A Novel Three Dimensional Immune Oncology Model for High Throughput Testing of Tumoricidal Capability

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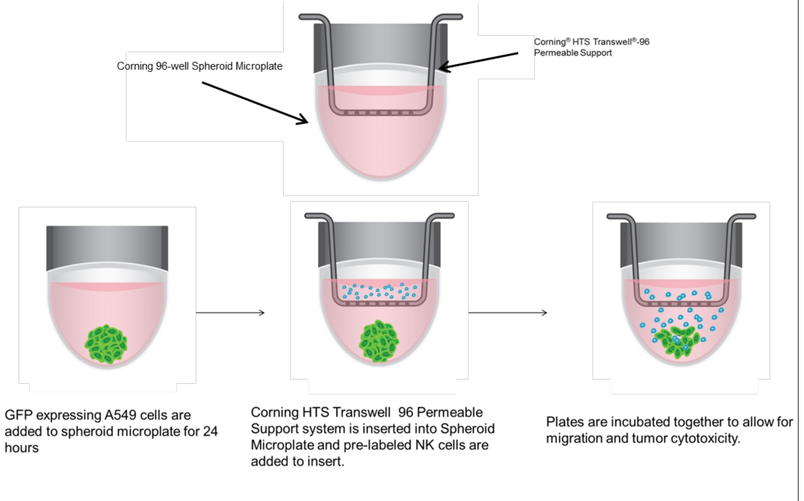
ABSTRACT

There has been increasing interest in utilizing immune cells for cancer treatment, with therapy involving activating a patient's own T-cells to attack their tumors. However, the effectiveness of immunotherapy is not equivalent for all patients or cancer types, which has led to the need for better models for scientists and researchers to understand why. Traditionally, tumoricidal activity and immune evasion have been studied by utilizing two dimensional (2D) systems, which may not accurately reflect the complexity of a three dimensional (3D) tumor. The barriers immune cells need to overcome in a 3D system are much greater than those of 2D. The immune cells not only need to migrate to the tumor site, but also need to infiltrate a 3D structure in order to attack the target cells. Beyond the physical differences between a 2D and 3D system, it has been shown that phenotypic differences also occur when tumor cells are cultured in 3D that allow for higher resistance to cytotoxicity[1-2]. Here, we demonstrate a 3D model to study cancer/immune cell interactions, by combining two commercially available products; Corning® 96-well spheroid microplates and the Corning HTS Transwell®-96 tissue culture system. Corning spheroid microplates are multiple well, cell culture microplates with round well-bottom geometry that are coated with Corning Ultra-Low Attachment surface, resulting in the formation of highly reproducible, single multi-cellular tumor spheroids centered in each well. Corning HTS Transwells-96 are permeable support systems commonly used for drug transport and migration/invasion studies. By replacing the standard 2D flat-bottom Transwell receiver plate with a Corning spheroid microplate, the ability to investigate immune cell homing, tumor cytotoxicity, and tumor immune evasion, in an easy-to-use, 3D, high throughput assay is achieved.

METHODS/MATERIALS

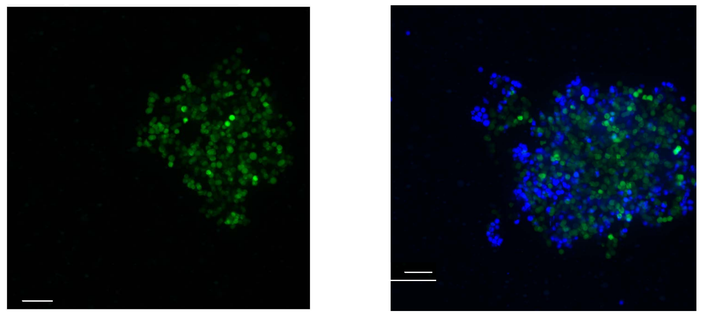
- A549/GFP Cells (Cell Biolabs Cat. No. AKR-209) were seeded into 96-well spheroid microplates (Corning Cat. No. 4515) at 2,000 cells per well in 100 µL of Iscove's Modification of DMEM (IMDM) (Corning Cat. No. 10-016-CM) supplemented with 10% fetal bovine serum (FBS) (Corning Cat. No. 35-010-CV) and cultured for 24 hours.
- **Infiltration assays:** 100 µL of medium or NK-92MI (ATCC® Cat. No. CRL-2408) or MOLT-4 (ATCC Cat. No. CRL-1582) cells, pre-labeled with CellTracker™ Blue CMHC Dye (Thermo Fisher Scientific Cat. No. C2110), were added to A549 spheroids for 24 hours. Infiltrated spheroids were dissociated using TrypLE™ Select Enzyme (10X) (Thermo Fisher Scientific Cat. No. A1217701) and analyzed via flow cytometry utilizing a Miltenyi Biotec MACSQuant®.
- **Migration assays:** 150,000 pre-labeled NK-92MI cells were added to each insert of a Corning HTS Transwell-96 tissue culture system (Corning Cat. No. 3387) and allowed to migrate overnight towards media in the receiver plate containing Human SDF-1 alpha/CXCL12 (SDF) (Shenandoah Biotechnology Cat. No. 100-20). Migration was enumerated via flow cytometry.
- **2D and 3D immune oncology models:** 2,000 A549/GFP cells per well were seeded into either 96-well spheroid microplates or tissue culture-treated HTS Transwell-96 receiver plates (Corning Cat. No. 3382) for 24 hours. Medium was replaced with 200 µL of IMDM, 10% FBS containing 30 ng/mL of SDF or vehicle control. Pre-labeled NK92-MI cells were treated with 2 µg/mL prostaglandin E_2 (PGE) (Tocris Cat. No. 2296) or vehicle control in IMDM without serum for an hour. HTS Transwell 96-well permeable supports were placed in 96-well spheroid plates or HTS Transwell-96 receiver plates. NK-92MI cells re-suspended in serum-free IMDM were seeded into inserts at 50,000 cells/well. After 24 hours, inserts were discarded and the spheroid and receiver plates were processed for migration and cytotoxicity via flow cytometry.

