ID: 02674
Type: Poster
Topic: Mechanism of tumour initiation and progression

ANAPLASTIC LYMPHOMA KINASE CONTRIBUTES TO THE IMMUNOEVASIVE AND METASTATIC PHENOTYPE OF PANCREATIC DUCTAL ADENOCARCINOMA

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INTRODUCTION: we have recently published that anaplastic lymphoma kinase (ALK) contributes to PDAC aggressiveness by supporting pancreatic cancer stem cell (PaCSC) features such as self-renewal, tumorigenicity and chemoresistance through ligand-dependent activation (*Parejo-Alonso et al., Biomed Pharmacother. 2023*). In addition to these characteristics, PDAC aggressiveness is also attributable to its metastatic and immunosuppressive landscape. The latter is characterized, among others, by infiltration of M2-polarized protumoral macrophages (TAMs). However, ALK implication in both the immunoevasive and metastatic processes has not been linked to date.

OBJECTIVES: to evaluate the contribution of ALK in the immunoevasive and invasive potential of pancreatic cancer (stem) cells.

METHODS: *primary cultures:* PDAC patient-derived xenografts (PDXs) and patient circulating tumor cells-derived xenografts (CTCs). PDAC murine cell line: CHX2000 cells isolated from a liver metastasis generated in the KPC (*Kras$^{G12/+}$; Trp53$^{R172H/+}$; P48-Cre*) PDAC GEMM. *Treatments:* MCM (M2-polarized macrophages conditioned medium), ALK ligands (Midkine and Pleiotrophin), clinically-approved ALK inhibitors (Crizotinib, Ensartinib and Alectinib), chemotherapy (Gemcitabine and Paclitaxel). *Gene expression:* transcriptomic bioinformatic analyses, real time quantitative PCR (RTqPCR). *Signaling:* Western Blot. *CSC-enriching cultures:* PDXs grown as spheroids. *Pro-metastatic abilities:* scratch wound healing assay, invasion assay in microfluidic devices, invasion assay in Boyden Chambers. *In vivo treatment exploratory assay:*orthotopic injections of murine PDAC cells in immunocompetent mice. *Experimental metastasis assay:* human PDAC cells injected in the spleen of immunocompromised mice. *Statistical analyses:* data are represented as mean ± SEM and analysed using t test or one-way ANalysis Of VAriance (ANOVA) of, at least, three independent experiments (unless otherwise specified). * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

RESULTS: first, an *ALK* overexpression (OE) gene signature previously described was robustly associated with TAMs infiltration and *PD-L1* expression in PDAC samples from the TCGA and GTEx datasets. Interestingly, incubation of PDAC cells with TAMs conditioned medium (MCM), previously shown to enhance CSC properties in PDAC *(Sainz et al., Cancer Res. 2014),* resulted in a rapid and potent phosphorylation of ALK receptor and increased *PD-L1* mRNA expression levels. In addition to this, treatment with ALK inhibitors, alone or in combination with Gemcitabine, decreased the expression levels of some immunoevasion markers *in vitro*. Consistently, an exploratory *in vivo* experiment in immunocompetent mice bearing orthotopic PDAC revealed decreased M2 macrophages infiltration after a combined treatment of chemotherapy and Crizotinib, while the infiltration of CD4 and CD8 T lymphocytes increased. These results are in line with previous *in vitro* results showing increased expression of some immunoevasion markers in CSC-enriching conditions, as the main cell population responsible for scaping the immune system and resist chemotherapy. On the other hand, our previously published gene set enrichment analysis showed upregulation of epithelial-to-mesenchymal transition (EMT) pathway in PDAC patients with high *ALK* levels compared to those with low *ALK* expression. In addition, the *ALK* OE signature mentioned above correlated with the expression of an EMT signature composed by *SNAIL*, *SLUG*, *ZEB1* and *LOXL2*. Incubation of PDAC cells with the ALK ligands Midkine (MDK) and Pleiotrophin (PTN) accelerated migration. Conversely, ALK targeting reduced invasion trajectories of PDAC cells in microfluidic devices, as well as decreased invasiveness of both local and metastatic PDX models. Importantly, ALK inhibition with Crizotinib and Alectinib

reduced metastatic incidence in intrasplenic PDAC-bearing immunocompromised mice.

MAIN CONCLUSIONS: targeting ALK receptor with clinically approved compounds may reeducate the immunosuppressive microenvironment and impair metastatic dissemination in PDAC.