Rapid, label-free, functional potency assay predicts ROR1 CAR-T effectiveness in solid tumor microenvironment

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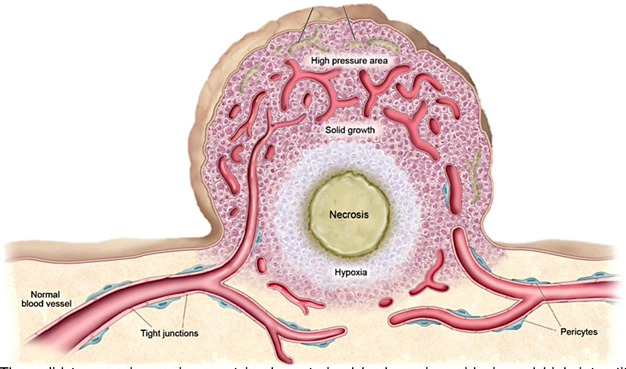
Abstract: Predict CAR T potency in solid tumor microenvironment

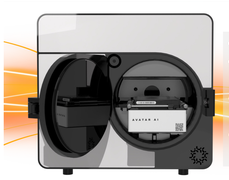
Chimeric antigen receptor (CAR) T cell therapy holds great promise for the treatment of various cancers, including solid tumors. However, attempts to model the behavior and effectiveness of CAR-T cell therapies for solid tumors have been challenging due to the unique tumor microenvironments in which these cancer cells are found. The solid tumor microenvironment (TME) is often characterized by hypoxia, increased acidity and high interstitial fluid pressures, allowing cancer cells to effectively evade immune surveillance. This immunosuppressive TME also contributes to CAR-T cell exhaustion, thereby limiting its antitumor activity and function. To address these concerns, we have developed a novel cell-based assay to measure CAR-T cell potency and cytotoxic function in immunosuppressive tumor microenvironments. Utilizing a custom-built electrical impedance and capacitance reader, we measured tumor cell viability without the use of labels or fluorescent reporters. This non- invasive method allows for sensitive and rapid assessment of tumor cell kinetics and is well suited for cytotoxic screening experiments involving CAR-T cells. The potency assay is conducted under hyperbaric (pressurized) and hypoxic culture conditions to mimic the TME, achieved through the application of a proprietary incubation technology. Data analysis was performed using custom-built data acquisition and analysis software. Proof-of-concept experiments were performed using anti-receptor tyrosine kinase-like orphan receptor 1 (ROR1) CAR-T cells targeting the ovarian adenocarcinoma cell line, SKOV3. Defined ratios of effector T cells to tumor cells was assessed (1:2, 1:1, 2:1, 5:1, 10:1, respectively) to model CAR-T potency. Multiple pressure and oxygen settings were examined to model the cross-section of a solid tumor (0 PSI to 5 PSI, 1% to 10% O2). Initial results from these screening experiments show significant decline in ROR1 CAR-T mediated cytotoxicity when performed under TME conditions. Interestingly, acclimating and expanding ROR1 CAR-T cells to high pressure and decreased oxygen culture (AVATAR) conditions improved potency levels and warrants further investigation. In conclusion, we describe a rapid, label-free potency assay that incorporates a proprietary incubation technology to predict the behavior of cell therapies in immunosuppressive tumor microenvironments.

Background: Key features of the solid tumor microenvironment



The solid tumor microenvironment is characterized by hypoxia, acidosis and high interstitial fluid pressure. This complex ecosystem comprised of cancer cells, stroma and infiltrating immune cells create an immunosuppressive environment leading to treatment resistance and disease progression. Xcellbio has developed technologies to identify cell therapies that work effectively in tumor microenvironments. Figure ref: Sun et al., Acta Pharmacol Sin., 2020

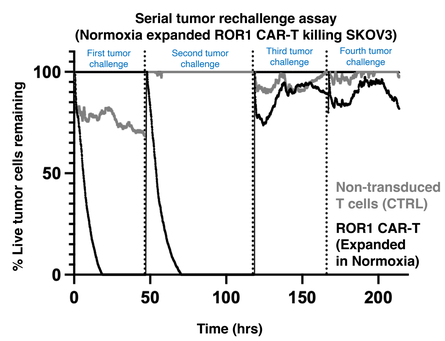
Technology: Introducing the AVATAR AI. Predict cell therapy potency in TME

