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Imaging-based spatial phenotyping:

From target discovery to preclinical evaluation

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Background

Imaging-based spatial phenotyping is a powerful tool that can be applied to several steps of the drug discovery pipeline, from the early identification of putative protein targets and biomarkers to late stage, preclinical characterization of cellular responses to therapeutics. However, current technologies can only provide information for a few selected proteins, with limited spatial and single-cell resolution, thereby offering a restricted insight into the complexity of the diseases.
To overcome these limits, Miltenyi Biotec developed the MACSima™ Platform, which enables fully automated, imaging-based spatial phenotyping of hundreds of proteins, providing detailed information about their localization, quantification, and interactions down to single-cell level on several types of samples.

In this study about the discovery of targets for chimeric antigen receptor (CAR) T cell–based immunotherapy for pancreatic cancer, we applied the MACSima Platform to validate the tumor specificity of previously screened targets[1], as well as to better understand the biology behind the therapeutic responses in preclinical models. This application note is based on the linked reference[2].

Methods

Sample collection and preparation

Tumor tissues from pancreatic ductal adenocarcinoma (PDAC) patients undergoing surgical resection of tumor mass were collected at the University Medical Center Göttingen. All preclinical mouse models were obtained from Charles River Discovery Research Services Germany GmbH. All specimens were embedded in Tissue Freezing Medium (Leica) and stored at –70 °C until further use. Afterwards, 8 µm sections were cut on a CM3050 S cryostat (Leica), collected on SuperFrost® Plus slides (Menzel) and stored no longer than two weeks at –70 °C. On the day of use, sections were thawed in –20 °C acetone and further processed. In order to identify neoplastic regions of interest, the hematoxylin and eosin staining was performed on thawed sections, and areas were evaluated by pathologists with light microscopy.

Cyclic immunofluorescence staining

After identification of a region of interest, the fixed section of the same specimen was stored briefly in autoMACS® Running Buffer and then transferred to the MACSima Platform.

The MACSima Platform enabled fully automated, imaging-based spatial phenotyping of hundreds markers on the very same biological samples thanks to Miltenyi Biotec's MACSima Imaging Cyclic Staining (MICS) technology, which is based on a reiterative cyclic staining process. This process is composed of three steps that are all conducted in the MACSima System in a fully automated manner: to begin, the sample is stained with multiple fluorochrome-conjugated antibodies, produced and pretested by Miltenyi Biotec; this is followed by acquisition and subsequent processing of an image with a widefield microscope; to complete the cycle, the fluorescent signal is erased.

This cycle is repeated automatically for as many times as needed, allowing the staining of hundreds of markers on a single sample. The MACSima System can analyze different types of samples fixed with various methods, ranging from tissue sections to adherent cells (fig.1). For the majority of the markers, REAfinity™ Antibodies were used in order to guarantee a background-free and robust analysis. Images were generated and analyzed according to the manufacturer's instructions.

