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Original article

Monitoring and functional characterization of the lymphocytic compartment in pancreatic ductal adenocarcinoma patients

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ABSTRACT

Background/Objectives: Pancreatic ductal adenocarcinoma (PDAC) still has a poor prognosis and current treatments including immunotherapy often fail. This might be due to the pronounced immunosuppressive milieu impairing infiltration and function of immune effector cells. This study aimed at a comprehensive analysis of immune cells in PDAC patients by determining absolute and relative peripheral blood cell numbers of immune cell subsets along with their functional capacity.
Methods: Whole blood cells or isolated peripheral blood mononuclear cells were characterized by flow cytometry. PDAC tissues were analyzed by immunohistochemistry. Anti-tumor activity of immune effector cells was determined by RTCA system.
Results: Our data demonstrate that relative $CD4^+$ memory- and regulatory T cell numbers were enhanced, whereas determination of absolute cell numbers revealed generally lower immune cell numbers in PDAC patients compared to healthy controls. γδ T cells accumulated at higher numbers compared to αβ T cells in the malignant ductal epithelium of PDAC tissues indicating that γδ T cells infiltrate into the tumor. Cytotoxicity against tumor cells of even small numbers of T- and NK cells could be induced by a bispecific antibody targeting $CD3^+$ T cells to human epidermal growth factor receptor (HER)2 expressing PDAC cells or Trastuzumab. Importantly, a critical number of γδ T cells was required for significant tumor cell killing by a bispecific antibody engaging the γδ T cell receptor on γδ T cells and HER2 on tumor cells.

Abbreviations: ADCC, antibody-dependent-cell mediated cytotoxicity; bsscFv, bispecific single-chain fragment variable; HD, healthy donor; HLA, human leukocyte antigen; NK cells, natural killer cells; n-BP, aminobisphosphonates; PDAC, pancreatic ductal adenocarcinoma; PBMCs, peripheral blood mononuclear cells; rIL-2, recombinant Interleukin-2; Treg, regulatory T cells.

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Conclusion: Monitoring immune cells along with the determination of their functional capacity provides a comprehensive assessment as a prerequisite for a personalized immunotherapeutic PDAC treatment.

1. Introduction

In western countries pancreatic ductal adenocarcinoma (PDAC) is the 4th most lethal tumor with an increasing prevalence [1]. With ~6%, the overall 5-year-survival rate is still low which is partly due to the fact that the disease is generally diagnosed in an advanced stage, limiting curative therapeutic options to fewer than 20% of the patients. Additionally, most PDAC patients exhibit a profound resistance to radio- or chemotherapy, thus further worsening the patient prognosis [2]. Thus, an improved diagnosis allowing the detection of the disease at earlier stages as well as more efficient therapeutic strategies are urgently needed. In the latter context, several novel therapeutic concepts including targeted therapies or immunotherapies either alone or in combination with chemotherapy have been evaluated in clinical studies but as yet fail to considerably improve the prognosis and survival rate of PDAC patients [3–5].

One reason for the failure of therapeutic strategies including immunotherapy might be the immunosuppressive stromal microenvironment of PDAC accounting for up to 80% of the whole tumor mass. The PDAC stroma is mainly composed of extracellular matrix, myofibroblasts and various immune cells, such as macrophages and T cells [6,7]. Several reports indicate that these non-neoplastic stromal cells are largely immunosuppressive and create a tumor-promoting environment for tumor cells [6,8–14]. Accordingly, high numbers of tumor associated immunosuppressive macrophages, regulatory T cells (Treg) and Th2 cells have been found to correlate with advanced tumor stages, reduced patient survival and poor prognosis [8–14]. Moreover, a high number of stromal $CD4^+$ and $CD8^+$ T cells along with a concomitant low number of Treg was correlated with longer survival of PDAC patients [13,14]. Therefore, the immune status of PDAC patients is important not only for predictive purposes but also as a prerequisite for immunological treatments. Gustafson et al. recently demonstrated a novel flow cytometry based approach by which the absolute leukocyte cell number is determined in whole blood of cancer patients resulting in defined and more accurate immune profiles [15].

