we describe the development and careful optimization of an in vivo mouse CRISPR-screening platform to identify knock-out targets in primary T cells, with the goal of increasing T cell abundance and persistence in tumors with different TMEs. Using a mouse retroviral system to express single-guide RNA (sgRNA) libraries in T cells from Cas9 transgenic mice, we performed in vivo screens in syngeneic, fully immune-competent mouse tumor models.

**Results** We identified both known and potential novel regulators of T cell activation and persistence. Importantly, we have discovered knock-out targets that accumulate in multiple, distinct TMEs and other targets that are TME-specific. The use of sub-genomic-focused libraries allowed us to rapidly screen in multiple tumor model systems and reproducibly identify hits across individual mice.

**Conclusions** We have developed a fully optimized an in vivo genetic screen, which could be a rich source for target discovery, and can enable identification of functional regulators of T cells for rapid incorporation into CRISPR-engineered T cell therapies for different solid TMEs.

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**AN EX VIVO TUMOROID MODEL OF FRESH PATIENT TUMORS (3D-ACT) TO ASSESS EFFICACY OF CELLULAR THERAPY IN IMMUNO-ONCOLOGY**

Stephen Iwanowycz*, Jared Erhart, Mibel Pabon, Tina Pastoor, Jenny Kreahling, Soner Altiok. Nilogen Oncosystems, Tampa, FL, USA

**Background** Adoptive T cell therapy (ACT) strategies have achieved substantial advances in the treatment of malignant tumors. Some of the unique challenges posed to ACT by solid tumors include locating target cells, as well as entering and surviving the complex tumor microenvironment. To develop better ACT applications and identify combination therapies to enhance tumor cell killing efficacy of ACT it is imperative to develop preclinical platforms that recapitulate the complexity of patient tumor microenvironment (TME). The goal of this study was to develop an integrated confocal-based high-throughput, high-content real time imaging platform to assess immunogenic tumor cell killing (TCK) activity of ACT applications such as CAR-T and TCR using fresh patient tumor samples.

**Methods** All patient tumor samples were obtained with patient consent and relevant IRB approval. For the confocal imaging platform, unpropagated 3D tumoroids with intact TME measuring 150 micron in size were prepared from fresh tumor samples of renal cell carcinoma (RCC), colorectal carcinoma (CRC) and non-small cell lung cancer (NSCLC) using proprietary technology developed at Nilogen Oncosystems. Engineered T-cells were labeled with different fluorescent cell tracker dyes to monitor cell migration and locations within tumoroids by confocal analysis. Comprehensive flow cytometry analysis was performed to corroborate confocal imaging findings from TCK and multiplex cytokine release assays used to assess changes in the TME.

**Results** Our studies demonstrated that the confocal-based high-content real time imaging platform described here, combined with a custom image analysis algorithm, allowed for monitoring of treatment-mediated tumor cell killing with structural and functional analysis of engineered T-cells in intact 3D tumoroids. The penetration rate of CAR-T and TCR cells into tumoroids as well as associated tumor cell death varied significantly between different tumor types. Flow cytometry analysis allowed for monitoring of the activation status and viability of engineered T-cells, and treatment-mediated changes in tumor resident immune cell populations.

**Conclusions** Our data indicated that the immunosuppressive tumor microenvironment may have implications for the application of ACT. Use of the ex vivo platform described here (3D-ACT) may aid in the validation of combinatorial therapies that block or deplete suppressive factors present within the TME, allowing these therapies to overcome mechanisms associated with dysfunction in CAR-T and TCR cell applications.

Ethics Approval The study was approved by Chesapeake IRB Pro00014313.

