as measured by flow cytometry. The efficiency of ROR1 directed CAR T-cell lysis was tested by co-incubating ATC cells together with ROR1-CAR T-cells in 2D cultures and 3D spheroid models and untransduced T-cells served as controls. To exclude cell line-based effects and to prove that the CAR T-cells specifically target ROR1-expressing cells, CRISPR/Cas9 was used to knock out ROR1 in three ROR1-positive ATC cell lines. Cytobead arrays were used to determine CAR T-cell activity.

Results Our data demonstrates an overexpression of ROR1 in ATC/PDTC compared to non-malignant thyroid tissue. In addition, three different ATC cell lines showed overexpression of ROR1, and we could show that pre-treatment with kinase inhibitors like Lenvatinib or Sorafenib does not affect ROR1 surface expression. ROR1-positive ATC cell lines were efficiently lysed by ROR1-CAR T-cells in different 2D- and 3D cell culture models, while untransduced T-cells had no effect. The cytokine profile including IFN-γ, TNF-α and GM-CSF showed a specific activation of ROR1-CAR T-cells in the presence of ROR1 positive ATC cell lines. ROR1 knock out ATC cells were not affected by ROR1-CARTIs and did not induce cytokine activation, thus proving specificity for the target. Animal models are ongoing and will be finalized at presentation.

Conclusions In summary our data proof ROR1 as a viable and specific target for several types of thyroid carcinoma which is the basis for the design of a clinical phase I trial using ROR1-CAR T-cells in metastasized ATC and PDTC patients.


Materials and Methods Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy-coats by density gradient centrifugation using Ficoll-Paque PLUS and activated with a polymeric nanomatrix agonist for CD3 and CD28 T cell receptors, in IL-2-containing culture medium. Activated PBMCs were transduced with produced CAR-encoding lentivirus - transient transfection of 293E/T cells with different ratios of CAR-transgene, packaging, rev and envelope plasmids - either in flat- or round-bottom 96-well plates, in the presence or absence of the activation reagent. Transduction efficacy in T cells was assessed by both tdTomato fluorescence (reporter gene) and CAR surface expression through flow cytometry.

Results The transgene: packaging:rev:envelope plasmid ratio enabling the highest transduction rate - 50% of both tdTomato and surface CAR expression - was chosen for further CAR-transductions. The presence of T-cell-activating nanomatrix in transduction-media has demonstrated to influence CAR-transduction efficacy. Moreover, differences in tdTomato fluorescence levels between T cells transduced in flat- or round-bottom microplates were also observed.

Conclusions Overall, the presentwork provides insight on relevant experimental parameters that impact anti-nucleolin CAR-transduction in human T cells.

This work was funded by the FCT R&D Project EXPL/MED-FAR/1512/2021 and by CIBB contract programmes UIDB/04539/2020, UIDP/04539/2020 and LA/P/0058/2020.

REFERENCES


Background Triple-negative breast cancer (TNBC) is an aggressive cancer with low survival rates, still seeking for efficient targeted therapies. Genetic modification of human T cells to express chimeric antigen receptors (CAR-T cells) redirects T cells activity towards pre-established tumor-associated antigens (TAAs). Despite highly successful against hematological malignancies, CAR-T cells efficacy against solid tumors has been limited, owing to lack of accessible TAAs and tumor microenvironment (TME)-mediated immunosuppression. In this context, nucleolin, a membrane-nucleus shuttling phosphoprotein, has been demonstrated to be overexpressed on both TNBC cancer and endothelial cells from angiogenic blood vessels, emerging as an accessible target. In this respect, this work focuses on the first steps of anti-nucleolin CAR-T cells manufacturing, aiming at optimizing lentiviral-based CAR-transduction conditions.

Materials and Methods Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy-coats by density gradient centrifugation using Ficoll-Paque PLUS and activated with a polymeric nanomatrix agonist for CD3 and CD28 T cell receptors, in IL-2-containing culture medium. Activated PBMCs were transduced with produced CAR-encoding lentivirus - transient transfection of 293E/T cells with different ratios of CAR-transgene, packaging, rev and envelope plasmids - either in flat- or round-bottom 96-well plates, in the presence or absence of the activation reagent. Transduction efficacy in T cells was assessed by both tdTomato fluorescence (reporter gene) and CAR surface expression through flow cytometry.

Results The transgene: packaging:rev:envelope plasmid ratio enabling the highest transduction rate - 50% of both tdTomato and surface CAR expression - was chosen for further CAR-transductions. The presence of T-cell-activating nanomatrix in transduction-media has demonstrated to influence CAR-transduction efficacy. Moreover, differences in tdTomato fluorescence levels between T cells transduced in flat- or round-bottom microplates were also observed.

