

**Progress report**  
**IAP-APA Young Investigator Grant in Pancreatology**

**Neuroimmune crosstalk regulates anti-tumor  
immune response in pancreatic cancer**

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## **Title: Neuroimmune crosstalk regulates anti-tumor immune response in pancreatic cancer**

Starting on the 1<sup>st</sup> of August 2024, the IAP-APA founded the following proposed project:

**WP 1: Phospho-proteomic analysis using LC/MS-MS to explore cytokine-related pathways and comprehensively characterize intracellular signaling and post-translational modifications in fresh-frozen tumor-altered DRG neurons from different transgenic mouse models of PCa.** Pancreas-innervating DRG neurons (Th8-12) will be dissected from the newly established neuroinvasive TPAC (Ela-TGF $\alpha$ ; Ptf1a-Cre; Trp53fl/fl; RelAfl/fl, n=6, 48-50 weeks old) mice, the Kras-based KPC (Ptf1a-Cre; LSL-Kras+/G12D; Trp53+/fl, n=6, 20-22 weeks old) and age-matched littermates without Ptf1a-CRE allele mice (Ela-TGF $\alpha$ ; Trp53fl/fl; RelAfl/fl, n=5, 48-50 weeks old for TPAC and LSL-Kras+/G12D; Trp53+/fl, n=5; Trp53+/fl, n=5 and LSL-Kras+/G12D; n=5, 20-22 weeks old for KPC). In cooperation with the Proteomics Core Facility at TUM, we plan to validate immune-related targets identified by spatial transcriptomics and analyze phosphorylation of downstream genes by phospho-proteomic profiling. The TMT11-plex labeled full proteom and IMAC-enriched phospho-peptides will be measured in data-dependent acquisition mode using a nanoflow LC-MS/MS by coupling a Dionex Ultimate 3000 UHPLC + system to an Eclipse Tribid mass spectrometer (Thermo Fisher Scientific).

**WP 2: Modulation of neuronal chemokine expression by generation of DRG-neurons-specific knock-outs and subsequent orthotopic implantation of KPC cells.** Treatment of AvCreERT2; Ccl2fl/fl mice with tamoxifen results into conditional depletion of Ccl2 gene in Avil (advillin) cells, namely DRGs and sensory neurons. Tumor cells from a primary cell line from KPC mutant mice (Kras+/G12D; Trp53fl/+Ptf1a+/Cre) will be orthotopically implanted into the pancreas of 6-8 week old AvCreERT2; CCL2fl/fl mice (n=10) and control AvCreERT2 mice (n=10) after tamoxifen treatment. Tumor size will be monitored weekly via abdominal ultrasound. Organ retrieval is planned on day 85 after transplantation. The following parameters will be quantified: tumor size, metastasis, composition of infiltrating immune cell and neural invasion. Here, we hypothesize that chemokine depletion in sensory neurons will reduce neutrophil chemotaxis into the perineural niche, thereby restoring the anti-tumor immune response and slowing PCa progression. Tumor growth and metastasis will be examined macroscopically and histologically. The composition of infiltrating immune cells into pancreatic tumors will be analyzed by flow cytometry of dissociated solid tumors with enzymatic digestion in the gentleMACSTM Octo Dissociator (Milteny). The following established antibody panel will be used: CD45-FITC, CD8a-PB, CD4-PE, B220-PECy7, CD11b-APC-Cy7, Gr1-APC (all from eBioscienceTM). In addition, for multiplex immunofluorescence staining (VectraPolaris/Akoya), we have developed a 7-plex immunopanel for spatial characterization of TAN (CD11b+ Gr1+) in relation to nerves (PRPH+, GFAP+), tumor-associated fibroblasts ( $\alpha$ SMA+) and tumor cells (PanCK+). We will also analyze the immunosuppressive role of tumor innervating DRGs in vitro. For this, we will sort CD45+CD8a-CD4-CD11b+Gr1+ neutrophils from murine spleens on MoFlo Astrios EQ sorter (CyTUM Core Facility) and analyse migration of these cells in a 3D-neural migration assay to DRG neurons isolated from the AvCreERT2; CCL2fl/fl mice and AvCreERT2 control mice. In this assay cells are suspended in an extracellular matrix/ECM gel drops that are connected with a 7mm ECM bridge. Cell migration will be quantified by digital time-lapse microscopy with an observation interval of 15 minutes with Zeiss Observer D1 system during a total of 72 hours.