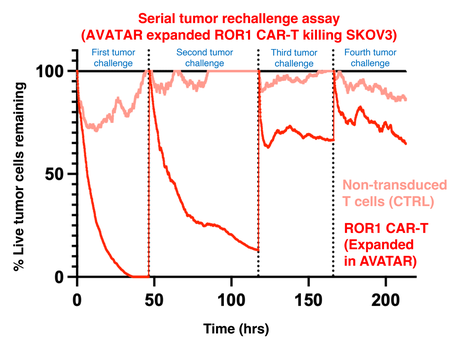


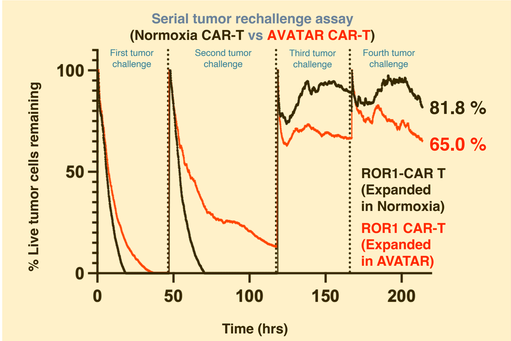
- **Designed for CAR-T cell potency assays**
- **Proprietary incubation system is built-in to the AVATAR AI to replicate the TME**
- **Modular system configuration allows for multiple systems to pair and run in parallel.**
- **Real-time, label-free system measures tumor cytolysis, allowing for on-the-fly analysis of CAR-T potency**

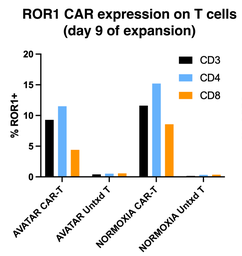
AVATAR AI effector cell cytotoxicity assay workflow

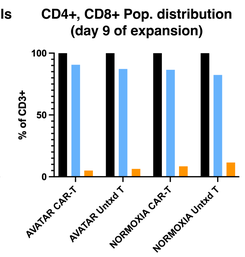
Case study: AVATAR AI makes serial tumor challenge assays easy to set up and analyze. CAR-T expansion conditions contribute to enhanced potency