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**A FAS-4–1BB IMMUNOMODULATORY FUSION PROTEIN CONVERTS A PRO-DEATH TO A PRO-SURVIVAL SIGNAL, ENHANCING T CELL FUNCTION AND EFFICACY OF ADOPTIVE CELL THERAPY IN MURINE MODELS OF AML AND PANCREATIC CANCER**

1Shannon Oda, 2Kristin Anderson, 3Philip Greenberg, Nicolás García, Pranali Ravikumar, Patrick Bonson, Cody Jenkins, Summer Zhuang, Andrew Daman, Shannon*, Seattle Children’s Research Institute, Seattle, WA, USA; 4University of Washington, Seattle, USA; 5Fred Hutchinson Cancer Research Center, Seattle, WA, USA

**Background** Adoptive cell therapy (ACT) with genetically-modified T cells has shown impressive results against some hematologic cancers, but limited efficacy against tumors with restrictive tumor microenvironments (TMEs). FasL is a particular obstacle for ACT; it is expressed in many tumors and TMEs, including AML, ovarian and pancreatic cancers, and upregulated on activated T cells, where it can mediate activation-induced cell death (AICD).

**Methods** We engineered T cells to boost function with novel immunomodulatory fusion proteins (IFPs) that combine an inhibitory ectodomain with a costimulatory endodomain. Like current checkpoint-blocking therapies, IFPs can abrogate an inhibitory signal, but also provide an often absent costimulatory signal. Additionally, IFP-driven signals are delivered only to the T cells concurrently engineered to be tumor-specific, thereby avoiding systemic T cell activation. For FasL-expressing TMEs, we developed an IFP that replaces the Fas intracellular tail with costimulatory 4-1BB. We tested the Fas-4-1BB IFP in primary human T cells and in immunocompetent murine models of leukemia and pancreatic cancer.

**Results** Fas-4-1BB IFP expression enhanced primary human T cell function and enhanced lysis of Panc1 pancreatic tumor cells in vitro. Fas-4-1BB IFP-engineered murine T cells exhibited increased pro-survival signaling, proliferation, antitumor function and altered metabolism in vitro. Notably, the Fas ectodomain is trimeric and the 4-1BB intracellular domain requires trimerization to signal. In contrast, the CD28 domain is dimeric and did not enhance function when paired with 4-1BB. In vivo, Fas-4-1BB increased T cell persistence and function, and Fas-4-1BB T cell ACT significantly improved survival in a murine AML model. When delivered with a mesothelin-specific TCR, Fas-4-1BB T cells prolonged survival in the autochthonous KPC pancreatic cancer model, increasing median survival to 65 from 37 days (with TCR-only, “P=0.0042). Single-cell RNA sequencing revealed differences in the endogenous tumor-infiltrating immune cells, including changes in cell frequency and programming.
Conclusions We developed an engineering approach to enhance the in vivo persistence and antitumor efficacy of transferred T cells. Our targeted, two-hit strategy uses a single fusion protein to overcome a death signal prevalent in the TME of many cancers and on activated T cells, and to provide a pro-survival costimulatory signal to T cells. Our results suggest that this fusion protein can increase T cell function when combined with murine or human TCRs, and can significantly improve therapeutic efficacy in liquid and solid tumors, supporting clinical translation.

REFERENCES

EVALUATING THE SAFETY OF TUMOR TREATING FIELDS (TTFIELDS) APPLICATION TO THE TORSO – IN VIVO STUDIES
Shiri Davidi, Roni Blat, Mijal Munster, Anna Shteingauz, Shay Cahal, Uri Weinberg, Yoram Palti, Moshe Giladi*, Novocure, Haifa, Israel

