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

Application Note

CORNING

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Introduction

There has been increasing interest in utilizing immune cells for cancer treatment by activating a patients' T-cells to attack their tumors. However, the effectiveness of immunotherapy is not equivalent for all patients or cancer types, which has created a need for efficient, *in vitro* screening models for targeted therapy. Traditionally, tumoricidal activity and immune evasion have been studied by utilizing two dimensional (2D) systems, which may not accurately reflect the complexity of a tumor in a three dimensional (3D) system. The barriers that immune cells need to overcome in a 3D system are much greater than those in 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 HTS Transwell®-96 tissue culture system. Corning spheroid microplates have a round well-bottom geometry and are coated with Corning Ultra-Low Attachment surface, which results in the formation of highly reproducible, single multicellular tumor spheroids centered in each well. The HTS Transwell-96 permeable support systems are commonly used for drug transport and migration/invasion studies. By replacing the standard 2D flat-bottom Transwell receiver plate with a Corning spheroid microplate, researchers are enabled to investigate immune cell homing, tumor cytotoxicity, and tumor immune evasion in an easy-to-use 3D high throughput assay.

Materials and Methods

For tumor spheroid formation, 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).

For immune infiltration studies, A549/GFP cells were allowed to form spheroids over 24 hours. After which, 100 µL of medium with various concentrations of pre-labeled NK-92MI (ATCC® Cat. No. CRL-2408) or MOLT-4 (ATCC Cat. No. CRL-1582) cells were added to A549/GFP spheroids for overnight incubation. Effector cells (NK-92MI and MOLT-4) were stained for one hour with 80 µM CellTracker™ Blue CMHC dye (Thermo Fisher Cat. No. C2110) prior to addition. In order to determine the impact of effector cell type and number on A549/GFP spheroids, medium was removed and replaced with 150 µL TrypLE™ select enzyme (10X) (Thermo Fisher Cat. No. A1217701) and incubated at 37°C until spheroids dissociated into single cells with minimal pipetting. Cells were then analyzed via flow cytometry utilizing a Miltenyi Biotec MACSQuant®.

For the migration component of the study, 150,000 pre-labeled NK-92MI cells in IMDM without FBS were added to each insert of a Corning HTS Transwell-96 tissue culture system (Corning Cat. No. 3387) and allowed to migrate overnight towards various concentrations of human stromal-cell derived factor-1 (SDF-1α/CXCL12; Shenandoah Biotechnology Cat. No. 100-20) in IMDM supplemented with 10% FBS. Migration was enumerated via flow cytometry.

Finally, for 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 flat-bottom HTS Transwell-96 receiver microplates (Corning Cat. No. 3382). The next day, medium was replaced with 200 µL of IMDM supplemented with 10% FBS, containing 30 ng/mL of SDF-1α or vehicle control. NK-92MI cells were stained, as previously described, while simultaneously being treated with 2 µg/mL prostaglandin E2 (PGE2) (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 microplates (a schematic is shown in Figure 1) or HTS Transwell-96 receiver microplates. NK-92MI cells were then resuspended in serum-free IMDM and seeded into inserts at 50,000 cells/well. After 24 hours, inserts were removed and both spheroid and flat-bottom receiver microplates were processed for flow cytometry.

