Advanced Models for 3D Screening: Immune Oncology Applications

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Common methods for 3D multicellular tumor spheroid formation

Parameters to consider

- Physiological relevance
- Cell-cell interactions
- Cell-ECM interactions
- Complexity
- Uniformity and reproducibility
- Scalability
- Compatibility with high-throughput screening
- Equipment investment
- Reagent volume
- Cost
- Long term culture

Breslin & O’Driscoll, 2013, Drug Discov Today. 18(5-6), 240-9
Multicellular tumor spheroids: importance of the microenvironment

Challenges with solid tumor immune cell therapy: Hostile tumor microenvironment

Overcoming counter-attack and local immunosuppression

- Oxidative stress, nutrient depletion, low pH, hypoxia
- Suppressive soluble factors and cytokines
- Suppressive immune cells (Tregs, MDSC, M2-TAM / N2-TAN)
- “On target-off tumor”

Newick et al., 2016, Mol. Ther. Oncolytics, 3, 16006
Advanced assay for detecting CAR-T mediated tumor spheroid cytotoxicity

**Flat-bottom microplate**

**2D Assay**
- Target cells seeded into 384-well microplate
- 24-48 hr.
- CAR-T cells added
- 24 hr.
- KILR Detection Reagent added
- 1 hr.
- Detect luminescence

**3D Assay**

**Spheroid microplate**
CAR-T cell invasion of multicellular tumor spheroids

**HCC827 spheroids with CAR-T cell invasion**

- **EGFR CAR-T E/T 0**
- **EGFR CAR-T E/T 10**
- **EGFR CAR-T E/T 40**

HCC827 spheroids formed using spheroid microplate for 48 hours. Twenty-four hours after ProMab Biotechnologies EGFR scFv-CD28-CD3ζ CAR-T cell addition, spheroids were stained for cytokeratin-7 (green) and CD3ε (red), with Hoescht nuclei counterstain (blue). As E/T ratio is increased from 10:1 to 40:1, invasion of the CAR-T cells into the HCC827 tumor spheroid and subsequent tumor cell lysis is visible. Images obtained using Thermo Fisher CellInsight CX7 in confocal mode using 10X objective.
Sensitivity of tumor cells to CAR-T cell mediated cytotoxicity shifted for 3D compared to 2D cell culture

- Affinity-tuned scFvs exhibit higher anti-tumor efficacy to cells with higher expression of target receptor and no anti-tumor efficacy to cells exhibiting normal target receptor levels.
- HCC827 cells (lung adenocarcinoma) exhibit EGFR copy number amplification
- Second generation CAR-T cells targeting EGFR were used to target breast and lung cancer cell lines. Mock scFv Control CAR-T cells were used as negative control.
Challenges with solid tumor immune cell therapy: Migration and invasion

Homing and infiltration

Ager et al., 2016, Biochem. Soc. Trans., 44(2), 377-85
Combining technologies to enhance 3D microenvironments

A549 cells added to spheroid microplate for 3D multicellular spheroid formation

Transwell 96-well permeable support is inserted into spheroid microplate; NK cells added to insert.

Plates incubated together to allow for migration, spheroid infiltration, and cytotoxicity.
Immune cell migration and tumor spheroid infiltration in a single high-throughput screening amenable assay

- NK cell migration towards A549 multicellular tumor spheroids in the presence and absence of SDF-1α (SDF) and/or prostaglandin E2 (PGE) in the medium

- NK cell induced cytotoxicity of A549 multicellular tumor spheroids after migration. Percent cytotoxicity was calculated via flow cytometry by enumerating GFP positive A549 cells.
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Appendix
Supplementary figures and slides
Clinical Trials of CAR-T Therapy

- 374 clinical trials all over the world
- CAR targets include cancer, diabetes, AIDS, vascular diseases, etc.
CAR-T Cell Therapy

- T-cell therapy activates a patient's T cells against cancer with a chimeric antigen receptor (CAR) that recognizes an antigen expressed on the cancer cells.

- CARs are single-chain antibodies coupled to a transmembrane region and an intracellular signaling domain (e.g., from CD28 or 4-1BB).

- CARs against many tumor-associated antigens have been constructed and tested pre-clinically, and some have entered clinical trials.

https://www.mskcc.org/blog/car-t-cell-therapy-growing-area-research

CAR-T Cell Therapy

- CAR T cells face extra challenges with solid tumors:
  - Have to be made specific for an antigen whose expression clearly identifies tumor from normal tissue.
    - “on-target, off-tumor” cytotoxicity
  - Must be able to home and penetrate the fibrous connective tissue that surrounds the tumor.
  - Once within the tumor they must expand, persist and mediate cytotoxicity in a hostile environment with immunosuppressive modulators.

http://www.nature.com/nbt/journal/v32/n7/full/nbt0714-604.html
Affinity-tuned CAR-T Cells from ProMab Biotechnologies

- ProMab Biotechnologies supplies second and third generation CAR-T cells targeting a variety of cell-surface receptors.