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REFERENCES


Background CAR-T cell-based therapies hold great promise for neuroblastoma, because of its potential for targeted effects and already promising results in adult solid cancers. However, neuroblastoma tumour cells can escape T cell mediated killing by inhibition of T cells through secretion of immunosuppressive factors. Here, we have identified Macrophage Migration Inhibitory Factor (MIF) and Midkine (MDK) by scRNA-seq analysis to have a negative effect on the cytotoxicity of T cells. Next to that, we have found both MIF and MDK in high abundance in the secreteme of neuroblastoma patient derived organoids. These factors have a suppressive effect on T cells and blocking their function might increase the efficacy of T cell based therapies for neuroblastoma.

Materials and Methods To study immunoregulatory interactions in neuroblastoma, single-cell RNA-sequencing of 25 tumours
were analysed using the CEL-seq2 platform. Interactions between tumour and immune cells were predicted using an unbiased ligand-receptor interaction analysis. Proteins secreted by tumour cells were analysed by performing mass spectrometry on conditioned medium from patient derived organoid cultures. The conditioned medium was concentrated using a 3kDa Millipore filter and prepared for LC-MS. Mass spectrometry data were acquired in data-dependent acquisition mode. For determining suppression, healthy donor PBMCs were stimulated with anti-CD28/anti-CD3 Dynabeads in the presence of recombinant proteins or concentrated conditioned medium from neuroblastoma organoids. In vitro killing assays were performed with GFP and luciferase transduced organoids and healthy donor PBMCs.

Results Analysis of scRNA-seq of 25 neuroblastoma tumours showed a negative correlation between MIF and MDK expression and the cytotoxicity of NK cells, CD8 and γδT cells within the tumour. These findings could be confirmed with a previously published SEQC bulk-RNAseq dataset containing 498 patients. Next to that, a higher expression of MIF and MDK correlated with poor survival in the same dataset. In the secretome from cultured neuroblastoma organoids, we have used mass spectrometry to identify MIF and MDK amongst the top 100 most abundant proteins from a total of ~1200 proteins. In vitro, we showed that rMIF and rMDK have a suppressive effect on the activation of T cells and the amount of cytotoxic factors such as granzyme B and Perforin are produced by the T cells. This confirms our hypothesis that MIF and MDK negatively influence the cytotoxicity of T cells.

Conclusions Using two different unbiased analyses -scRNA-seq analysis of tumours and mass spectrometry of neuroblastoma organoid secretome-, we have identified MIF and MDK as immunosuppressive factor in neuroblastoma. Currently, we are testing several MIF and MDK inhibitors to test if T cell mediated killing of neuroblastoma can be increased if the immunosuppressive MIF and MDK are blocked.


P07 Cell therapy in haematologic diseases

P07.01 THE POTENTIAL ROLE OF EXTRACELLULAR VESICLE-DERIVED SMALL RNAs IN AML RESEARCH AS NON-INVASIVE BIOMARKER

Background Antileukemic responses of immune reactive cells in AML-patients need to be improved. Combinations of blast-modulatory kitM (GM-CSF+PGE1) (vs control) convert myeloid blasts into dendritic cells of leukemic origin (DCleu), that effectively activate immune-cells against leukemic blasts. DC-derived EVs express molecules and carry nucleic acids (eg. miRNA) with immune regulatory functions.

Materials and Methods 1) DC/DCleu-generation of blast containing AML patients’ (n=5) and of healthy volunteers(n=5) relevant TAAs were determined by Fluorospot and protein-bound bead assays. Digital image analysis was used to evaluate the correlation of TAAs and T-cell abundance. T-cell receptor sequencing, in-vitro expansion with autologous CD40-activated B cells (CD40Bs) and in-vitro cytotoxicity assays were applied to determine specific expansion, clonality and cytotoxic activity of expanded T cells.

Results 68.3% of patients expressed ≥5 TAAs simultaneously with co-regulated clusters, which were similar to data from The Cancer Genome Atlas (n=505). Endogenous cellular or humoral responses against ≥1 TAA were detectable in 75.0% and 53.7% of patients, respectively. We found a correlation of T-cell abundance and the expression of TAAs and genes related to antigen-presentation. TAA-specific T-cell responses were polyclonal, could be induced or enhanced using autologous CD40Bs and were cytotoxic in-vitro. Despite the frequent expression of TAAs co-occurrence with immune responses was rare.

Conclusions We identified the most relevant TAAs in EGA for monitoring of clinical trials and as therapeutic targets. Antigen-escape rather than missing immune response should be considered as mechanism underlying immunotherapy resistance of EGA.

Disclosure Information M. Thelen: None. D. Keller: None. J. Lehmann: None. K. Wennhold: None. H. Weitz: None. E. Bauer: None. B. Gathof: None. M. Brüggemann: None. M. Kotrova: None. A. Quaas: None. C. Mallmann: None. S. Chon: None. A.M. Hillmer: None. C. Bruns: None. M. von Bergwelt-Baidlon: B. Research Grant (principal investigator, collaborator or consultant and pending grants as well as grants already received); modest; Astellas, Roche, SD. F. Consultant/Advisory Board; modest; Bristol Myers Squibb. M.A. Garcia-Marquez: None. H.A. Schlößer: B. Research Grant (principal investigator, collaborator or consultant and pending grants as well as grants already received); modest; Astra Zeneca.