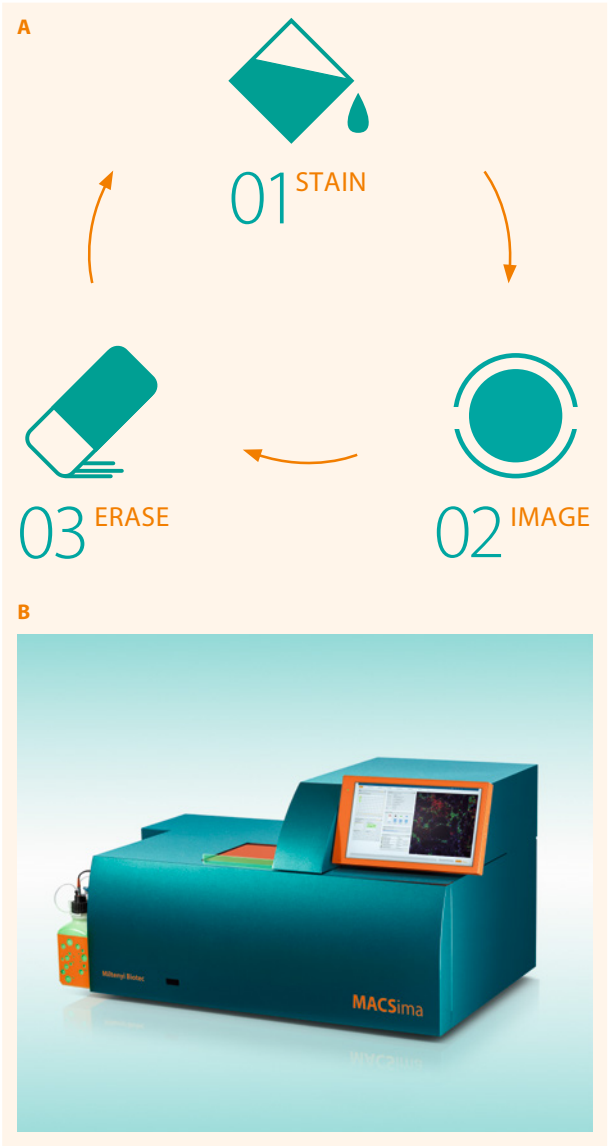

Figure 1: Schematic of the MICS principle (A) and MACSima System (B). The MACSima System performs the three steps of MICS technology in a fully automated manner: 1) The sample is stained, 2) imaged, and 3) the signal is erased. After a cycle is completed, the process automatically continues with the next cycle, enabling the analysis of hundreds of markers on a single sample.

Results

Validation of target candidate expression and tumor specificity

A total number of 50 target candidates was identified via early flow-cytometric screenings[1] on mouse xenografts and PDAC, and further prioritized regarding off-tumor expression based on a bioinformatic ranking (data not shown).

Afterwards, in order to characterize their spatial distribution within the tumor tissues, imaging-based spatial phenotyping was utilized. First, two runs of cyclic IF imaging on two different human PDAC tumor tissues were performed. Observations were centered on identifying target candidates showing colocalization with tumor cells and no or low expression on non-tumor cells.

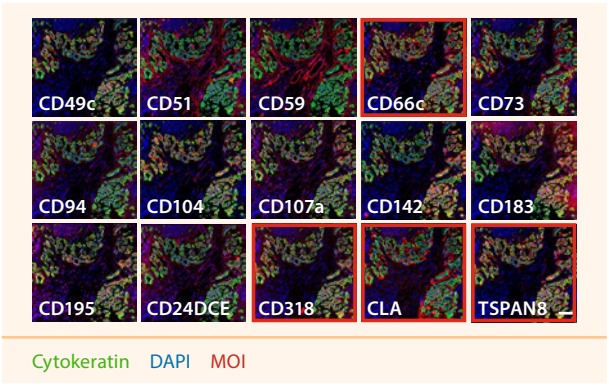

Figure 2: Analysis of target expression and spatial distribution on human PDAC biopsies. Selection of 15 cyclic immunofluorescence images of a representative human PDAC tissue to evaluate co-expression of the marker of interest (MOI) with cytokeratin-positive tumor cells. Images are representative for two MICS runs on two different PDAC specimens. Scale bar = 100 µm.

Putative targets that were ranked promising through previous bioinformatic analysis and then showed specific expression on tumor cells from patients were CLA, TSPAN8, CD318, and CD66c (fig.2). Other candidates, such as CD240DCE, CD195, and CD183, exhibited strong expression on non-tumor cells. Some candidates appeared to be specific to the tumor cells, although they did not belong to the group of high-ranked targets, such as CD49c, CD73, CD104, and CD142, suggesting a generic epithelial reactivity. CD51, CD59, and CD107a had low bioinformatical rank and presented massive expression offside of tumor cells, nicely confirming the value of this approach.

Next, observations were confirmed on preclinical xenograft models, as well as on 17 healthy tissues, the latter with regards to off-tumor target expression (fig.3).

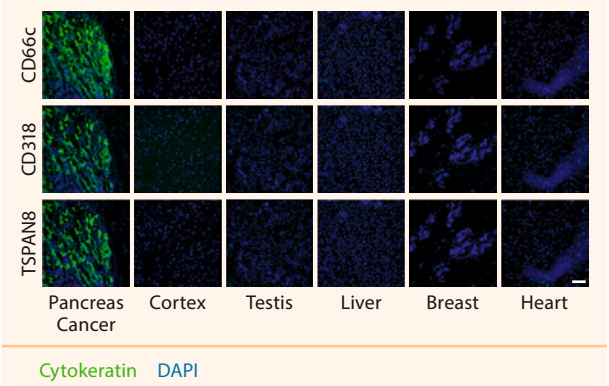

Figure 3: Analysis of target expression on healthy tissues. Representative cyclic immune fluorescence images of several healthy tissues stained with a PE-conjugated marker of interest. Scale bar = 100 µm. Images are representative for at least two regions of interest from one tissue. Regions of interest were chosen based on manual DAPI and Cytokeratin prestaining, and in dependency to the respective tissue size.

Based on these results, CAR constructs for the most promising target candidates were designed and their functionality validated in terms of cytotoxicity, cytokine release, and cell phenotype *in vitro* (data not shown).