Background Tumor Treating Fields (TTFields) are a noninvasive, antineoplastic treatment delivered locoregionally to tumor bed via low intensity (1–3 V/cm), intermediate frequency (100–500 kHz), alternating electric fields. This treatment modality has been shown to be cytotoxic to rapidly dividing cells, with highest efficacy demonstrated at different optimal frequencies depending on tumor cell-type. TTFields therapy is FDA-approved for the treatment of newly diagnosed and recurrent glioblastoma (GBM), with the overall tolerable safety profile (EF-11 and EF-14 clinical trials) attributed to the low rate of mitotic events in normal, quiescent brain cells. Further evaluation of the safety profile of TTFields is needed for treating cancer in different body regions where there are high rates of cellular proliferation, i.e. torso. Many solid malignant tumors may reside in the torso region – mesothelioma and non-small cell lung carcinoma (NSCLC) in the thoracic segment; pancreatic cancer, hepatocellular carcinoma, and gastric cancer in the abdomen; and ovarian cancer in the pelvis. Hence, we investigated the safety of delivering TTFields to the torso of healthy rats at conditions previously deemed effective for treating the aforementioned cancer cell types. Method TTFields were applied using the Novo-TTF100L system at frequencies of 150 or 200 kHz and intensities of 1–2 V/cm RMS to torsos of Sprague Dawley (SD) female rats for a duration of 2 weeks. Throughout treatment, animals underwent daily clinical examinations. Blood samples and comparative histological evaluation of major internal organs were performed at treatment cessation. Results No significant differences were observed for the TTFields treated groups in comparison to control groups for the following parameters: activity level, food and water intake, stools, motor neurological status, respiration, weight, complete blood count, blood biochemistry, and pathological findings. Conclusions These results demonstrate the safety of 150 and 200 kHz TTFields when delivered to torsos of healthy rats, where there are normal tissues with high cellular proliferation rates. Overall, TTFields delivery to the torso demonstrated safety and feasibility for the treatment of thoracic and other abdominal and pelvic cancers. TTFields are currently being investigated in clinical studies for the treatment of solid tumors located in the torso, including locally advanced pancreatic cancer (PANOVA-3 Study, NCT03377491), ovarian cancer (INNOVATE-3 Study, NCT03940196), lung cancer (LUNAR Study, NCT02973789), hepatocellular carcinoma (HEPANOVA Study, NCT03606590) and gastric cancer.

A SEVERE CYTOKINE RELEASE SYNDROME WITH RESPIRATORY FAILURE IN RECURRENT MESOTHELIOMA INDUCED BY EPCAM CAR-T CELLS INFUSION: A CASE REPORT
Sitong Wang*, Juemin Fang, Hui Wang, Qing Xu. Shanghai Tenth People’s Hospital, Shanghai, China

Background With the development and maturity of chimeric antigen receptor T (CAR-T) cells therapy-related technologies, the application of CAR-T therapy has progressed from blood tumors to solid tumors, and its potential risks and side effects have been more widely recognized. As the most common complication of CAR-T therapy, cytokine release syndrome (CRS) is an inflammatory syndrome caused by the activation and proliferation of T-cell and the increased levels of multiple cytokines. Epithelial cell adhesion molecule (EpCAM) is overexpressed in a variety of tumors and has been used as one of the targets of CAR-T therapy. Case reports of severe CRS due to the use of anti-EpCAM CAR-T cell therapy are very rare. Methods A 45-year-old malignant mesothelioma woman with EpCAM-positive whose disease progressed after chemotherapy was enrolled into our study (ChiCTR2000030274). The patient received a total of 1.8×107 autologous T cells which contained sequences encoding single-chain variable fragments (scFv) specific for EpCAM after cyclophosphamide lymphodepletion. After the infusion of CAR-T cells, the patient developed typical CRS reactions such as fever, hypoxemia, pulmonary edema, and elevated inflammatory factors. The patient’s condition did not improve after the use of anti-inflammatory and antipyretic drugs. After administration of tocilizumab (4 mg/kg, day 6 and day 17) combined with glucocorticoid (40 mg q12h, decreasing gradually), the patient’s general condition gradually improved, and chest computed tomography (CT) showed that pulmonary edema was absorbed. Results The patient’s CRS was successfully eliminated after the use of IL-6 inhibitor tocilizumab combined with glucocorticoid. Conclusions Although EpCAM CAR-T is safe in general, serious complications still happen possibly requiring close monitoring and timely treatment. Our findings suggest that tocilizumab combined with glucocorticoid can be an effective therapeutic method for severe CRS caused by CAR-T cells therapy in solid tumor.