**WP 3: Multispectral imaging and subcellular mapping of immune-related RNA targets in selected human PCa FFPE tissue cores with neural invasion using spatially resolved PhenoCyclerTM and Xenium in Situ (10x Genomics) technology.** Neural- and immunophenotyping in human PCa will be performed using multispectral imaging in collaboration with PD Dr. Katja Steiger (Head of the Core Facility Comparative Experimental Pathology/CEP) at the Institute of Pathology at TUM. We customized a 19-plex antibody panel

(Table 1) to characterize the immune landscape populating the perineural niche in PCa and to characterize the subtypes of nerves associated with PNI. Whole-slides digital images obtained with a Phenocycler<sup>TM</sup> (Akoya) will be analyzed using object-based automated cell detection analysis with QuPath/Cell Profiler software. In addition, we will examine the transcriptional profile of tumor-invaded nerves using the Xenium in Situ platform (10x Genomics), also located in the CEP Core Facility. A tailored panel of 300 genes for immune target validation will be customized after integration of data obtained in the proteomic and spatial transcriptomic approach of tumor-activated DRG neurons. TMA blocks with 2mm diameter tissue cores of selected areas containing nerves with and without neural invasion from 25 PCa patients have been prepared from the archived FFPE blocks and are available for further processing.

Immunophenotype	Immune checkpoint	Tumor markers	Neural markers
CD3 (EP449E, Abcam), CD4 (EPR6855, Abcam), CD8 (C8/144B, BioLegend), FOXP3 (236A/E7, Thermo Fisher), CD19 (LE-CD19, BioRad), CD20 (riGEL/773, Novus Biologicals), CD68 (KP-1, BioLegend), HLA-DR (EPR3692, Abcam)	PD-1 (RM309, RevMab), PD-L1 (RM320, RevMab), OX40 (, LAG3 (D2G40, CST), IDO1 (V1NC3IDO, Thermo), ICOS	PanCK (C11, BioLendend)	NeuN (14H6L24, Thermo Fisher), S100 (S100A4, BioLegend), TH (NB300-109, Novusbio), ChAT (PA1-9027, Thermo Fisher)

Table 1. Customized 19-plex panel designed for immunophenotyping of pancreatic neural invasion via multiplex immunofluorescence

**To point 1:**

An optimized protocol for DRG isolation compatible with the TMT11-plex labelled phosphoproteome analysis was established. As our study focuses on tumor-innervating DRGs located between Th8 to Th12, the maximum number of pancreas-innervating DRGs that we can obtain per mouse for the phosphoproteome analysis is 10 DRGs. On a first approach, to achieve a high protein yield from this relatively small amount of tissue, we optimized our DRG collection protocol by adding a cocktail of phosphatase and protease inhibitors, in contrast to PBS in standard proteomics protocols, into ProteinLoBind tubes. Moreover, to determine the minimum amount of DRGs necessary for the phosphoproteome analysis, we analyzed the DRG-specific protein content and phosphoproteome profile based on the number of DRGs analyzed per sample. We included 3 groups with 4 DRGs, 8 DRGs, or 16 DRGs per sample. As our cut-off value for future phosphoproteome analyses, we determined the minimum protein amount as 20ug/ sample. After running the TMT11-plex labelled phosphoproteome assay, the results showed that all samples in the 8 DRG group had a protein amount higher than the cut-off value of 20ug (Figure 1A, 1B). Based on these results, we will proceed with the experimental cohort using 10 DRGs per mouse.