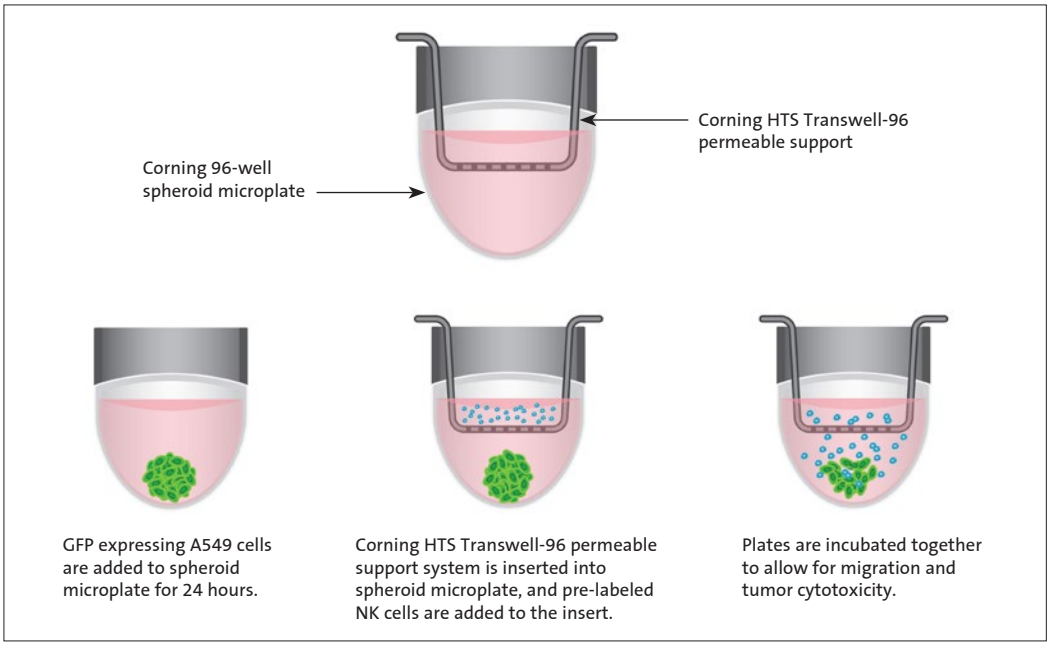

Figure 1. HTS Transwell 96-well permeable supports were placed into 96-well spheroid microplates for 3D assays.

Results and Discussion

Migration

An important component to any immune response is the activation of immune cells and subsequent relocation to the target area requiring defense. *In vitro* migration studies allow for evaluating immune cell recruitment to determine conditions which enhance or suppress this activity. In this study, cells from a Natural Killer (NK) cell line, NK-92MI, were placed in the apical compartments of Transwells® with 5.0 µm pore size and allowed to migrate overnight towards medium containing various concentrations of SDF-1α (a CXC chemokine that signals through the CXCR4 receptor and is a known chemoattractant for lymphocytes[3]). A dose response of NK-92MI chemotaxis was generated (Figure 2) resulting in an EC_{50} value of 1.76 nM, which is consistent with values for NK cell lines and primary NK cell chemotaxis reported in literature[4].

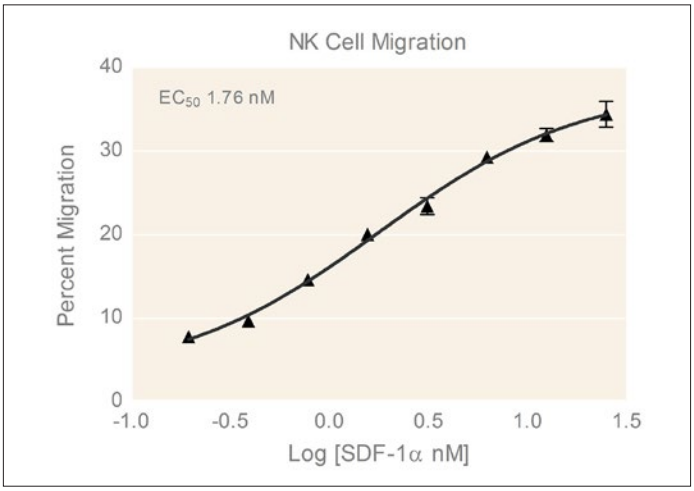

Figure 2. Dose-dependent migration of NK-92MI cells towards SDF-1α over a period of 24 hours. NK-92MI (NK) cells were evaluated for migration to chemokine SDF-1α at varying concentrations. Cells in the basolateral chambers were quantified by flow cytometry. Data are plotted as the ratio of migration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

