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A myeloid and lymphoid infiltrate in murine pancreatic tumors exposed to plasma-treated medium

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ABSTRACT

Metastatic pancreatic cancer often is fatal in patients. Palliation can include disseminating large amounts of chemotherapeutic liquid in the peritoneal cavity to slow tumor growth. We have previously demonstrated that repeated application of plasma-treated medium performed well in decreasing peritoneal tumor burden in mice and prolonging animal survival. We here extend on this study by detailed immune-related analysis of cryo-conserved tumor nodes of mice that had received either cell culture medium alone or plasma-treated cell culture medium. Animals of the treatment group had significantly fewer lesions, which were characterized by an increased influx of macrophages. The staining intensity of CD206, a murine M2 macrophage marker associated with tumor promotion, was decreased in tissue sections, while iNOS (M1 marker associated with inflammatory macrophages) was not changed. In the infiltrate, other myeloid cells such as neutrophils and dendritic cells were in tendency increased and decreased, respectively. Further, we observed a significant increase in T cells and calreticulin staining, suggesting an involvement of immunogenic cancer cell death in plasma-treated medium therapy of pancreatic tumor lesions. In summary, exposure to plasma-treated medium not only decelerates tumor growth but also serves as immunomodulatory agent with a possible relevance for therapeutic outcome.

1. Introduction

Diffuse metastatic spread of pancreatic cancer in the peritoneal cavity presents only few therapeutic options. During palliation, standard therapy of peritoneal lavage with gemcitabine or other drugs is of only limited advantage for patients [1]. Together with an increasing incidence and number of pancreatic cancer deaths [2], the need for new therapeutic avenues becomes apparent. We recently reported that tumor-bearing mice suffering from metastatic spread of pancreatic cancer benefited from repeated intraperitoneal application of plasma-treated medium [3]. Such an innovative route of application was recently proposed also by others for the use against cancer [4–6]. In mice, promising effects of plasma-treated medium were observed also against peritoneal spread of gastric cancer [7].

Despite encouraging results of this novel therapy, mechanistic understanding in how this solution propagates its effect in tissues is only poorly understood [8]. Plasma-treated medium may contain oxidation products (proteins and lipids) and long-lived oxidative reagents such as hydrogen peroxide and nitrite [9–11]. While plasma-treated medium is relatively easy to investigate, intratumoral cellular dynamics pose larger experimental challenges. Especially the contribution of immune cells in anticancer responses is receiving increasing scientific attention as it is a sensitive predictor of therapeutic outcome [12]. In pancreatic cancer, the intratumoral dominance of tumor-supportive M2 macrophages over pro-inflammatory M1 macrophages is associated with poor survival [13]. This balance and the presence of other immune cells infiltrating the tumor is linked to whether therapy-induced apoptosis is of tolerogenic or immunogenic nature [14]. Especially ecto-calreticulin (CRT) is a key protein facilitating immunogenic cancer cell death (ICD) and subsequent induction of tumor-reactive T cells [15].

In this study, we investigated the myeloid and lymphoid infiltrate in murine tumors repeatedly exposed to plasma-treated medium. Marker expression associated with M1 and M2 macrophages as well as CRT as predictor of ICD was studied and linked to therapeutic efficacy.

2. Materials and methods

2.1. Animal experiments and tumor nodes

6606PDA murine syngeneic pancreatic tumor cell lesions were

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Table 1
Overview of cell surface marker labeling in this study.

Name	Function	Typically expressed on
Calreticulin	Quality control chaperone of proteins in the endoplasmic reticulum, prevents misfolded proteins from export, binds calcium ions, pro-phagocytic signal [50,51]	Cells dying the immunogenic cell death
CD3	T cell co-receptor important in recognizing peptides presented on major histocompatibility complexes [52]	Cytotoxic and T helper cells
CD11c	Leucocyte Function Associated (LFA) molecule, role in cell-cell interaction and recognition of stimulated endothelial cells [53]	High levels on dendritic cells, also found on the surface of other myeloid cells
CD206	Mannose receptor clearing glycoproteins and recognizing protein glycans on the surface of microorganisms [54],[55]	M2 macrophages and tumor-associated macrophages
F4/80	Endothelial growth factor-like domain receptor (EMR1) [56]	Marker for macrophages in mice
iNOS	Inducible nitric oxide synthase catalyzing the production of nitric oxide from L-arginine [57]	(M1) macrophages, endothelial cells
Ly6G	Lymphocyte antigen 6 complex locus G6D is a developmental marker in myeloid cells [58]	Differentiated and matured Neutrophils

