In PB-type tumors, stromal immune cell signature
1, and not 5, was associated with a prolonged survival, and this
association remained significant even after adjustment for clinic-
opathological factors. Stromal immune cell signatures 1 and 5 were
similar in composition, but in contrast to stromal immune cell
signature 5, stromal immune cell signature 1 was also character-
ized by low levels of FoxP3+CD45ROhigh cells. This could imply
that there is a difference between the two morphologies regarding
which immune cells constitute an effective antitumor response.
The results from our study indicate that there is a subgroup of
patients with immunogenic tumors with low levels of immuno-
suppressive lymphocytes but high levels of effector lymphocytes,
and that these patients have a better prognosis. These results are
in line with previous research in pancreatic cancer.32,44

Finally, we investigated spatial localization of the immune
cells in relation to tumor cells and to other lymphocyte sub-
populations. We identified single-positive CD8+ cells being of
particular importance when localized in immediate vicinity to
cancer cells. We managed to demonstrate this observation by
different methods, evaluating single-positive CD8+ cell enrich-
ment in the tumor versus stromal compartment, and by ana-
lyzing their potential direct interaction with cancer cells. The
potential interaction zone between lymphocytes was set to a
radius of 15 μm, based on previous publications, indicating
15 μm to be most appropriate distance to analyze potentially
predictive features of cell-to-cell interactions.45 Overall, our
results demonstrate that single-positive CD8 cell interaction
with cancer cells is an independent prognostic factor in per-
ipancreatic adenocarcinoma. These findings are in line with
previous studies on pancreatic cancer.32,46 A recent study by
Masugi et al., could not establish a significant association
between CD8+ T-cell proximity to cancer cells and patient
survival, however, our study used a different methodology.47
They did, however, find a significant association between
patient survival and CD8+ T-cell infiltration in the tumor cen-
ter, further supporting that the topographic infiltration pat-
terns of CD8+ cells are of prognostic importance.47 Further,
Conclusions
The present study provides a first detailed description of the lymphocyte infiltration patterns in periampullary adenocarcinoma. The data presented demonstrate that the composition, topography and clinical impact of lymphocyte infiltration differ by morphological type as well as by localization. The multiplex technique with spatial cell mapping indicates specific mechanisms of the immune regulation in human tumors, and the findings may help to stratify patients in the context of immunotherapy or suggest combined modalities to overcome resistance.

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Conflict of interest
The authors declare that they have no conflict of interest.

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