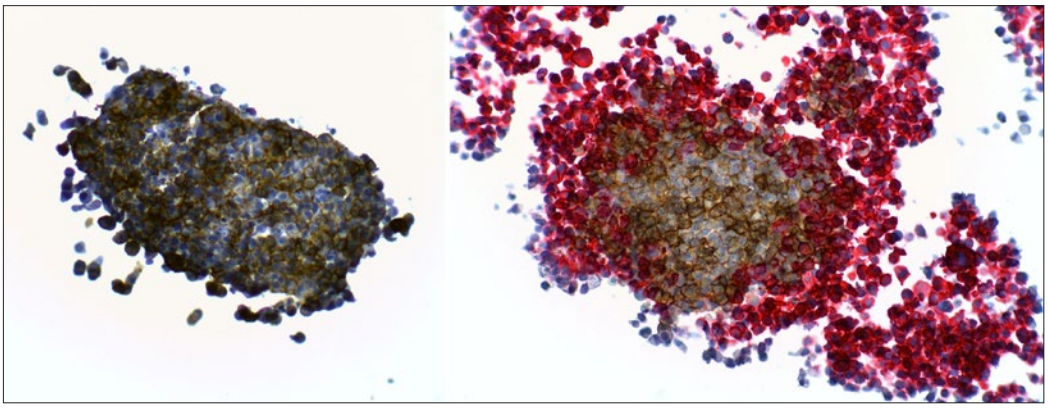

Figure 3. Histological analysis of NK-92MI cell infiltration into A549/GFP tumor spheroids. 200x imaged CD45 (red) and E-cadherin (brown) stained sections of A549/GFP spheroids alone (left) or after exposure to NK-92MI cells for 4 hours (right).

Infiltration

The presence of certain immune cells in a malignant structure has been shown to correlate with increased patient survival[5]. Unlike more commonly used 2D *in vitro* models for studying immune cytotoxicity, 3D models can be utilized to observe immune cell infiltration into tumor spheroids. Two common methodologies for accomplishing this are histology and confocal microscopy. Figure 3 shows E-cadherin stained A549/GFP spheroids alone or those exposed to NK-92MI cells for 4 hours and stained with CD45. Infiltration can also be observed via confocal microscopy (Figure 4), where A549/GFP cells are shown in green, and NK-92MI cells shown in blue. These studies confirm that the immune cells in this model are not only able to reach the target tumor cells, but are also capable of infiltrating the 3D spheroid structure.

3D Cytotoxicity

To investigate the immune induced cytotoxicity of A549 cells, both NK-92MI and MOLT-4 suspension cells were added to spheroid microplates containing A549 tumor spheroids. NK92-MI cells are a natural killer cell line derived from peripheral blood known to be cytotoxic to a wide range of malignant cells. MOLT-4 cells were used as a negative control since they are a T-cell leukemia cell line with no known cytotoxic effect on other malignant cells. Figure 5 demonstrates the dose dependent effect NK-92MI cells have on A549/GFP viability, as compared to the limited effect the MOLT-4 cells show at only the highest concentrations. These data support the specific targeting of the A549/GFP cells by NK-92MI cells, and resulting tumor spheroid cell death.

Migration with Cytotoxicity

To evaluate the migration and cytotoxic effects of NK-92MI cells in 3D on A549/GFP tumor spheroids in one single, easy-to-use high throughput assay, the HTS Transwell®-96 tissue culture system and Corning® spheroid microplate were combined, as previously described (Figure 1). Figure 6 demonstrates how immune cell migration can be enhanced by the addition of chemokines, such as SDF-1α, as well as suppressed by the addition of inhibitors, such as PGE2. NK-92MI cell migration in both 2D and 3D systems was significantly increased when the chemoattractant SDF-1α was present in the receiver or spheroid

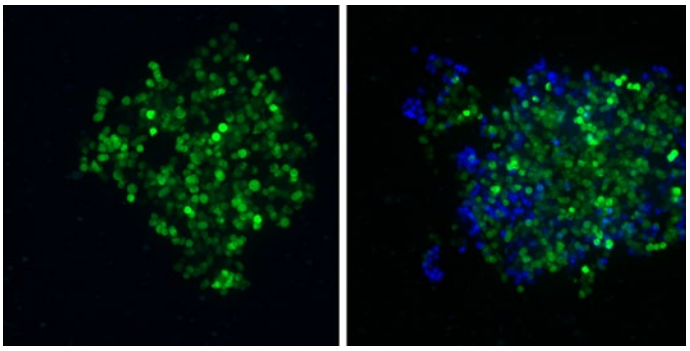

Figure 4. Confocal analysis of NK-92MI cell infiltration into A549/GFP tumor spheroids. Representative photomicrographs of A549/GFP spheroids (green) with (right) and without (left) NK-92MI cell (blue) infiltration. Images taken at 20X objective with a Z stack height of ~125 µm via Thermo Fisher CellInsight™ CX7.

