Identification of circulating myeloid cells as a potential diagnosis and recurrence marker of pancreatic ductal adenocarcinoma through the single-cell analysis

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Research Article
Abstract

Background:
Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy with a poor survival rate, largely due to the lack of biomarker for early detection. Given the crucial roles of circulating and tumor-infiltrating myeloid cells in PDAC progression, the identification of specific subsets of them can be a biomarker in liquid biopsies for diagnosis and prediction of recurrence risk of PDAC.

Methods:
We analyzed PDAC tissue microarray by immunohistochemistry to measure cytokine expressions. Peripheral blood cells of PDAC patients were subjected to define distinct cell population of PDAC patients by single-cell RNA sequencing and flow cytometry. In addition, the presence of myeloid cells was analyzed by murine xenograft/orthotopic models of PDAC cell lines. Finally, we performed a clinical study to evaluate the correlation of IL-10R2 expression and PDAC diagnosis or recurrence.

Results:
We found enriched IL-10R2+/IL-22R1 + myeloid cells in peripheral blood from PDAC patients, and that they display a signature association with tumor-educated monocytes. In addition, we verified the positive correlation of pancreatic tumor growth with increased IL-10R2+/IL-22R1 + myeloid cells through the murine xenograft/orthotopic models. Most importantly, the IL-10R2 + myeloid cells signaled tumor recurrence 130 days faster than CA19-9 in post-pancreatectomy patients.

Conclusions:
Enriched IL-10R2 + myeloid cells in PDAC patient's blood may benefit uncomplicated and effective diagnostic marker and indicator of recurrence.

Backgrounds
Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, comprising 90% of all malignant pancreatic neoplasms. Its frequency is increasing worldwide and is predicted to become the second leading cause of cancer deaths by 2030 (1, 2). The high rate of mortality due to PDAC is mainly driven by limited early detection methods and the lack of curative therapies for late-stage cancers (1, 2). Moreover, the tumor microenvironment (TME) of PDAC is non-immunogenic (3, 4), contributing to therapy resistance. This immunosuppressive TME and low prevalence of immunotype tumors shields PDAC lesions from various immunotherapies that have been proven successful in other cancers, and from traditional treatments such as chemotherapy (5). Hence, characterization of the
mechanisms of early immune responses, including the identification of specific cell subtypes infiltrating the PDAC TME, in association with PDAC progression is critical for more sensitive diagnostic strategies for early detection and more effective immune therapy options to improve the survival rates in the affected patients.

Myeloid cells infiltrating the tumor are predominant in the PDAC TME and have been recognized as crucial mediators of immune evasion in this cancer in association with its poor clinical outcomes (6). The PDAC tumor cells that escape from host immune surveillance create a TME suitable for enhanced tumorigenesis, disease development, and metastasis. During tumorigenesis, PDAC tumor cells recruit various myeloid cells including tumor-educated monocytes (TEMs), tumor-associated macrophages (TAMs), tumor-associated neutrophils, and myeloid-derived suppressor cells, together with regulatory T cells (Tregs), thereby establishing an immunosuppressive TME (3–5). The tumor-associated myeloid cells induce T-cell dysfunction and recruit Tregs that promote tumor growth by suppressing tumor immunity (7–9). Especially, monocytes are educated by the TME through cellular communication by interacting with each other, which consist of a small subset of tumor-associated of myeloid cells, TEMs (10). Circulating TEMs activate and differentiate to TAMs that contribute to tumor progression by enhancing protumor activity in TME (11), implying the tumor-immune reactions. It was therefore of considerable interest to identify whether tumor-reactive specific subtypes of myeloid cells in PDAC patients could be used to diagnose progression of PDAC.

Single-cell RNA sequencing (scRNA-seq), a technology to analyze transcriptome in a single-cell level, enabled identification of new cell types based on expression profile of multiple genes(12). This technique can be a powerful tool for cancer immune research to define and characterize highly heterogenous cell subtypes of TME (11, 13, 14) and “TEM signature genes” in several cancers including breast cancer, lung cancer (11, 15). Due to a specific regulation of myeloid cell heterogeneity by tumor cells to form “TEM signature genes” and its circulation subsequently (10, 13), it is recognized that distinct expression profiles in circulating myeloid cells reflects TME conditions (14). Thus, there is urgent need to identify specific circulating myeloid cell types for PDAC diagnosis and treatment monitoring by scRNA-seq approach in peripheral blood, one of sample sources for non-invasive biomarkers (14).

Several lines of evidence to date have demonstrated that Interleukin-22 (IL-22) is highly expressed in PDAC (16, 17). IL-22 secreted by immune cells such as Th17, innate lymphoid cells, and myeloid cells (18, 19) has been suggested to play a role in the malignant disease progression of colon (20), lung (18, 21), and breast tumors (22), and lymphomas (23). In addition, recent evidence has shown that IL-22 promotes pancreatic fibrosis (24) and PDAC tumor development (17, 25). The IL-22 receptor is also elevated in gastric cancer (26), hepatocellular carcinoma (27), and PDAC (16). However, the clinical benefits of detecting elevated IL-22 and its receptor in the context of an early diagnosis or recurrence indicator for PDAC remains largely unknown.

In our present study, we identified IL-22 receptor (IL-10R2/IL-22R1)-expressing myeloid cells as an abundant cell population in PDAC patient blood samples through the scRNA-seq approach. We found
that IL-10R2+/IL-22R1+ cells expressed tumor-educated monocyte signature genes by scRNA-seq, and infiltrated in PDAC tissues. We further verified these cells in murine PDAC models, and found that they correlate with pancreatic tumor progression from the early phase, and infiltrate to pancreatic tumor tissues. Finally, we found that IL-10R2+/IL-22R1+ myeloid cells in blood cells can be a reliable indicator for PDAC prognosis, and a predictive marker of PDAC recurrence.