Assay Schematic

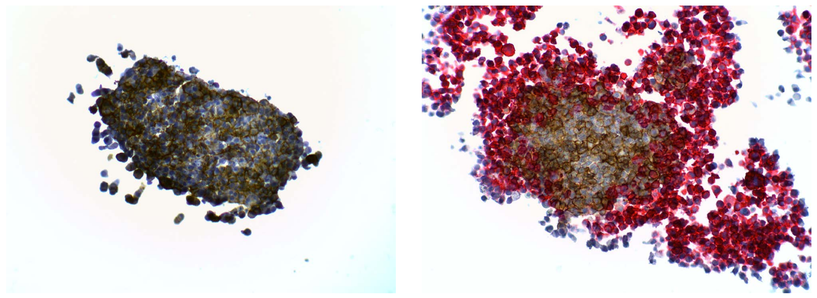


RESULTS

Spheroid Formation/NK Infiltration

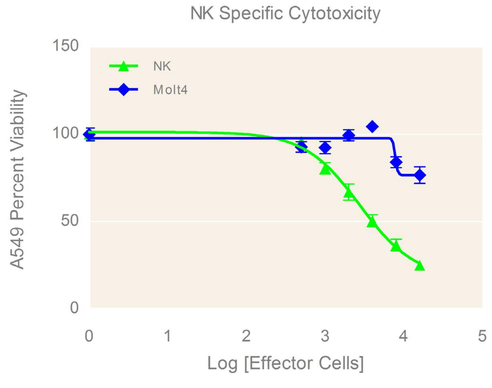


Representative photomicrographs of A549/GFP spheroids with (right) and without (left) NK infiltration (20X). A549/GFP cells shown in green and NK-92MI cells shown in blue. Images taken at a Z stack height of -125 µm via Thermo Fisher Scientific CellInsight™ CX7. Scale bar is 100 µm.



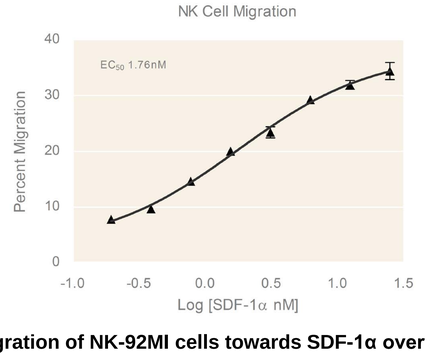
Histological analysis of NK-92MI cell infiltration into A549/GFP tumor spheroids. 200X imaged CD45 (red) and e-cadherin stained (brown) sections of A549/GFP spheroids alone (left) or that were exposed to NK-92MI cells for 4 hours (right). Spheroids were fixed in 4% paraformaldehyde (Boston BioProducts Cat. No. BM-155) for cryostat sectioning and H&E staining (carried out at the University of New England, Biddeford, Maine).