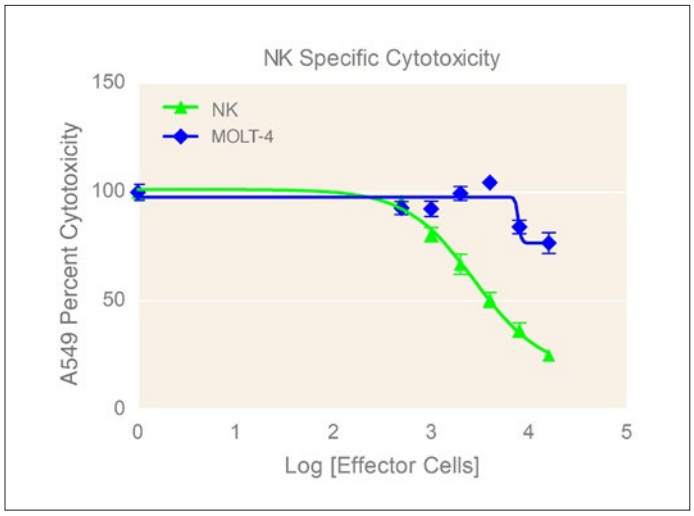

Figure 5. Specific and dose-dependent effector function demonstrated by NK-92MI cells. NK-92MI (NK) cells added at various concentrations to A549/GFP (A549) tumor spheroids displayed dose-dependent cytotoxicity that was significantly left-shifted compared to that observed with MOLT-4 cells, a T-cell 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 ±SEM. N = 12 per concentration.

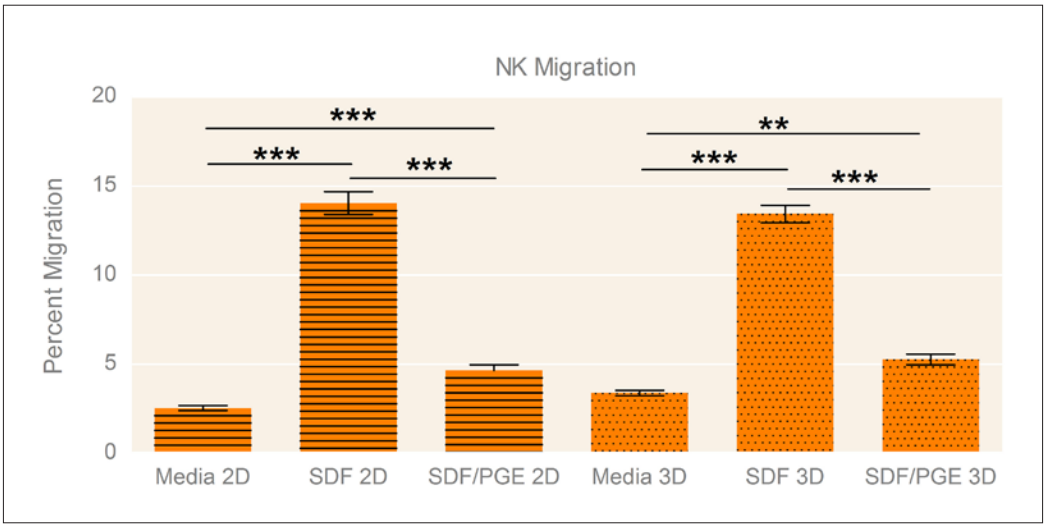

Figure 6. NK-92MI cell migration towards A549/GFP cells cultured in 2D and 3D. NK-92MI (NK) cell migration was evaluated in the presence and absence of SDF-1α (SDF) and/or prostaglandin E2 (PGE) in the medium. Percent migration was calculated as a ratio of cells in basolateral chamber compared to total cells seeded in insert. Horizontally lined columns indicate data generated in 2D and dotted columns indicate data generated in 3D. Data shown as the average of 2 independent studies ±SEM, N = 24 with 1-way ANOVA with Boneferroni's post test. *** =p<0.0001 and **=p<0.001.