**Methods**

**Patient inclusion and sample collection**

We collected peripheral blood samples from patients with the following diagnoses: PDAC, acute pancreatitis, chronic pancreatitis, intraductal papillary mucinous neoplasm (IPMN), and other systemic cancers. PDAC and other cancers were diagnosed by cytology through endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) or using surgical specimens. Acute pancreatitis, chronic pancreatitis, and IPMN were diagnosed through the evaluation of clinical symptoms and by imaging studies including CT, EUS, and/or MRI.

Initial blood samples were obtained at the time of diagnosis in all subjects including PDAC (n = 154), acute (n = 32) or chronic pancreatitis (n = 39), bile duct cancer (n = 57), and IPMN (n = 9) cases. Samples from healthy controls (n = 159) were obtained from non-cancer participants. In PDAC patients, further samplings were performed under the following schedule: 1) in patients with a resectable PDAC, blood samples were collected every month after surgery for 12 months and at the time of recurrence during follow-up; 2) in unresectable PDAC cases, blood samples were collected every three months during chemotherapy/radiotherapy. Patients with evidence of serious illnesses, immunosuppression or on immunosuppressive drugs, or autoimmune/infectious diseases, were excluded. Approximately 10 mL of blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes for each subject and transferred to the laboratory.

In addition to the patient clinical data, laboratory findings and tumor markers including CA19-9 were collected. The clinical tumor staging was determined in accordance with the TNM classification system for PDAC (40). These clinical studies were approved by the appropriate committee as described in Declaration.

**Reagents And Antibodies**

A detailed description of the reagents and antibodies used in this study can be found in Supplementary table 1.

**Flow Cytometry**
Human and mouse PBMCs were isolated from whole blood using Histopaque® 1077 or Histopaque® 1083, both with 1119 density gradient centrifugation. The interphase layers of PBMCs and granulocytes were washed in PBS containing 0.5% bovine serum albumin. All sequence steps were carried out at 4°C. Data were analyzed using FlowJo (Ver10).

**mRNA Collection And Quantitative Rt-pcr**

mRNA collection and quantitative RT-PCR

Total RNA was extracted from human PBMCs using QIAzol reagent and reverse transcribed to cDNA using a cDNA Synthesis Kit. Quantitative real-time PCR was performed using a PCR detection system (StepOnePlus Real-Time PCR) and a commercial detection kit (SYBR Premix Ex Taq Kit). The primer sequences used in this study are listed in Supplementary Table 2.

**Facs Cell Sorting**

Single cell suspensions of PBMCs were stained with fluorochrome-conjugated antibodies and appropriate isotype controls for 60 minutes at 4°C. After the staining protocol, cells were filtered through a 30-µm cell strainer before sorting. Stained cells were sorted on the basis of fluorescence intensity using a BD FACSARia III cell sorter.

**Scrna-seq Experiments**

IL-10R2+ cells were enriched from the PBMCs of PDAC patients using a FACSARia III flow cytometer. To count the number of cells and check for viability, sorted cells were stained with trypan blue and diluted at a concentration of 1×10^5~2×10^6 cells/mL. The cell viability was estimated to be ~90%. scRNA-seq libraries were generated using the Chromium system (10x Genomics) with the Chromium Single Cell 3’ Library & Gel Bead Kit v2.

**Scrna-seq Data Analysis**

Raw FASTQ files were processed with the Cell Ranger software suite (v2.2.0) using default mapping options. Reads were mapped to the human reference genome (GRCh38) using STAR (v2.5.1b), and quantified with an Ensembl GTF file (release 91). Cell barcodes corresponding to empty droplets were filtered out using the DropletUtils (v1.2.2) package for R with an FDR < 0.01. Poor-quality cells that had a greater than 10% unique molecular identifiers (UMIs) mapped to mitochondrial genes, less than 1,000 total UMI counts, and less than 102.5 expressed genes were excluded. The thresholds were chosen by visually inspecting outliers in the two-dimensional PCA plot on all quality control metrics calculated by the Scater (v1.10.1) package for R. The filtered count matrix was normalized using the Seurat (v3.0-alpha) package for R. In each dataset, the top 2,000 highly variable genes were selected as a subset of
feature genes using the FindVariableFeatures function of the Seurat package with default options. Batch effects were removed using the Seurat package on 30 canonical correlation vectors. The integrated expression matrix was scaled, and then visualized in the two-dimensional UMAP plot using the RunUMAP function of the Seurat package on 30 principal components. For cell type annotation, we used the CreateSingleSeuratObject function of the SingleR package (v.0.2.2) (25) for R on the raw UMI count matrix by setting npca = 15, min.cells = 0, min.genes = 0, and regress.out = NULL. Differentially expressed genes between P5 and P5(-) or cell type marker genes were identified using the Wilcoxon rank sum test implemented in the Seurat package with the options of adjusted P-value< 0.01. The gene-by-cell count matrices of PBMCs from healthy donors (2.1.0/pbmc8k, 1.1.0/pbmc6k, 1.1.0/frozen_pbmcdonor_a, 1.1.0/frozen_pbmcdonor_b, 1.1.0/frozen_pbmcdonor_c) were downloaded from the 10X Genomics website and processed without filtering out cells.

**Laboratory Animals**

Eight- to 10-week-old BALB/c nude mice were obtained from Charles River Laboratory. The animals were maintained in a specific pathogen-free environment, were acclimated for at least 1 week before any experiments were conducted, and randomly assigned to the treatment groups. All procedures used in this study were approved by the appropriate committee as described in Declaration.

**Cell Lines And Culture**

Human pancreatic cancer cell lines (AsPC-1 & Panc-1 cells) were purchased from ATCC, cultured in DMEM or RPMI 1640 (containing 20mM HEPES and L-glutamine, without sodium bicarbonate) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2.