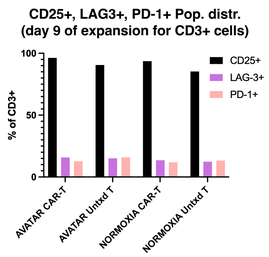


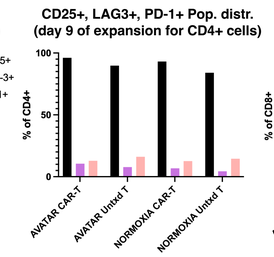


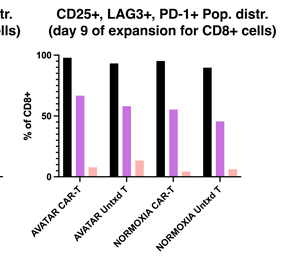


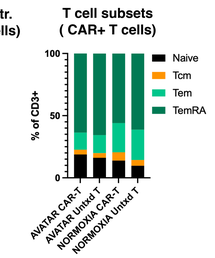




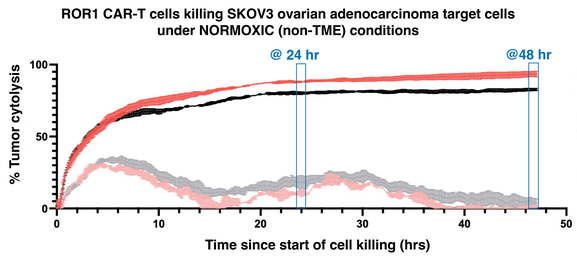


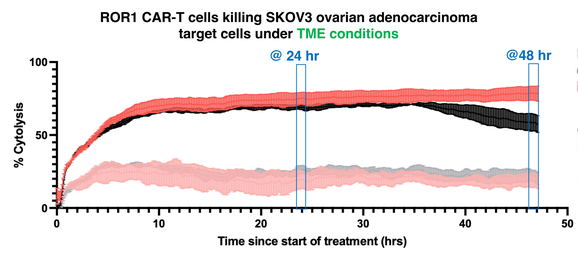


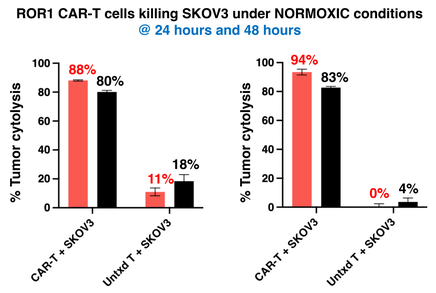


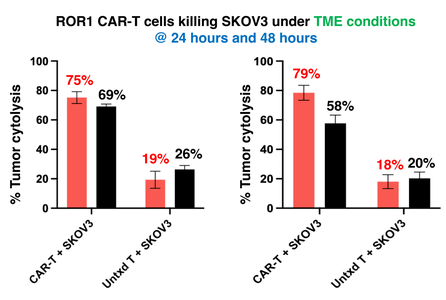


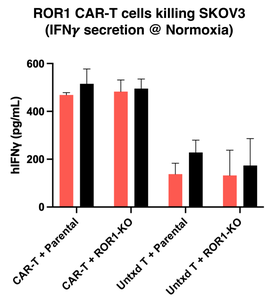
Case study: AVATAR AI is designed for functional potency release assays. Predict CAR-T cell potency and effectiveness in a solid tumor microenvironment