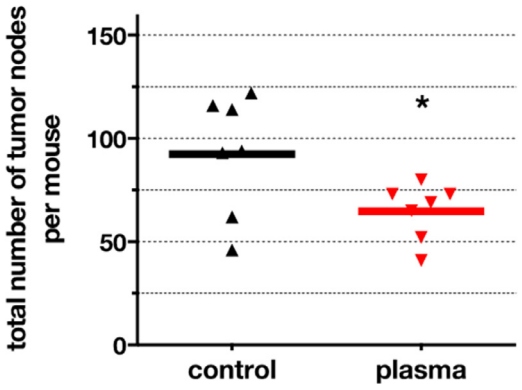

Fig. 1. Plasma-treated medium decreased the total number of intraperitoneal tumors. Seven days after tumor challenge, tumor-bearing mice were repeatedly exposed to 1 ml of plasma-treated or control medium over 28 days. On day of sacrifice, the number of tumor nodes of 7 mice were counted. The treatment group exhibited significantly lower number of intraperitoneal tumors.

retrieved from a previous study in C57BL/6 mice (12 animals per group), where 21 daily intraperitoneal injections of 1 ml DMEM F12 cell culture medium exposed to the plasma of a certified atmospheric pressure plasma jet (kINPen MED, 5 standard liters per minute of argon) was performed in mice seven days after tumor challenge. Specifically, 5 ml of cell culture medium were exposed to plasma for 10 min. Experiments were approved by the State agency for agriculture, food safety, fishery Mecklenburg-Vorpommern (LALLF-MV, application number 7221.3-1.1-003/14) [3]. After animal sacrifice, tumor nodes were collected and either immediately cryo-conserved or embedded in paraffin.

2.2. Tissue sections and staining

Paraffin sections of 4 µm were generated followed by fixation with 4% paraformaldehyde (Sigma, Germany), permeabilization with 0.5% Triton X-100 (Sigma), and peroxidase block (Dako, USA). Slides were washed and stained for macrophages using an anti-F4/80 antibody (BioRad, Germany). An anti-rat HRP-labelled antibody and DAB substrate was added before hematoxylin staining and sample mounting on microscopy slides using Faramount (all Dako). For fluorescent microscopy of immunologically relevant molecules, tissue sections were stained with either of the following anti-mouse antibodies: CD11c Alexa Fluor 488 (BioLegend, UK); calreticulin (Enzo, Germany) and anti-rabbit superboost Alexa Fluor 647 (life technologies, USA) according to the manufacturer's instructions; CD206 Alex Fluor 488; and inducible nitric oxide synthase (iNOS) Alexa Fluor 594 (Gentaur, Belgium). Nuclei and DNA were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

2.3. Tissue section imaging and quantification

DAB staining was imaged using a Keyence BZ-9000 microscope. Results were evaluated with BZ-II-Analyzer 4.6.2.2 software (Keyence, Japan). Fluorescence staining was imaged using an Operetta CLS high content imaging system (PerkinElmer, Germany). To retrieve whole tissue section images, the slide was pre-scanned at low resolution (1.25x NA 0.03) and DAPI areas were re-scanned at higher resolution (20x NA 0.4) with different fluorescent channels in parallel. To detect DNA extrusion at the boarder of tissue sections, images were acquired on an Axio Observer Z.1 (Zeiss, Germany) using a 63x objective. To obtain whole tissue images, individual images were stitched with Harmony 4.6 software (PerkinElmer), which was also utilized for quantitative image analysis. For the latter, cells within individual images were segmented by identifying the nuclear (DAPI) region and slightly increasing the segmentation border. $CD206^+$ or $CD11c^+$ cells were counted by calculating the mean fluorescent intensity of this marker channel for each cell, and thresholding the final count to remove background signals. To compare signal intensities for iNOS and CRT, the mean contrast of segmented cells was calculated (in $CD206^+$ cells for iNOS), as a partially lower staining intensity was not always compatible with the thresholding algorithm. The advantage of quantitative image analysis is its unbiased nature, as the same analysis sequence is applied to all samples and fields of views, yielding quantitative and unsupervised data. In this study, over 10.000 fluorescent images were acquired and analyzed in this fashion. An overview of the markers used in this study is given in Table 1.

2.4. Flow cytometric analysis of immune infiltrate in tumor nodes

Total weight-corrected tumor mass from cryo-conserved material, stemming from three different animals per group, were digested using the mouse gentle macs tumor dissociation kit and the gentle macs octodissociator with heaters (both Miltenyi Biotec, Germany) according to the manufacturer's instructions. Cells suspensions were incubated for 10 min with red blood cell lysis buffer, washed, and fixed and permeabilized with fixation buffer and permeabilization buffer (all BioLegend), respectively. After washing, suspensions were pooled and incubated with FC-block (BioLegend) prior to addition of monoclonal antibodies conjugated with the following fluorochromes and directed against the following epitopes: F4/80 PE-Dazzle, CD3 PE-Cy7, CD45 Alexa Fluor 700, and Ly6G APC-fire (all BioLegend) as well as DAPI. After washing, suspensions were filtered, and analyzed by flow cytometry (Gallios; Beckman-Coulter, USA) using dedicated optics for multicolor flow cytometry. Data analysis was performed with Kaluza 2.0 software (Beckman-Coulter). An overview of the markers used in this study is given in Table 1.

2.5. Statistical analysis

Statistical analysis was performed using prism 7.04 (GraphPad