**Animal Model For Cancer Induction (Xenograft, Orthotopic Model)**

Mice were placed individually in an anesthetizing chamber and anesthesia was induced with 2 ~ 3% isoflurane in 100% oxygen. 1x10⁷ tumor cells were then subcutaneously injected in mouse back skin, or 2x10⁶ tumor cells were orthotopically implanted into the mouse pancreas. The mice were sacrificed on a scheduled date.

**Immunohistochemical (Ihc) Staining**

The IHC staining methods for histologic sections (5µm) have been described previously (28). Trichrome and Picro Sirius Red staining was also performed following the standard protocol provided by the manufacturer.
**Statistical analysis**

The Chi-square test was used to compare frequencies. Continuous variables were examined for normality using a Shapiro-Wilk test. Variables that had not deviated from normal were presented as a mean and standard deviation (SD), and were compared using an independent two sample t-test, paired t-test or one-way analysis of variance. Variables that were not normally distributed were presented as a median and interquartile range, and assessed using the Mann-Whitney U, Wilcoxon signed rank, or Kruskal-Wallis test. Post-hoc p-values were corrected with the Bonferroni method. Correlation coefficients between two continuous variables were calculated by Pearson correlation analysis.

The independent predictors of PDAC were determined using multivariable logistic regression: variables showing p values < 0.05 by univariable logistic regression were entered. The AUC for an ROC curve was used to determine the predictive ability of IL-10R2, IL-22R1, and IL-22. A combination of IL-10R2, IL-22R1, and IL-22 for PDAC was compared using the DeLong method. Kaplan-Meier survival curves were created using overall survival data and groups were compared using the log-rank test. Two-sided p-values < 0.05 were considered significantly different. All statistical analyses were performed using SAS (version 9.4, SAS Inc., Cary, NC) and SPSS for Windows, version 25.0 (SPSS, Chicago, IL).

**Results**

**Concomitant elevation of IL-22 in PDAC tissues and IL-10R2+/IL-22R1+ cells in patient blood samples**

To evaluate the association between the expression of tumor-driven immunosuppressive cytokines and PDAC, we determined the expression levels of IL-10 family cytokines by immunohistochemistry (IHC) using PDAC tissue microarray (TMA) blocks. Cancer tissues from 183 out of 184 patients (99.5%) exhibited strong staining for at least one of these cytokines (Supplementary Fig. 1). IL-22 was the most frequently expressed in this family (96.8% of patients, Fig. 1A and Supplementary Fig. 1) and showed strong staining in tumor tissues as well as peritumorous matrix tissue (strong expression (ST): 82.3%) (Fig. 1A and Supplementary Fig. 1). In contrast, positive staining for IL-10, IL-17, and IL-23 was observed in 47.2%, 29.3%, and 69.4% of patients, respectively. Moreover, staining of below 10% was found for IL-20 and IL-26 in PDAC patients (ST: 9.3% and 2.9%, respectively) (Fig. 1A), further demonstrating the prominent expression of IL-22 in PDAC tissues.

IL-22 signals through a heterodimeric receptor composed of two subunits, IL-10R2 and IL-22R1 (29). Given the significant elevation of IL-22 in PDAC tissues, we next determined whether the population of IL-10R2+/IL-22R1+ cells, which respond to this cytokine, was increased in PDAC patient peripheral blood mononuclear cell (PBMC) samples by detecting the mRNA expression of IL-10 family receptors in comparison to healthy controls. We observed an approximately 7-fold increase in the IL-10R2 and IL-22R1 mRNA levels in the PBMCs of PDAC patients compared to the healthy controls (Fig. 1B). IL-10R1, IL-20R1, IL-22R2, and IFNR-λ mRNA levels were also significantly elevated in the PDAC patients (Fig. 1B). These
results show that mRNA expressions of IL-22 receptors including IL-10R2 and IL-22R1 are highly elevated in PBMC of PDAC patients compared to that of healthy controls.

Given the elevated mRNA expression of IL-10R2 and IL-22R1 in PDAC PBMC, we reasoned that the surface protein expression of these receptors in PDAC PBMC may be much higher than in non-disease controls (CTL) or other disorders. Using flow cytometry analysis, the levels of IL-22+, IL-10R2+ or IL-22R1+ cells in PBMC from PDAC patients were compared with those from non-disease controls (CTL) and from other disorders including acute pancreatitis (AP), chronic pancreatitis (CP), alcohol induced acute pancreatitis (AIAP), cholangitis (Cho), common bile duct stone (CBDS), and gall bladder stone (GBS). Surprisingly, IL-10R2+ cells expressing CD45 (IL-10R2+CD45+ cells) were specifically elevated in PDAC compared to all of the other non-tumor diseases (Fig. 1C). When the three markers were compared to other malignancies including Ampulla of Vater carcinoma (AoVCa), biliary carcinoma (BCA), neuroendocrine tumor of pancreas (NET), pancreatic cyst or pseudocysts (PC), lung cancer (LC), colon cancer (CoCa), and intraductal papillary mucinous neoplasm (IPMN), only IL-10R2+ cells were significantly elevated in PDAC compared with other malignancies (Fig. 1D). In contrast, the number of IL-22R1+ cells were not significantly different between PDAC and the other tested diseases in both tumorous and non-tumorous conditions although IL-22R1+ cells were significantly elevated in the PDAC patients compared to the CTL group ($p = 0.0021$ and $0.0003$, Fig. 1C, D). Taken together, these results indicate a correlation between elevated IL-22 expression in PDAC patient tissues and an abundant population of IL-10R2+ or IL-22R1+ cells in PDAC patient peripheral blood. More importantly, IL-10R2+ cells were identified as a specific marker that can distinguish PDAC from other types of malignancy.