***Ex vivo* preclinical evaluation of therapeutic functionality**

Given the *in vitro* functional evaluations, three CAR candidates, TSPAN8, CD318, and CD66c, were selected and further evaluated in a preclinical setting. To this end, firefly luciferase $(Luc)^+$ PDAC cells (AsPC1) were engrafted in NSG mice and, after tumor growth, CAR T cells were injected intravenously. In bioluminescence analysis, potent but heterogeneous anti-tumor responses were detected in the treated group. CAR T cell numbers were then quantified via *ex vivo* flow cytometry and it was observed that this parameter alone was insufficient to explain the therapeutic efficacy (data not shown).

In order to better understand the biology behind the therapeutic functionality, *ex vivo* cyclic immunofluorescence analysis was then performed, and it was concluded that unresponsiveness was not linked to target down-regulation (fig. 4) but possibly rather to low (CAR) T cell and macrophage infiltration (fig. 5).

Indeed, mice treated with CD318 CARs or TSPAN8 CARs achieved heterogeneous but overall potent levels of tumor eradication – with the former being the most promising from a therapeutic perspective – and showed in parallel increased infiltration of (CAR) T cells (CD4, CD8, and LNGFR) and macrophages (mCD68) as compared to those treated with CD66c CAR T cells, which conversely exhibited poor therapeutic outcomes.

Based on this analysis, the therapeutic efficacy of our CAR constructs were verified at different therapeutic magnitudes with an overall high correlation among *in vivo* studies.

NB: Please note that all microscopy images have been compressed for web format and therefore do not reflect the resolution obtained by the instrument.

Conclusion

- The MACSima Platform enables the fully automated, imaging-based spatial phenotyping of an unlimited number of markers on a single sample in terms of marker expression, quantification, and spatial distribution.
- The present data show that the MACSima Platform is a powerful and versatile tool that can support the development of novel therapeutics at different stages of the discovery pipeline, from the early identification and in-depth characterization of targets to the late-stage, preclinical evaluation of biology underlining therapeutic efficacy.

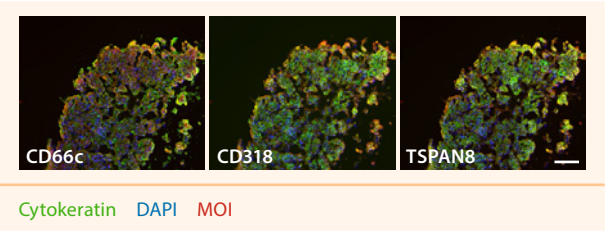

Figure 4: Analysis of target expression upon xenotransplantation of AsPC1 cells *in vivo*. Tumors were grown for 30 days, resected, and analyzed for cyclic immunofluorescence. Scale bar = 100 µm. Images are representative for tumor triplicates.

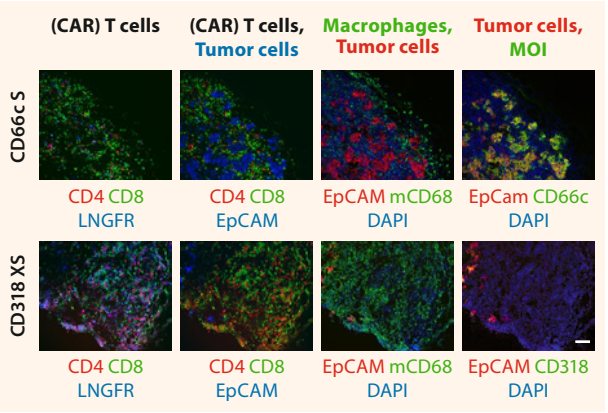

Figure 5: Staining of AsPC1-derived tumor tissues upon treatment with CAR T cells. Cyclic immunofluorescence analysis of (CAR) T cell and macrophage tumor infiltration, and target expression. Staining was performed on one tumor of the respective treatment group and each image is representative for at least two regions of interest. Regions of interest during cyclic IF were chosen based on manual prestaining of DAPI and EpCAM. Scale bar = 100 µm.

References

1. Application note: Integrated workflow solutions for the early screening of target candidates for immunotherapy of pancreatic cancer. https://static.miltenyibiotec.com/asset/150655405641/document_cnla5f47ip2d76ksm0hd81ed7r?content-disposition=inline
2. Schäfer, D., Tomiuk, S., Küster, L.N. *et al.* Identification of CD318, TSPAN8 and CD66c as target candidates for CAR T cell based immunotherapy of pancreatic adenocarcinoma. Nat Commun 12, 1453 (2021). https://doi.org/10.1038/s41467-021-21774-4

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