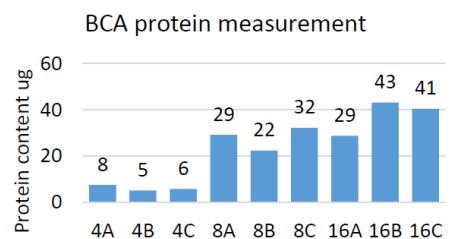
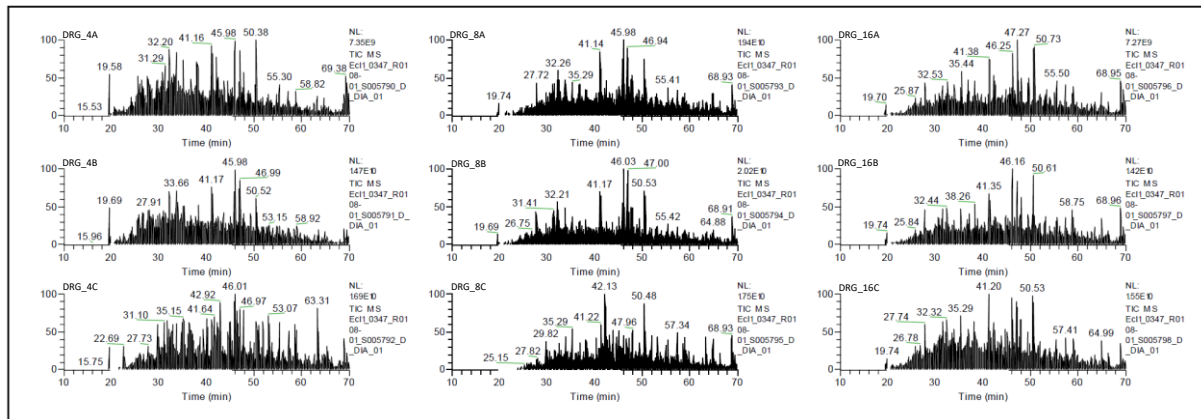


Figure 1. BCA protein measurement of DRG samples. The cut-off value was exceeded with the 8 DRG group.



**Figure 2. Total ion current and protein content of DRG samples. A.** Triplicates for 4 DRG, 8 DRG and 16 DRG groups each.

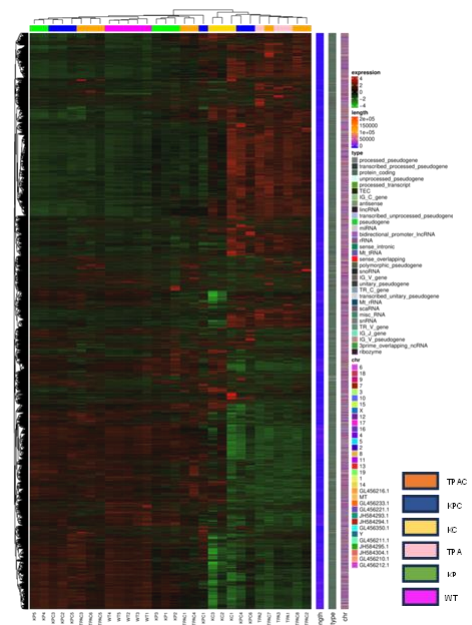
During the first funded period, we were also able to collect most of the samples necessary for the definitive experiment which is planned for the end of May 2024, which include isolated pancreas-innervating DRGs from 6 mice per group, as stated in Table 1.

Mouse model	Genotype	N° of mice
TPAC	Ela-TGF $\alpha$ ; Ptf1a-Cre; Trp53fl/fl;RelAfl/f	6
TPA	Ela-TGF $\alpha$ ; Trp53fl/fl;RelAfl/fl	6
KPC	Ptf1a-Cre; LSL-Kras+/G12D; Trp53+/fl	6
KP	Ptf1a-Cre; Trp53+/fl	6
KC	Ptf1a-Cre; LSL-Kras+/G12D	0