**Characterization of IL-10R2+ myeloid cells in the peripheral blood of human PDAC patients by scRNA-seq and their infiltration of PDAC tissues**

Given our finding of IL-10R2+ PBMC enrichment in PDAC human patients, we next analyzed these cells by scRNA-seq to further identify their specific enriched subtype, considering their heterogeneity (Supplementary Fig. 2). scRNA-seq was performed using IL-10R2+ PBMCs from five PDAC patients (Supplementary Fig. 3A) and IL-10R2- PBMCs from control subjects. A total of 24,819 cells (18,608 IL-10R2+ and 3,721 IL-10R2− cells) passed our stringent quality control criteria and were represented in a low-dimensional space using the uniform manifold approximation and projection (UMAP) algorithm (30) (Fig. 2A). We applied the SingleR algorithm (31) to identify four major immune cell types: B, T, natural killer cells, and monocytes/macrophages, and these were confirmed by the expression of cell type-specific markers (Fig. 2B, Supplementary Fig. 3B, C). The composition of the IL-10R2− cells in the PDAC patients was similar to those of healthy donors (Fig. 2C). Compared to cells from healthy donors and IL-10R2− cells (P5(-)) in the PDAC patients, the most prominent differences in the IL-10R2+ cells in the PDAC patients were an expansion of monocytes/macrophages and a reduction in B and T cells (Fig. 2C).

Given the strong expansion of the monocyte/macrophage compartment in IL-10R2+ PBMCs, we further dissected and characterized this subpopulation. We analyzed the expression of CD14 and CD16, two
surface markers that categorize monocytes into classical (CD14^+CD16^-), intermediate (CD14^+CD16^+), and nonclassical (CD14^-CD16^+) types, to determine whether this subset belongs to myeloid population. In addition, the monocyte/macrophage subsets from IL-10R2^+ cells were distinguished by the expression of CD16, which indicated enrichment of nonclassical monocytes compared to IL-10R2^- cells (P5(-)) (Fig. 2D and Supplementary Fig. 4A, B). This subset in IL-10R2^+ cells exhibited high levels of TNFSF10, which belong to a signature gene set associated with the recently characterized TEM (11). Additionally, CSF1R, CX3CR1, CXCL16, IL1B, IL15, IL1R1, IL2RG, TNFRSF8, which are suggested to be involved in further differentiation to TAM (10, 32, 33), were also detected with high intensity in this subset (Supplementary Fig. 4C). The TEM score for IL-10R2^+ monocytes from the PDAC patients was significantly higher than that for the total monocytes from healthy donors or IL-10R2^- monocytes (p<10^-8, Fig. 2E), which could be explained by the increased proportion of non-classical (CD14^-CD16^+) monocytes in the IL-10R2^+ monocyte/macrophage compartment (Fig. 2F and Supplementary Fig. 4D). Taken together, these results indicated that the IL-10R2^+ PBMCs of PDAC patients are enriched for myeloid-derived non-classical monocytes expressing the TEM signature.

The enriched population of IL-10R2^+ myeloid cells displaying the TEM signature in the PDAC patient peripheral blood samples raised the possibility that these cells are predisposed to migrate from the peripheral blood and relocate to PDAC tissues during cancer progression. To test this possibility, we compared the level of cells expressing IL-22 binding receptors (IL-10R2^+ and IL-22R1^+) between non-disease normal and PDAC patient tissues using flow cytometry. From flow cytometry analysis of human tumor tissue, the total CD45^+ cell population was found to represent 2.8%-14.3% of the total live cells (Fig. 2G). Among the CD45^+ cells in the PDAC tumor, IL-22R1^+ (15.9% of CD45^+) or IL-10R2^+ (13.4% of CD45^+) cells were abundantly increased and scattered within the tumor tissues compared to the normal control (2.3% of IL-22R1^+CD45^+ and 3.2% of IL-10R2^+CD45^+) (Fig. 2H). In addition to cells positive for a single receptor, 8.6 ± 6.4% (range 0.0 ~ 12.2%) of cells were found to have both receptors (IL-22R1 and IL-10R2) i.e. double-positive cells (DPCs) (Fig. 2H), demonstrating the increased infiltration of IL-10R2^+ cells in PDAC tissues.

**Enrichment of IL-10R2^+/IL-22R1^+ myeloid cells infiltrating the tumor in a murine model of pancreatic cancer**

To verify whether the population of IL-10R2^+/IL-22R1^+ myeloid cells was augmented in a PDAC murine model in a similar manner to human PDAC patients, a xenograft murine model of pancreatic cancer was created by subcutaneously injecting PANC-1 cells into nude BALB/c mice (Fig. 3A). IL-10R2^+ or IL-22R1^+ cells were detectable in the peripheral blood of these animals within a week of tumor inoculation and persisted until day 14 (the end of the observation period). Interestingly, the numbers of both cell types correlated well with the tumor volume (Fig. 3B). However, IL-10R2^+ cells were much more abundant (5.2-fold greater number than IL-22R1^+ cells) (Fig. 3C), indicating that IL-10R2^+ or IL-22R1^+ cells are enriched in PBMC of PDAC murine model.
To assess if the infiltration of IL-10R2⁺/IL-22R1⁺ cells in pancreatic tumor tissue in murine model is similar to human PDAC patients, we evaluated tumor infiltration of two myeloid subsets of cells, IL-10R2⁺CD45⁺ or IL-22R1⁺CD45⁺ cells, by FACS after direct injection of AsPC-1 or PANC1 cells into the pancreas of nude BALB/c mice. We compared the mRNA expression levels of IL-10 family receptors and found that IL-10R1, IL-10R2, and IL-22R1 mRNA levels in the PBMCs of PNAC-1 tumor bearing mice were higher than those of WT mice (Fig. 1D). Depending on the cell line, the patterns of induction were varied (Fig. 3D): i.e. IL-22R1 expression was more prominent in the tumor tissues of the PANC1-injected mice at day 14 whereas the increase in IL-10R2 expression was significantly greater in the AsPC-injected group at day 14 and 21 (Fig. 3D). Nevertheless, IL-10R2 and/or IL-22R2⁺ myeloid cell infiltration of tumor tissues increased in a time-dependent manner (Fig. 3D and 3E). Interestingly, not only the single positive cells but also the IL-10R2⁺IL-22R1⁺ DPCs, which were rarely found in PBMCs, were significantly increased in the tumor tissues (Fig. 3F). These results indicate that pancreatic tumor progression in a murine model is also correlated with an increased number of IL-10R2⁺/IL-22R1⁺ myeloid cells and their infiltration in tumor tissues as seen in human PDAC tumor tissues.