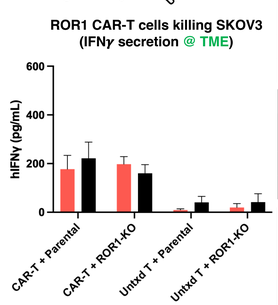


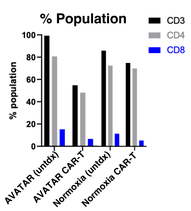


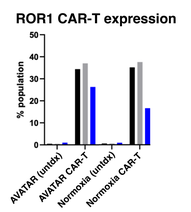


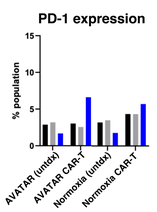


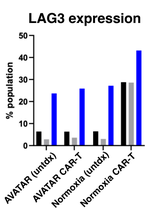


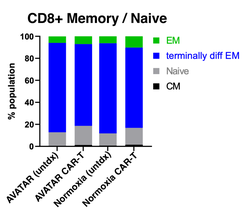


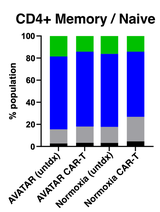


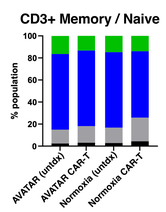












Experimental details

Serial challenge assay: To measure CAR-T exhaustion in vitro, we have developed a serial tumor challenge assay using AVATAR AI to repeatedly 'challenge' CAR-T cells against tumor cells. This repeated stimulation of CAR-T cells allow for assessment of their cytolytic function and durability, mimicking what occurs in vivo. Four serial tumor challenges were performed, each challenge demarcated by the dotted line, with recovery periods of either 48 or 72 hours between tumor challenges (X-axis). Target cells, SKOV3 ovarian adenocarcinoma cell line (ATCC) was seeded onto an AVATAR AI E-plate at 5K cells per well (96 well format). Target cells were allowed to adhere for 24 hours before effector cells (ROR1 CAR-T cells) were introduced at 10:1 effector to target ratio (50K). After the 'first tumor challenge,' 50% of the media in each well containing the effector cells was removed and collected for cytokine analysis. The remaining 50% of media containing effector cells was replenished with an equal amount of fresh culture media (50uL, RPMI + 10% FBS), and the entire volume (100uL) containing effector cells was moved to an adjacent well with 5K cells that were pre-seeded 24 hours prior. This process was repeated for subsequent tumor challenges. Serial tumor challenge assays were conducted at 21% oxygen, 0 psi, and at 37 degrees in an AVATAR AI System. ROR1 CAR-T cells were generated from cryopreserved PBMCs (single healthy donor) transduced and expanded for a period 9 days (2 days of transduction/recovery and 7 days of expansion). ROR1 CAR-T cells were expanded in RPMI + 10% serum and activated with CD3/CD28 Dynabeads (with no IL2 added). ROR1 CAR-T cells were expanded in a CO2 incubator (Thermo Heracell Vios) or in an Avatar System (Xcellbio). AVATAR expanded ROR1 CAR-T cells was cultured at 4 PSI, 3% oxygen, 5% CO2 and at 37 degrees Celcius (noted in **RED**). ROR1-CAR T expanded in 'Normoxia' was cultured in a CO2 incubator at 37 degrees Celcius and 5% CO2 (noted in **BLACK**).

Experimental details & conclusions

Tumor cytolysis assay: Target cells, SKOV3 (ATCC) were seeded onto an AVATAR AI E-plate at 5K cells per well (96 well format). Target cells were allowed to adhere for 24 hours before effector cells (ROR1 CAR-T cells) were introduced at 10:1 effector to target ratio (50K). Cell killing assays were performed under **Normoxia** (21% O2 & 0 PSI) or **TME** (1% O2 & 2 PSI). ROR1 CAR-T cells were transduced and expanded for 9 days, either in a **CO2 incubator** or an **AVATAR System** (Ambient O2 + 4 PSI). IFNγ secretion assays was performed at 48 hours after co-culture (BD cat# 555142). **Flow cytometry** was performed using a BD Fortessa X-20. Naïve cells (CCR7+CD45RA+), Central Memory (CCR7+CD45RA-), Effector Memory (CCR7-CD45RA-), Terminally Differentiated Effector Memory (CCR7-CD45RA-). Details on flow cyto. antibodies used are found below.

Normoxia expanded CAR-T potency		
Target Killing Environment	Tumor Cytolysis % @ 24 hr	Tumor Cytolysis % @ 48 hr
Normoxia	80.2	82.7
TME	**69.1**	**57.7**

AVATAR expanded CAR-T potency		
Target Killing Environment	Tumor Cytolysis % @ 24 hr	Tumor Cytolysis % @ 48 hr
Normoxia	88.1 (+7.9%)	93.5 (+10.8%)
TME	**75.2 (+6.1%)**	**78.5 (+20.8%)**

FLUOR	MARKER	CLONE	ISOTYPE	VENDOR	CAT
FITC	CD3	HIT3a	mIgG2a	BioLegend	300306
n/a	ROR1-huFc (anti-hFc)	n/a	hIgG1-Fc	RnD Systems	9490-RO-050
PE	anti-hFc Secondary	M1310G05	rIgG2a	BioLegend	410708
PE/Cy5	CD25	BC96	mIgG1	BioLegend	302608
PE/Cy7	CD223 (LAG3)	11C3C65	mIgG1	BioLegend	369310
PE/TxRd (PE-Dazzle)	CD4	OKT4	mIgG2b	BioLegend	317448
PerCP/Cy5.5	CD197 (CCR7)	G043H7	mIgG2a	BioLegend	353220
APC	CD279 (PD-1)	EH12.2H7	mIgG1	BioLegend	329908
APC/Cy7	Live/Dead	n/a	n/a	BioLegend	423105
AF700	CD127 (IL-7Ra)	A019D5	mIgG1	BioLegend	351344
BV421 (Pac Blue)	CD45RA	HI100	mIgG2b	BioLegend	304130
BV510 (AmCyan)	CD8	SK1	mIgG1	BioLegend	344732
BV605	CD103	Ber-ACT8	mIgG1	BioLegend	350218

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