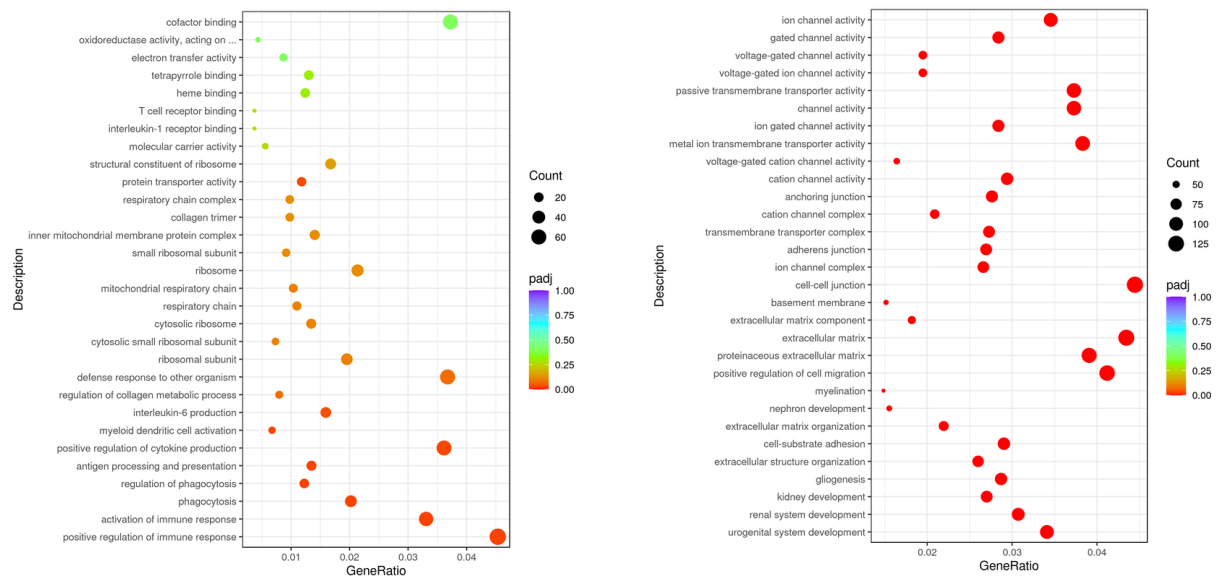
**Table 1. Mouse models planned for phosphotranscriptomic analysis of DRGs in PDAC.**

Using this optimized protocol, we will supplement our spatial transcriptomic RNA sequencing-based characterizations of DRG neurons to elevate our understanding of sensory neurons' role in PDAC carcinogenesis and neural invasion.

Furthermore, we also performed bulk-RNAseq analysis on the same mouse models included in our former spatial transcriptomic analysis and on our phosphoproteom analysis. The results of the bulk RNA sequencing was confirmatory in nature to our spatial transcriptomic analysis. Here, DRGs collected from the tumor models TPAC and KPC formed a converged transcriptomic cluster (Figure 3). In gene-set enrichment analyses, most significantly upregulated pathways in KPC mice compared to their age-matched controls were related to activation and positive regulation of immune response (Figure 4). Here, chemokines CXCL14 ( $p < 0,0005$ ), CCL8 ( $p < 0,005$ ), CCL6 ( $p < 0,05$ ), CXCL9 ( $p < 0,05$ ), CXCL12 ( $p < 0,05$ ) and were significantly upregulated. This chemokine-mediated activation of immune response was reflected in TPAC mice with a significant upregulation of CCL21a ( $p < 0,05$ ) and CCL25 ( $p < 0,05$ ) compared to their age-matched controls.



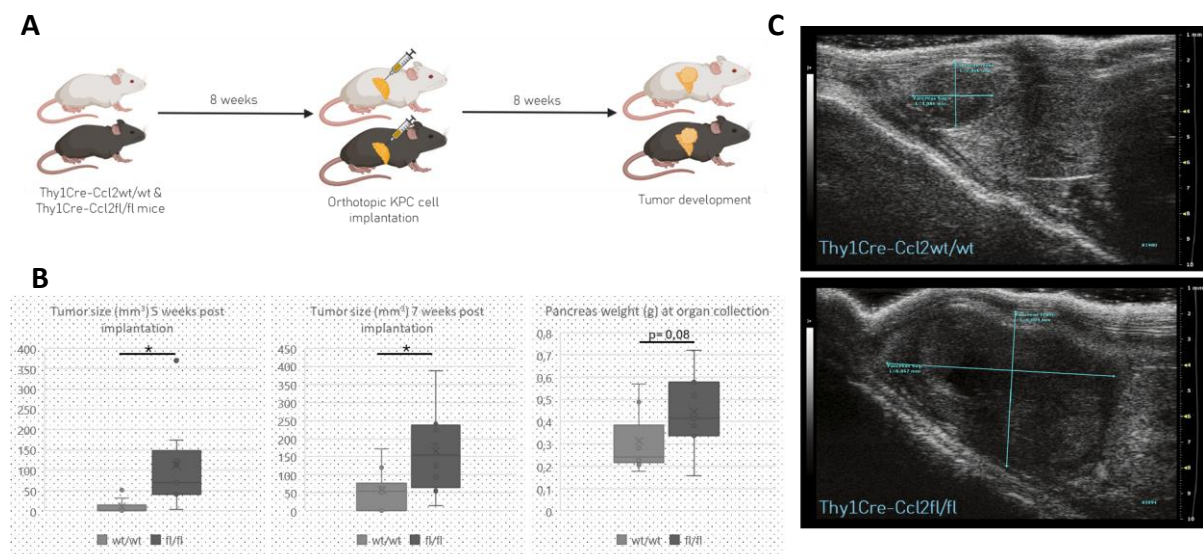
**Figure 3. Bulk RNA sequencing of DRGs- Heat map.**