**Clinical significance of the IL-10R2⁺ myeloid population of PBMCs for the early detection of PDAC onset and recurrence**

We speculated that the elevated numbers of IL-10R2⁺ or IL-22R1⁺ cells in the peripheral blood of PDAC patients may provide an effective diagnostic marker for these cancers. Notably, the patients with high IL-10R2 or IL-22R1 expression had a lower survival rate than the patients with low IL-10R2 or IL-22R1 expression (p = 0.003 or 0.00008, respectively; Supplementary Fig. 5), indicating an inverse relationship between IL-10R2 or IL-22R1 expression and patient survival. To evaluate IL-22R1⁺ or IL-10R2⁺ cells as potential diagnostic biomarkers of PDAC, we determined the clinical significance and value of IL-10R2 and IL-22R1 expression in peripheral blood cells for PDAC detection by measuring the sensitivity and specificity for positive mRNA expression in patients through a hospital-based prospective study. In the analyses, besides the expression of IL-10R2 and IL-22R1, other IL-10 family cytokine receptors (e.g., IFNLR, IL-10R1, IL-20R1, and IL-22R2) were also detected. For IL-10R2 mRNA, the sensitivity and specificity were 0.677 and 0.763, respectively (area under curve (AUC) = 0.779, p < 0.0001); for IL-22R1, 0.569 and 0.789 (AUC = 0.695, p < 0.001); for IL-22, 0.338 and 0.947 (AUC = 0.634, p = 0.032); and for CA-19-9, 0.681 and 0.677 (AUC = 0.717, p < 0.001) (Fig. 4A). Interestingly, as determined by logistic regression, the combination of IL-22, IL-22R1, and IL-10R2 showed significant improvement in the AUC value (0.836) with a sensitivity of 0.692 and specificity of 0.895 (p < 0.0001) (Fig. 4A). The sensitivity and specificity values were similar between mRNA (as determined by qPCR) and protein analyses (cell frequencies determined by FACS analysis, data not shown). Notably however, the sensitivity and specificity were not improved by the addition of IFNLR, IL-10R1, or other receptor mRNA levels to the aforementioned combination (data not shown). These data indicated that the IL-10R2⁺ myeloid population size in the PBMCs alone was positively correlated with PDAC detection and that this correlation was more potent when combined with the IL-22R1⁺ and IL-22⁺ populations.
In addition to tumor detection, the availability of early markers of recurrence after surgery is also highly valuable. Accordingly, we compared the expression of the IL-10R2⁺ myeloid population of PBMCs from PDAC patients who underwent a margin-free pancreas resection. Initially, 61 of the patients who received surgery were recruited and recurrence was confirmed by MRI or CT scan in 12 cases who completed monthly analysis (Fig. 4B). The upregulated IL-10R2⁺ cells at the preoperative stage were significantly reduced or absent at 1 month after surgery (Fig. 4C), comparable to the results for CA19-9 which was previously reported as a recurrence indicator for PDAC (34) (data not shown). In contrast, IL-22⁺ and IL-17⁺ blood cells did not show different expression levels between the pre-and post-operative states (Fig. 4C). In addition to these cell surface markers, the mRNA levels of IL-10R2 in the total PBMC population was significantly reduced after surgery (Fig. 4D). Interestingly, as with IL-10R2, the initial reduction of IFNLR1 and hGPC1 was observed one month after pancreatectomy (Fig. 4D). However, neither IFNLR1 nor hGPC1 correlated with tumor recurrence until 12 months after surgery, unlike IL-10R2. Additionally, from serial observations and comparisons of IL-10R2 and CA19-9 in PDAC cases with recurrence, an earlier elevation of IL-10R2 was detectable compared to CA19-9 (Fig. 4E, F). At 6 months after surgery, all recurrence cases exhibited significantly elevated IL-10R2 even when the recurrent mass was not yet evident on an abdominal CT. In addition, 7 cases (58.3%) even showed normal CA19-9 levels. At the 9 month timepoint, whilst all recurrence cases continuously expressed elevated IL-10R2 + blood cells, four recurrent patients (33.3%) still exhibited normal CA19-9 levels. Moreover, the mean time to CA19-9 elevation after surgery was 239.7 ± 125.2 days whereas for IL-10R2, this period was only 109.9 ± 41.1 days (p < 0.0001, paired t-test) (Fig. 4F), indicating that an increased IL-10R2 + myeloid population of PBMCs may be a useful indicator of PDAC recurrence. Taken together, our current data indicate that an enriched IL-10R2⁺ myeloid population in the PBMCs of a PDAC patient can be used to not only diagnose this cancer at an earlier stage, but also detect tumor recurrence after pancreatectomy.