NK Specific Cytotoxicity



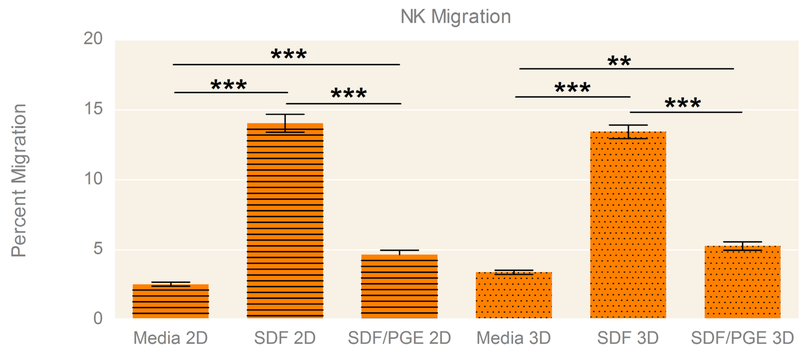
Specific and dose-dependent effector function demonstrated by NK-92MI cells. NK-92MI (NK) cells added at various concentrations to A549/GFP (A549) tumor spheroids displayed dose-dependent cytotoxicity that was significantly left-shifted compared to that observed with MOLT-4 cells, a T-cell leukemia cell line with no known cytotoxic effect on other malignant cells (P<0.0051, 2 way ANOVA). Data represents the average of 2 independent studies. N = 12 per concentration.

NK Migration

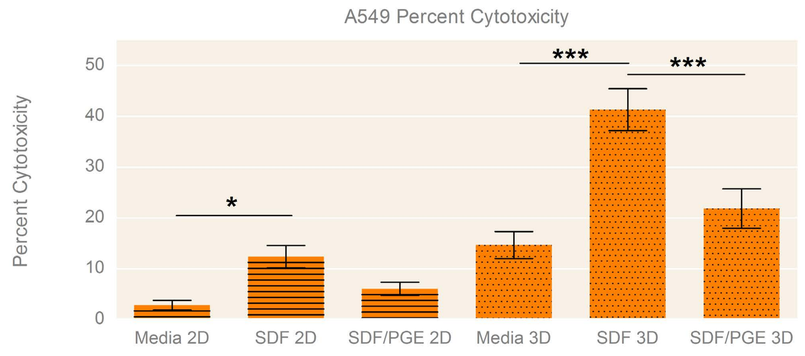


Dose-dependent migration of NK-92MI cells towards SDF-1α over a period of 24 hours. NK-92MI (NK) cells were evaluated for migration to chemokine SDF-1α at varying concentrations. Cells in the basolateral chambers were quantified by flow cytometry. Data are plotted as the ratio of migration induced by chemokine compared to total cells seeded in insert. Data represents the average of 2 independent studies. N = 24.

2D versus 3D Immune Oncology Model



NK-92MI cell migration towards A549/GFP cells cultured in 2D and 3D. NK-92MI (NK) cell migration was evaluated in the presence and absence of SDF-1α (SDF) and/or prostaglandin E2 (PGE) in the medium. Percent migration was calculated as a ratio of cells in basolateral chamber compared to total cells seeded in insert. Horizontal lined columns indicate data generated in 2D and dotted columns indicated data generated in 3D. Data shown as the average of 2 independent studies, N = 24 with 1-way ANOVA with Boneferroni's post test. *** = p<0.0001 and ** = p<0.001.



NK-92MI cell induced cytotoxicity of A549/GFP cells cultured in 2D and 3D. Cytotoxicity of A549/GFP cells was evaluated after exposure to NK-92MI (NK) cells in the presence and absence of SDF-1α and/or prostaglandin E2 in the medium. Percent cytotoxicity was calculated via flow cytometry by enumerating GFP positive A549 cells as they compared to A549 only spheroids. Horizontal lined columns indicate data generated in 2D and dotted columns indicate data generated in 3D. Data shown as the average of 2 independent studies, N = 24, with 1-way ANOVA with a Bonferroni's post test. *** = p<0.0001 and * =p<0.05.

SUMMARY/CONCLUSIONS

- Corning 96-well spheroid microplates allow for the formation of consistent sized, single spheroids in each well that are ideally suited for confocal microscopy.
- Natural killer cell infiltration into the formed spheroid can be demonstrated by way of confocal imaging and immunostaining of sectioned spheroids.
- 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 Corning spheroid microplates and the 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.

REFERENCES

1. Holmes TD, et al., A human NK cell activation/inhibition threshold allows small changes in the target cell surface phenotype to dramatically alter susceptibility to NK cells. Journal of Immunology 186(3):1538-1545 (2011).
2. Dangles-Marie V, et al., A three-dimensional tumor cell defect in activating autologous CTLs is associated with inefficient antigen presentation correlated with heat shock protein-70 down-regulation. Cancer Res. 63:3682-3687 (2003).