**Figure 4. Gene set enrichment analysis on KPCvsKP and TPACvsTPA DRGs**

**To point 2:**

As stated in the project proposal, we generated a sensory neuron-specific knockout of the chemokine *Ccl2*, termed *Thy1Cre-Ccl2fl/fl* mice. We then performed an orthotopic implantation with 500.000 KPC cells at 8 weeks of age on these mice (Figure 5A). As controls, *Thy1Cre-Ccl2wt/wt* mice were used. Abdominal ultrasound was performed weekly to monitor tumor size. Organ retrieval was performed 56 days after transplantation. No significant difference in metastatic formation was detected with one liver, colon, and spleen metastasis in the *Thy1Cre-Ccl2fl/fl* group and two spleen and one peritoneal metastasis in the *Thy1Cre-Ccl2wt/wt* group. Surprisingly, *Thy1Cre-Ccl2fl/fl* mice had significantly faster tumor engraftment and tumor growth than their counterparts. For instance, the mean tumor volume was 111,44mm<sup>3</sup> in the *Thy1Cre-Ccl2fl/fl* group compared to 11,25mm<sup>3</sup> in the *Thy1Cre-Ccl2wt/wt* group five weeks post-implantation. The same trend was observed at seven weeks, with 165,25mm<sup>3</sup> vs. 58,00mm<sup>3</sup> (Figure 5B). The ultrasound findings were confirmed via the pancreatic weight upon organ collection, with a mean weight of 0,44g in the *Thy1Cre-Ccl2fl/fl* group compared to 0,31g in the *Thy1Cre-Ccl2wt/wt* group (Figure 5C). In the following steps, we will get mechanistic insight into the significant functional role of *Ccl2* by quantifying the neuronal infiltration and different types of immune cells within the tumor microenvironment using immunohistochemistry and immunofluorescence stainings.