Discussion

We initially hypothesized that each cancer entity harbors specific circulating immune cells that migrate into the tumor tissue, play a role in establishing the TME, and actively interact with tumor cells during tumor initiation and progression, which is based on the hypothesis of “cancer immunoediting” where either innate or adaptive immune cells are able to monitor, detect and react with tumors, and may function in tumor elimination, especially in the early period (35). In support of this notion, a recent study has reported that macrophages alter their transcriptome for specific cancer subtypes (11). In the case of PDAC, CSF1R⁺F4/80⁺ macrophages exclusively infiltrate the cancer tissue and enhance tumor growth (36). Despite the fact that myeloid cells in cancer tissues originate from the bone marrow and pass through the peripheral circulation to finally reach the target tumor tissue, most previous studies have focused only on the characteristics and function of the tumor-infiltrating myeloid cells and not on changes in the blood cell subtypes or their specific markers.

It is well known that IL-22 is typically produced from immune cells, mainly from innate immune cells and some lymphoid lineages (18). Its receptors are usually expressed in normal tissues (e.g., IL-22R1 is
expressed in the intestinal and skin epithelium) (18, 19). Surprisingly however, the relationship between the IL-22 ligand and the expression patterns of its receptors was found to be reversed in PDAC carcinogenesis. Although IL-22 is not detected in the normal pancreas, we found in our current analyses, in agreement with previous studies, that PDAC tumors could actively produce this cytokine (16, 17, 25). Since myeloid cells expressing the IL-22-binding receptor (e.g., IL-10R2+CD45+) were found in PDAC tissues, we attempted to identify these cells in the peripheral blood of PDAC patients. As IL-22 has two heterodimeric receptors, IL-22R1 and IL-10R2, we initially attempted to assess IL-10R2+ IL-22R1+ DPCs in the peripheral blood of the patients. However, few DPCs were found in the blood of most of our PDAC study cases. In addition, unlike IL-10R2+CD45+ cells, which were found in most PDAC patient blood samples (92.4%, 244/264 of PDAC patients), IL-22R1+CD45+ cells were found in a comparatively lower number of these cases, i.e. just 15.6% (45 /264) of the patients. Another interesting finding from our current analyses was that the AUC value of the combination of the three markers was much higher than that for each single marker alone (Fig. 4A). These results may indicate that IL-10R2+ and IL-22R1+ myeloid cells are independently generated and separately contribute to the PDAC immune environment, thereby working synergistically to improve PDAC detection by enhancing the AUC value.

Previous studies have reported that some malignancies and inflammatory conditions can trigger IL-22R1+ lymphocytes and granulocytes, respectively (37, 38). Additionally, the aberrant expression of IL-22R1 in blood cells aggravated the inflammatory conditions in a mouse model (39). However, it remains uncertain why IL-22R1+ myeloid cells emerge but much less so than IL-10R2+ myeloid cells in the peripheral blood of PDAC patients. Since most PDAC patients had IL-10R2+ cells in their blood, we focused on and followed these cells to investigate their relationship with cancer progression. Besides PDAC tumors, colon and lung cancers have already been reported to express IL-22 (20, 21). However, we could not detect IL-10R2+ blood cells in the PBMCs of these tumor types (data not shown). In this current study, IL-10R2+ cells were not detectable either with other benign pancreatic or peripancreatic diseases, such as chronic pancreatitis or liver cirrhosis (Fig. 1C). Another interesting finding from our present investigation into the relationship between tumor tissue and peripheral blood was that IL-10R2+IL-22R1+ DPCs are rare in the blood, but are abundant in pancreatic tumor tissues in both human patients and mouse models (5.3–19.3% in the syngeneic model vs. 0–2.4% in naïve controls out of the total myeloid cells in the tumor). Although there is no direct evidence for the conversion of IL-10R2+ myeloid cells into DPCs, it is possible at the very least that DPCs may be generated under the control of the PDAC-specific TME and that these cells may not egress into the peripheral circulation.

Although CA19-9, carcinoembryonic antigen, and DUPAN-2 can be used to monitor PDAC patients, many clinicians agree that these markers are not useful for screening and diagnosing this cancer, especially for early cases (40, 41). Hence, extensive research to date has focused on the identification of new biomarkers based on biological, immunological, and genetic changes using blood, urine (42), and saliva (43) samples. Compared to these previous reports, we focused in or present study on cancer-specific immune cells and not serum factors that are often not identifiable in terms of their origins. Previously, most marker screening studies have been conducted using serum or tumor tissues. However, one of the
major caveats of these methods is that the detection threshold depends on the mass size. In another words, the marker level is elevated as the tumor mass is growing. Thus, for an early stage lesion with a small mass, the detection methods have been limited. However, the levels of immune cell markers do not hinge on the size of a tumor mass. We thus surmised that the use of these cells as biomarkers of a specific disease could be useful, especially for detecting small lesions. Hence, with the use of FACS sorting and scRNA-seq, several specific immune cells were successfully identified and found to be specific to PDAC as compared to other tumors. Besides IL-10R2 or IL-22R1, IL-10R2 + myeloid cells can express other specific markers following PDAC progression. Interestingly, some markers noted by our scRNA-seq data have been identified previously. We found CSF1R to be highly expressed in IL-10R2 + cells in our current study and it is notable that CSF1R + macrophages have been shown to contribute to pancreatic cancer growth through T cell suppression (36). Additionally, CX3CR1 was identified by our scRNA-seq analysis and Marchesi et al. (44) and we have also found that this gene is expressed in PDAC, observing that the CX3CR1–CX3CL1 axis contributes to tumor relapse. Recently, Romero et al. (45) reported that several chemokines are associated with T cell infiltration of PDAC tissue. Consistently, we here detected enhanced expression of CXCL9 and CXCL10 in IL-10R2+ cells, which may play a role in the immunosuppressive nature of PDAC tumors. Thus, future human and in vivo studies of these cells along with other PDAC-specific immune cell markers, in combination with IL-10R2, will further enhance the suite of effective markers for PDAC and also the range of detection.