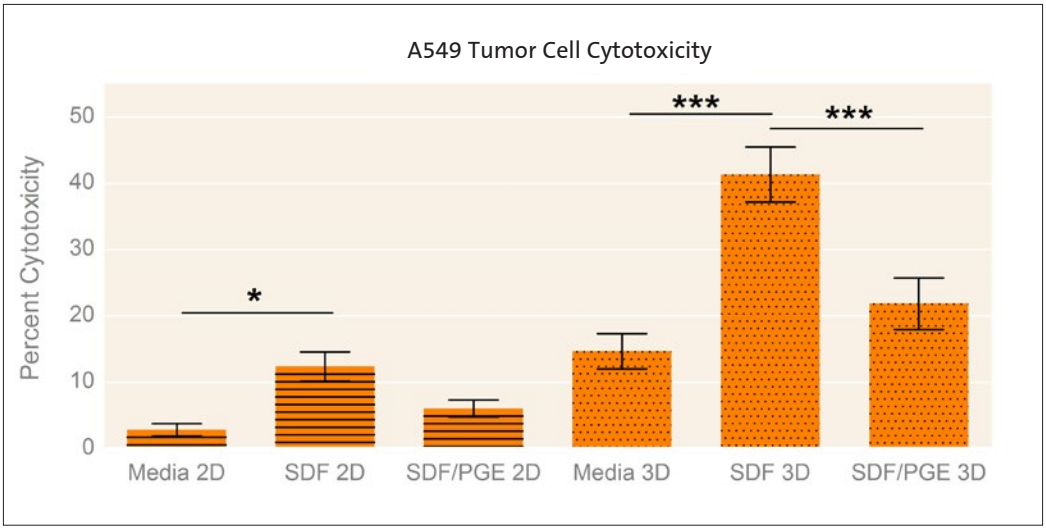

Figure 7. NK-92MI cell-induced cytotoxicity of A549/GFP cells cultured in 2D and 3D. Cytotoxicity of A549/GFP cells was evaluated after exposure to NK-92MI (NK) cells in the presence and absence of SDF-1α (SDF) and/or prostaglandin E2 (PGE) in the medium. Percent cytotoxicity was calculated via flow cytometry by enumerating GFP positive A549 cells after exposure to NK92-MI cells as they compared to A549/GFP only spheroids without NK-92MI cells. Horizontally lined columns indicate data generated in 2D and dotted columns indicate data generated in 3D. Data shown as the average of 2 independent studies ±SEM, N = 24, with 1-way ANOVA with a Bonferroni's post test. *** = $p<0.0001$ and * = $p<0.05$.

microplate. Conversely, migration was significantly decreased when NK-92MI cells were exposed to PGE2, a known inhibitor of immune cell function often secreted by cancer cells as a form of immune evasion[6]. Tumoricidal activity corresponds well with migration data, with the highest cytotoxicity of A549/GFP cells observed when NK-92MI cells were exposed to SDF-1α without PGE2 (Figure 7). An overall greater cytotoxic response can be observed with A549 cells cultured in 3D, as compared to 2D, which may be due to the faster doubling rate of the A549 cells in 2D compared to 3D. Differences in proliferation in 2D systems compared to 3D systems have been shown in several cell lines[7].

Conclusions

In order to create a model system to investigate immune cell homing, 3D tumor cytotoxicity, and tumor immune evasion, in a single, high throughput assay, Corning® 96-well spheroid microplates and the HTS Transwell-96 tissue culture system were combined. Previous studies have investigated separate components of this model by either looking at cell migration towards a 2D monolayer of target cells, or by looking at 3D tumoricidal activity independent of homing and immune evasion. The system described here allows for a more comprehensive immune oncology model by which individual aspects of cellular migration and cancer immune evasion can be studied, resulting in a more *in vivo*-like investigation.

References

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