Recently, as comprehensive stromal characterization of tumor tissues from 42 PDAC patients with the most frequently operated disease stage T3N1M0 revealed a low, but considerable accumulation of γδ T cells in the ductal epithelium as well as in the stroma close to the ductal epithelium [7,16]. Thus, these findings indicate that γδ T cells are able to infiltrate into PDAC and, moreover, enrich in close proximity to tumor cells even in late stage tumors pointing to a possible role of these cells in the immune response against PDAC. γδ T cells are of particular interest in current anticancer therapy because of their ability to kill tumor cells in a non-HLA-restricted manner [17–20]. However, the fact that PDAC development was not prevented might be explained either by their low frequency or their suppressed activity caused by the surrounding immunosuppressive microenvironment. Recently, we demonstrated that the adoptive transfer of γδ T cells in combination with the novel tribody [$(Her2)_2xV\gamma9$] potently reduced growth of PDAC cells subcutaneously inoculated into immune-deficient mice [16]. Albeit the composition of the tumor stroma in these mice does not fully mirror the situation in human PDACs, these findings clearly support the potential of these cells for immunotherapy of PDAC patients. However, a successful clinical application of this strategy will essentially rely on a simple but reliable method for i) immune monitoring in order to assess the immune status of PDAC patients and ii) functional characterization of patient-derived γδ T cells.

Accordingly, this study intended to monitor the immune status of PDAC patients by determining absolute and relative cell numbers of different immune cell subsets in small volumes of whole blood. Moreover, these findings were correlated with clinicopathological parameters and compared to the immune status of healthy donors. Finally, αβ and γδ T cells as well as NK cells from PDAC patients and healthy donors were functionally characterized by determining the cytotoxicity towards PDAC cell lines in a Real-Time Cell Analyzer.

2. Material & methods

2.1. Patients

Blood samples from 30 PDAC patients (median age: 67.5) were obtained from the Surgery Department of the Community Hospital Kiel and Department of General and Thoracic Surgery, UKSH Campus Kiel. As control, blood samples from 29 healthy controls (median age: 59.0) were analyzed with informed consent obtained. The research was approved by the ethics committee of the UKSH (reference number: D430/09). Pathological features of all tissues were assessed according to WHO classification and UICC TNM staging. Characteristics of patients and healthy donors are summarized in Supplementary Table 1. All patients had not been chemo- or radio-therapeutically treated before this investigation.

2.2. Isolation of PBMCs from blood

Human peripheral blood mononuclear cells (PBMCs) were obtained by Pancoll (Pan-Biotech GmbH, Aidenbach, Germany) or Ficoll-Hypaque (Biochrom, Berlin, Germany) density-gradient centrifugation of heparinized venous blood as described [16].

2.3. Monitoring of absolute cell number of leukocyte subpopulations

Whole blood samples (50 μl) from healthy donors or PDAC patients were analyzed using the BD Multitest™ 6-color TBNK reagent with BD Truecount™ tubes (BD Biosciences, Heidelberg, Germany) using the protocol provided by the supplier. In addition, γδ T cells were quantified by staining 50 μl of whole blood in BD TrueCount™ tubes as already described [16,21]. Additionally, we quantified peripheral blood monocytes according to their granularity and $CD45^{high}$ staining from these whole blood analyses. To determine the amounts of regulatory T cells (Treg) in whole blood samples, 50 μl of blood were stained with the following fluorochrome-conjugated antibodies: CD45-PECy7 clone HI30, CD4-FITC clone SK3 (both from BD Biosciences), CD25-APC clone CD25-361 (Thermo Fisher Scientific, Schwerte, Germany), and CD127-PE clone hIL-7R-M2 (BD Biosciences). After lysing red blood cells with 500 μl BD Lysing-solution, cells were spun down, washed with PBS containing 1% BSA and 0.1% sodium azide, and finally resuspended in 1% PFA prior to flow cytometric analysis on a FACS

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