For PDAC recurrences detected after cancer surgery, as shown in Fig. 4C, IL-10R2+ cells significantly decreased or even disappeared immediately after surgery and reappeared upon tumor recurrence at an early stage during tumor growth. The emergence of IL-10R2 + cells was detectable at 130 days sooner than that of CA19-9 in predicting tumor recurrence ($p < 0.001$, Fig. 4F) and imaging studies confirmed this result (CT or MRI, data not shown). Moreover, in an incidentally identified case with an extremely small tumor (6.3 mm in size), the patient already showed increased IL-10R2+ cells despite a normal CA19-9 level and negative findings on an imaging study. Taken together, our data indicate that IL-10R2+ myeloid cells might be a very effective "whistle blower" that signals PDAC development or recurrence before CA19-9 elevation or a positive imaging result, possibly because it has an important role in early cancer development. Although we identified elevated IL-10R2+ cells and typical mRNA signatures in our PDAC patient series, in cases showing recurrence, and also in a mouse model of pancreatic cancer, a large-scale population-based prospective study is still needed to confirm the clinical utility of these cells for screening the general population. Additionally, the mechanisms underlying the development of IL-10R2+IL-22R1+ cells and their functional roles were not determined in our present analysis. Hence, the mechanisms underlying the generation of these cells and their migration to tumor tissues should also be investigated in future studies.

In conclusion, IL-10R2+ circulating myeloid blood cells in combination with IL-22R1+IL-22+ cells are novel liquid biopsy markers for PDAC. These markers have potential applications for general screening, early diagnosis, and the development of treatment strategies for PDAC. In addition, as IL-10R2+ myeloid cells infiltrating the tumor tissues are highly correlated with the tumor stage and survival in PDAC, advanced
omics and exosome analysis techniques can be used to precisely investigate the heterogeneous IL-10R2+ myeloid cell population with respect to its activation period, functions, and interactions with tumor cells.

**List Of Abbreviations**

Pancreatic ductal adenocarcinoma (PDAC), tumor microenvironment (TME), tumor-educated monocytes (TEM), tumor-associated macrophages (TAMs), regulatory T cells (Tregs), Interleukin-22 (IL-22), T helper 17 (Th17), single-cell RNA sequencing (scRNA-seq), intraductal papillary mucinous neoplasm (IPMN), endoscopic ultrasound-guided fine needle aspiration (EUS-FNA), computerized tomography (CT), endoscopic ultrasound (EUS), magnetic resonance imaging (MRI), ethylenediaminetetraacetic acid (EDTA), cancer antigen 19-9 (CA19-9), tumor, node, metastasis (TNM), institutional review board (IRB), clinical research information service (CRIS), peripheral blood mononuclear cells (PBMCs), Fluorescence activated cell sorting (FACS), unique molecular identifiers (UMIs), principal component analysis (PCA), Immunohistochemical (IHC), standard deviation (SD), tissue microarray (TMA), strong expression (ST), non-disease controls (CTL), acute pancreatitis (AP), chronic pancreatitis (CP), alcohol induced acute pancreatitis (AIAP), cholangitis (Cho), common bile duct stone (CBDS), gall bladder stone (GBS), Ampulla of Vater carcinoma (AoVCa), biliary carcinoma (BCA), neuroendocrine tumor of pancreas (NET), pancreatic cyst or pseudocysts (PC), lung cancer (LC), colon cancer (CoCa), area under curve (AUC), double-positive cells (DPCs)

**Declarations**

*Ethics approval and consent to participate*

Clinical studies in this study were approved by the institutional review board (IRB) of Gangnam Severance Hospital (IRB No. 3-2018-0293, 3-2015-0012) and registered with the clinical research information service (CRIS, https://cris.nih.go.kr/cris/en/, KCT0004614), and informed consent was obtained from all participants. All the animal studies in this manuscript were approved by the Animal Care and Use Committee at Yonsei University College of Medicine and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, no. 85-23, 1996), and the ARRIVE guidelines.

*Consent for publication*

All the clinical data in this study have consent for publication.

*Availability of data and materials*

The datasets used and/or analyzed during the current study are included in this article and supplementary information files, or available from the corresponding author on reasonable request.

*Competing Interests*
The authors declare that they have no competing interests.

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**Authors’ Contributions**

HL: writing, conceptualization, experiments, data analysis, editing. SK.: writing, conceptualization, experiments, data analysis, editing. SC: writing, editing, BC: writing, data analysis, editing. SJJ: writing, conceptualization, experiments, data analysis, editing. JL: conceptualization, experiments, data analysis. HK: data analysis, editing. AY: conceptualization, experiments. JP: experiments, data analysis, editing. YC: data analysis. EC: writing, data analysis, editing. JK: writing, conceptualization, experiments, data analysis, editing. All authors contributed to the article and approved the submitted version.

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**References**


Figures
Concomitant elevation of IL-22 in PDAC tissues and IL-10R2+/IL-22R1+ cells in PDAC patient peripheral blood. (A) Pie charts representing the fractions of IL-10 family cytokines (ST: strong, INT: intermittent, NEG: negative staining), as evaluated by TMAs from PDAC patients (n=184). (B) Gene expression levels of receptors that bind to IL-10 cytokines were analyzed by qPCR from naïve (CTL) and PDAC PBMCs. qPCR experiments were repeated three times with triplicate samples (mean ± SD, **p < 0.01; ***p < 0.001; Student t-test). (Cand D) Comparison of the expression of IL-22, IL-10R2+, or IL-22R1+ CD45+ cells in PBMCs under non-tumor (C) or tumorous conditions of pancreas (D) by flow cytometry (one-way ANOVA with Tukey's post hoc multiple comparison tests; each p value for each disease were compared with
PDAC; NS: not significant). CTL; non disease control (N=98), AP; acute pancreatitis (N=25), CP; chronic pancreatitis (N=36), AIAP; alcohol induced acute pancreatitis (N=13), Cho; cholangitis (N=52), CBDS; common bile duct stone (N=31), GBS; gall bladder stone (N=42), PDAC; pancreatic ductal adenocarcinoma (N=180), AoVCa; Ampulla of Vater carcinoma (n=5), BCA; biliary carcinoma (n=91), NET; neuroendocrine tumor of pancreas (n=6), PC; pancreatic cyst or pseudocysts (n=23), LC; lung cancer (n=15), CoCa; colon cancer (N=15), IPMN; intraductal papillary mucinous neoplasm (N=13).