- This study used a second generation construct with affinity-tuned scFvs targeting epidermal growth factor receptor (EGFR) and an empty vector Mock Control.

https://doi.org/10.3389/fimmu.2016.00500
The KILR assay from DiscoverX is a highly specific, non-radioactive measure of target cell death in a co-culture.

KILR target cells are transduced to stably express a KILR reporter protein tagged with a β-gal fragment. This KILR reporter protein is released into the media upon cell death and lysis. Addition of detection reagents containing the other β-gal fragment results in a chemiluminescent output.
Affinity-tuned CAR-T Cells & EGFR Targets

- This study used a second generation construct with affinity-tuned scFvs targeting epidermal growth factor receptor (EGFR) and an empty vector Mock Control from ProMab Biotechnologies.

- For this study, 2 cell lines from the ATCC® EGFR Genetic Alteration Cell panel were selected:
  - HCC827 contains high EGFR copy number amplification
  - NCI-H460 contains no EGFR copy number amplification

Data from ATCC® Brochure
Corning® spheroid microplates enable improved spheroid assays for screening

- Corning Ultra-Low Attachment (ULA) surface and unique round well-bottom design enable the formation and growth of a single, uniform spheroid per well with reproducible size.

- Standard ANSI/SBS footprint dimensions for 96-well and 384-well formats

- Clear bottom for visualization and imaging

- Black sidewalls to reduce cross-talk and background noise in fluorescent- and luminescent-based assays
Conclusions

• The Corning Epic BT reader is capable of performing label-free kinetic CAR-T assays at physiologically relevant temperatures.

• In combination with KILR Cytotoxicity Assay, the Corning spheroid microplate provides a high throughput platform for culturing and screening tumor spheroids with CAR-T cell assays.

• Deliverables
  • Both assays were presented as posters at SLAS in January
  • Both assays will be published as Application notes
  • A webinar will be co-hosted with DiscoverX and ProMab Biotechnologies on March 30th
Methods: Demonstrate Immune Cell Tumoricidal Activity

• Day 1: Seed 2,000 A549/GFP cells (cancer cells) per well of 96 well spheroid plate in IMDM 10%FBS

• Day 2: Label effector cells with CellTracker Blue and add to A549 spheroids at various concentrations
  – NK92-MI: natural killer cell line derived from peripheral blood known to be cytotoxic to a wide range of malignant cells
  – MOLT-4: T-cell leukemia cell line with no known cytotoxic effect on other malignant cells

• Day 3: Aspirate medium and replace with 150 µL TrypLE™ Select Enzyme (10X) (Gibco™ Cat. No. A1217701) for 1 hour at 37°C or until spheroids could be broken up into single cells with minimal pipetting. Single cells were then analyzed via flow cytometry utilizing the Miltenyi Biotec MacsQuant®.
Results: Demonstrate Immune Cell Tumoricidal Activity

Dose dependent effector function was demonstrated with NK cells and not MOLT-4 cells when added at various concentrations to A549 spheroids.

Data represents the average of 2 independent studies. N=12 per concentration.
Results: Image Immune Cell Infiltration (Confocal)

Representative photomicrographs of A549/GFP spheroids with (right) and without (left) NK infiltration (200x). A549/GFP cells shown in green and NK-92MI cells shown in blue. Images taken at a Z stack height of -125 µm via Thermo Scientific™ CellInsight™ CX7. Scale bar is 100 µm.
Results: Image Immune Cell Infiltration (Histology)

200x CD45 (red) and e-cadherin stained (brown) sections of A549/GFP spheroids that were infiltrated by NK-92MI cells. Spheroids were fixed in 4 % paraformaldehyde (Boston Bioproducts Cat. No. BM-155) for cryostat sectioning and H&E staining (carried out at the University of New England, Biddeford, Maine).
Results: Demonstrate Immune Cell Chemotactic Response

Dose dependent migration of NK cells towards SDF over a period of 24 hours. Data represents the average of 2 independent studies. N=24.
Results: 2D versus 3D Immune Oncology Model

NK Migration towards 2D and 3D A549/GFP cells with or without SDF in the medium and with and without prostaglandin E2 inhibition of NK cells. Horizontal lines indicate statistical significance from a 1 way ANOVA with a Bonferroni’s multiple comparison post test. *** = p<0.0001 and ** = p<0.001. Data represents the average of 2 independent studies. N=24.
Results: 2D versus 3D Immune Oncology Model

NK induced cytotoxicity of A549/GFP cells grown in 2D and 3D with or without SDF in the medium and with and without prostaglandin E2 inhibition of NK cells. Horizontal lines indicate statistical significance from a 1 way ANOVA with a Bonferroni's multiple comparison post test. *** = p<0.0001, ** = p<0.001, and * = p<0.05. Data represents the average of 2 independent studies. N=24.
Summary

• Effector cell cytotoxicity and specificity can be assessed using a combination of the spheroid microplate and flow cytometry.

• Transwell permeable supports can be utilized to assess NK migratory response towards chemoattractants such as SDF.

• The combination of the spheroid microplate and HTS Transwell-96 Well Permeable Supports allows for a novel 3D model that combines immune cell migration, effector induced cytotoxicity, and immune cell evasion in one easy to use model.
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