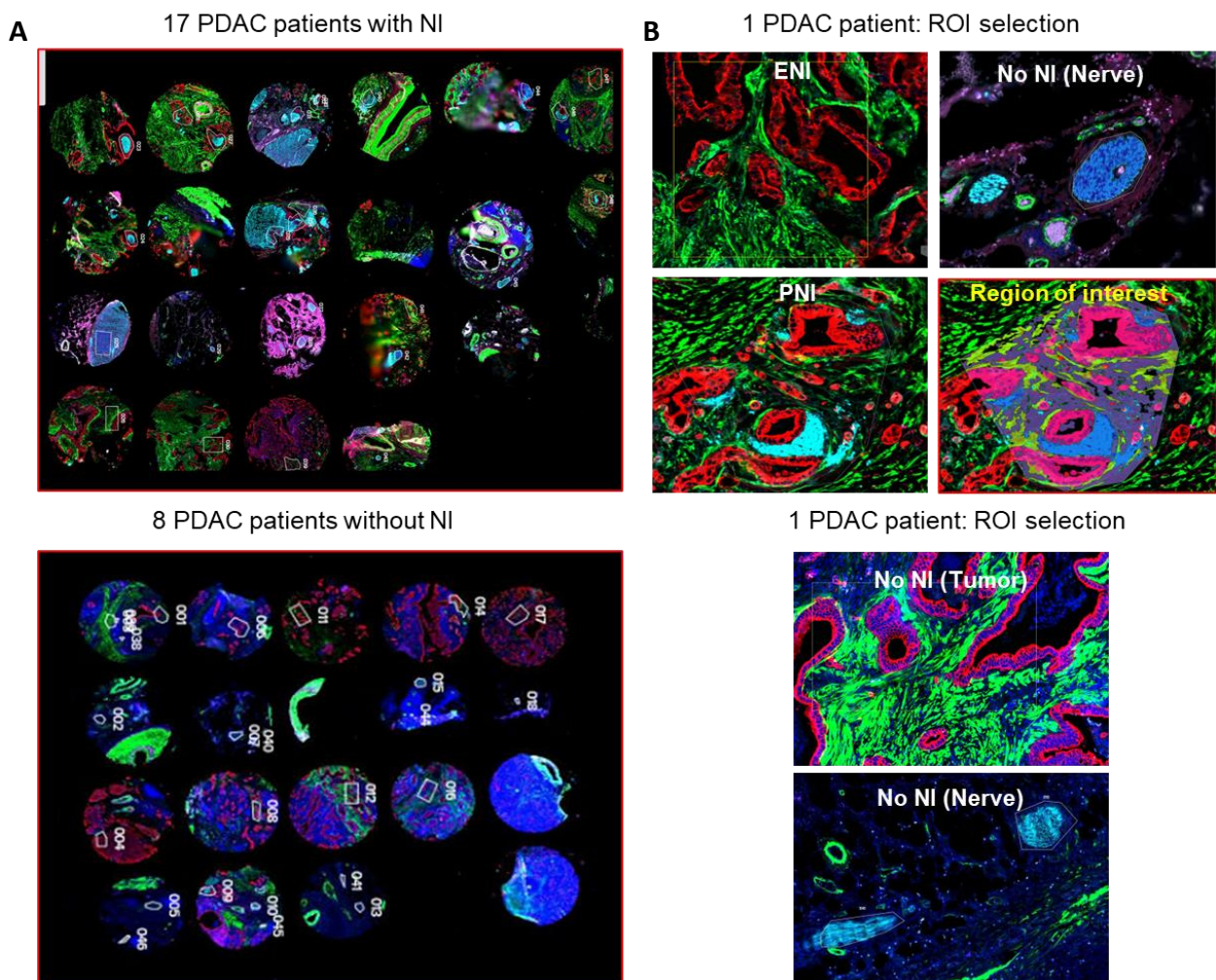




**Figure 5. Orthotopic transplantation of KPC tumor cells in Thy1Cre-Ccl2fl/fl mice. A.** Schematic representation of the experimental design. **B.** Boxplots showing the tumor size and pancreas weight on orthotopically transplanted wildtype and Thy1Cre-Ccl2fl/fl mice. **C.** Representative images of ultrasound monitoring of tumor growth throughout the experiment.

To point 3:

To customize the gene panel for Xenium analysis and determine the expression profile of pancreatic cancer cells and the nerves involved in neural invasion, we first conducted a spatial transcriptomic analysis using the Digital Spatial Profiler (DSP) and the Human NGS Whole Transcriptome Atlas. TMAs from human PCa had been selected based on the presence of perineural invasion. This approach enables to analyze gene expression in selected region of interest, however, in contrast to the Xenium technology, does not provide single cell resolution. Each slide was stained with fluorescence-labeled antibodies to allow identification of tissue: pan-cytokeratin for epithelial cells and B3-tubulin for nerves. Tumor and Nerve areas of interest (AOI) were selected as follows: (i) NI Tumor: nerve-invading pancreatic (N=34); (ii) no NI Tumor: non-invading tumor cells (n=23); (iii) NI Nerve: tumor-invaded nerve (n=34); (iv) no NI Nerve: non-invaded nerve (n=30). AOIs were drawn manually around nerves and tumor cells. Following the ROI selection, UV- photocleaved oligonucleotide barcodes from each ROI were collected and prepared by the NanoString GeoMx protocol. Sequencing was performed on the NextSeq 2000. The bioinformatic analysis is currently on-going and we expect the definitive results in Mid-June.



**Figure 5. Experimental design of spatial transcriptomic analysis of pancreatic cancer neural invasion using Nanostring DSP. A.** TMAs in PDAC patients with NI and without NI. **B.** Representative images of pancreatic neural invasion, the regions of interest are also exemplified.