Figure 2
Characterization of IL-10R2+ cells in PDAC peripheral blood by scRNA-seq and infiltration to PDAC tissues. (A) UMAP plots of 24,819 IL-10R2+ PBMCs for five patients (P1-5) with PDAC and of 3,721 IL-10R2- PBMCs for one patient with PDAC as a control (P5(-)). (B) UMAP plots of immune cell type. (C) Immune cell-type distribution for 33,981 PBMCs from five healthy donors (N1-5), 3,721 IL-10R2- PMBCs from one PDAC patient (P5(-)), and 18,608 IL-10R2+ PBMCs from five PDAC patients (P1-5). (D) Cell subtype distribution of PBMCs within the mono/macrophage compartment with CD14 and CD16. (E) Violin plots showing TEM signature scores for the monocyte/macrophage compartment in healthy donors and PDAC patients (left) as well as in IL-10R2- (P5(-)) and IL-10R2+ (P5) PBMCs from the PDAC patients (right) (***p < 10^{-8} using the Wilcoxon rank sum test). (F) Violin plots showing the TEM signature scores for the monocyte/macrophage compartment in CD14+ and CD16+ subtypes of IL-10R2+ PBMCs from PDAC patients (***p < 2.2\times10^{-16} using the Wilcoxon rank sum test). (G) FACS analysis strategies for human PDAC tissue samples. After 7AAD-gating, CD45+ or total cells were stained with IL-22R1 and IL-10R2. (H) IL-22R1+ CD45+ (left), IL-10R2+CD45+ (middle), and IL-22R1+IL-10R2+ CD45+ (double receptor positive myeloid cells, right) cell numbers between non-disease normal (normal) and PDAC pancreatic tissue (n=6) were determined by flow cytometry (Mann-Whitney U test).
Enrichment of IL-10R2⁺ myeloid cells in tumor tissues during pancreatic tumor progression in murine models. (A-C) A xenograft mouse model was established by subcutaneously injecting PANC-1 cells (1 × 10⁷ cells/mouse) into nude BALB/c mice. Mouse experiments were repeated at least three times with four mice in each group (A). Quantification of IL-22R1⁺CD45⁺ and IL-10R2⁺CD45⁺ cells by flow cytometry in representative PBMCs from naïve, and mice developed mass size of 0.5, 0.9, and 2.2 mL at 14 days after
tumor induction. (B). IL-22R1\(^+\) and IL-10R2\(^+\) cells were quantified by flow cytometry in representative PBMCs from naïve (CTL) and PANC-1 tumor-bearing mice (C) (Mann-Whitney U test). (D-F) Orthotopic pancreatic cancer mouse models were established using nude BALB/c mice by direct injection of PANC1 or AsPC-1 cells (2 × 10\(^6\) cells/mouse) into the pancreas. Mouse experiments were repeated more than three times with at least four mice. Gene expression level of IL-10-related receptors were analyzed by qPCR from CTL and PANC-1 tumor-bearing mice PBMCs. qPCR experiments were repeated three times with triplicate samples (mean ± SD, *p < 0.05; **p < 0.01; Student t-test). IL-22R1\(^+\) and IL-10R2\(^+\) cells were quantified by flow cytometric analysis of tumor tissues from naïve (CTL), PANC-1, and AsPC-1 tumor-bearing mice at two (Day 14) and three (Day 21) weeks after surgery (D). IL-22R1\(^+\)IL-10R2\(^-\), IL-22R1\(^-\)IL-10R2\(^+\), and IL-22R1\(^+\)IL-10R2\(^+\) cells were quantified by flow cytometry using tumor tissues from naïve (PBS) and PNAC-1 tumor-bearing mice at 7 days after surgery (n=4, Mann-Whitney U-test) (E and F).
**Figure 4**

**Clinical significance of the IL-10R2+ myeloid population in PBMCs early detection and recurrence of PDAC.** (A) Receiver operating characteristic (ROC) curve for IL-10R2, IL-22R1, IL-22, combination of IL-22 + IL-22R1 + IL-10R2, and CA19-9 in PBMCs from PDAC patients (n=180) versus non-cancer healthy controls (CTL, n=98). Multivariable logistic regression was used to determine the independent predictors of PDAC: variables showing p values <0.05 by univariable logistic regression were entered. A combination
of IL-10R2, IL-22R1, and IL-22 for PDAC was compared using the DeLong method. (B) Schematic illustration of the study flow and sample size. (C) Percentages of IL-10R2+, IL-22+, and IL-17+ cells in PBMC from the pre- and postoperative one month samples (paired t-test). (D) ΔCt values of IL-10R2, IFNLR1, GPC1, and IL-22 were measured by qPCR and compared between pre-and postoperative one month samples (paired t-test). (E) Fraction of biomarkers (IL-10R2 or CA19-9) detected among 12 radiologically confirmed recurrence cases in a time-dependent manner (red: over the cut-off value; green: under the cut-off value). (F) Comparison of the median number of days until the detection of tumor recurrence after surgery for each biomarker (Mann-Whitney U test).

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