Validation of an Image-Based 3D Natural Killer Cell Mediated Cytotoxicity Assay

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Introduction

Natural killer (NK) cells are a type of cytotoxic lymphocyte found in peripheral blood that play a role in host defense and immune regulation. Most recently, NK cells have become of particular interest in the field of immunotherapy due to their potential to target and destroy specific cancer cells, while leaving non-target healthy cells intact. The anti-cancer activity of NK cells has been shown to be associated with a better prognosis in several cancers such as colorectal cancer[1], non-small cell lung cancer[2], and clear cell renal cell carcinoma[3].

In order to properly study the interaction between NK cells and target tumor cells, an appropriate *in vitro* model system must be established. However, much of the data published to date has used cancer cells plated as a two dimensional (2D) monolayer on the bottom of microplate wells. A growing amount of data has shown that cells cultured in this manner lack the cell:cell and cell:matrix communication, metabolic gradients, and polarity demonstrated *in vivo*[4]. The ability to perform matrix infiltration studies is also eliminated with the use of 2D cell culture. By embedding cancer cells into a three dimensional (3D) matrix and allowing the formation of tumor spheroids, or tumoroids, the shortcomings of using 2D cultured cells can be overcome as communication networks and cellular gradients observed within *in vivo* tumors are reestablished.

With the incorporation of 3D cultured cells, however, traditional methods to monitor target and NK cell interactions, and subsequent target cell killing can become problematic. Microplate reader assays designed to detect signal from cell monolayers lack the sensitivity to quantify signal from spheroids surrounded by non-cell containing areas in the well with no signal generation. By incorporating microscopy-based detection and cellular analysis, signal emanating solely from spheroids is quantified, providing a highly robust method to detect induced toxicity within target cancer cells.

Here we describe a novel 3D NK cell mediated cytotoxicity (CMC) assay. HCT116 colorectal cancer cells were embedded within a collagen hydrogel of defined concentration and thickness, mimicking *in vivo* extracellular matrix (ECM). Following cell propagation to create tumoroids within the matrix, HCT116 and NK cells were labeled with individual cell tracking dyes, followed by NK cell addition. Fluorescent apoptosis and necrosis probes were also added to track cytotoxic events within the tumoroids. Cellular imaging and analysis were performed at regular intervals over a seven day period to monitor NK cell binding to the tumoroids and induced apoptosis and necrosis of the HCT116 cells making up each tumoroid. Experimental testing validated that the combined assay technique provides a sensitive, accurate, and repeatable *in vitro* method to determine the ability of NK cells to target and kill tumor cells.

BioTek Instrumentation

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 is a modular multi-mode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays, and is controlled by integrated Gen5™ Microplate Reader and Imager Software. The imager and software were used to capture brightfield and fluorescent images for 2D and 3D CMC assays and quantify the level of NK induced cytotoxicity.

BioSpa™ 8 Automated Incubator: The BioSpa 8 links BioTek readers or imagers together with washers and dispensers for full workflow automation of up to 8 microplates. Temperature, CO_2/O_2 and humidity levels are controlled and monitored through the BioSpa software to maintain an ideal environment for cell cultures during all experimental stages. Test plates were incubated in the BioSpa and automatically transferred to the Cytation 5 at designated time points to monitor cytotoxicity in 2D and 3D cultured cells.

2D and 3D Target and NK Effector Cell Preparation

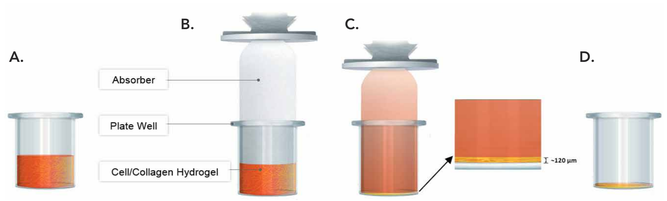

Figure 1. Creation of 3D cell/collagen hydrogel using RAFT™ system. (A) Cell/collagen mix dispensed to wells of tissue culture (TC) treated plate. **(B)** Absorber insertion into plate well. **(C)** Absorption of medium, concentrating collagen and cells to *in vivo* strength, creating an ~120 µm thick hydrogel. **(D)** Removal of absorber prior to dispense of fresh cell medium.

HCT116 target cells were mixed with the prepared RAFT™ collagen I suspension and dispensed to 96-well TC treated microplates (GBO Catalog No. 655090) in a volume of 240 µL to yield 1200 cells/well. Cells were propagated at 37 °C/5% CO_2 in the plates for seven days to allow spheroid creation through cell doubling, with media exchanges being performed every two days. On Day 6, HCT116 cells were added to additional 96-well cell culture treated microplates in 2D format at a concentration of 3000 cells/mL and allowed to attach overnight. Following the additional 24 hour incubation, negatively selected peripheral blood CD56+ CD16+ natural killer cells (Lonza Catalog No. 2W-501) were thawed and diluted in LGM™-3 Lymphocyte Growth Medium-3 (Lonza Catalog No. CC-3211) according to the manufacturer's protocol. The cells were then stained with the fluorescent CellTracker™ Deep Red dye (ThermoFisher Scientific Catalog No. C34565). Finally, NK cells were diluted to concentrations that equaled 10:1 or 20:1 ratios of final HCT116 populations per well in media containing either Abcam Kinetic Apoptosis Kit pSIVA™-IANBD and propidium iodide reagents (Catalog No. ab129817) and 500 U/mL human IL-2 IS (Miltenyi Biotec Catalog No. 130-097-744), or apoptosis kit reagents alone.

Automated Cell Mediated Cytotoxicity Process

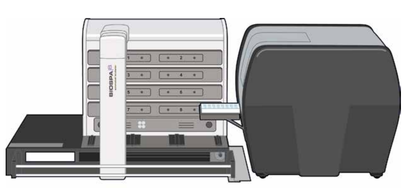

Figure 2. Automated cell mediated cytotoxicity system consisting of BioSpa 8 Automated Incubator (left) and Cytation 5 Cell Imaging Multi-Mode Reader (right).

Following addition of diluted NK cells to the 96-well 3D and 2D test plates, the plates were placed into the BioSpa 8 at 37 °C/5% CO_2 for an incubation period of 120 hours. The BioSpa method was programmed such that plates were automatically moved to the Cytation 5 every two hours, where brightfield and fluorescent imaging were carried out to monitor NK effector cell induced cytotoxicity. The imaging chamber of the Cytation was also maintained at 37 °C/5% CO_2 to ensure consistent environmental cell conditions. Imaging channels included in the experimental validation tracked as follows. Brightfield: whole cells and spheroids; GFP: pSIVA-IANBD fluorescent probe binding to externally exposed phosphatidyl serine (PS) on apoptotic cells; Propidium Iodide (PI): PI intercalating dye bound to necrotic cell DNA; CY5: CellTracker Deep Red stained NK cells.

3D NK Cell Mediated Cytotoxicity Imaging

Z-stacked images were automatically captured of HCT116 tumoroids every 2 hours over the entire 120 hour incubation period from NK treated positive control wells, as well as negative control wells containing no NK cells (Figure 3).

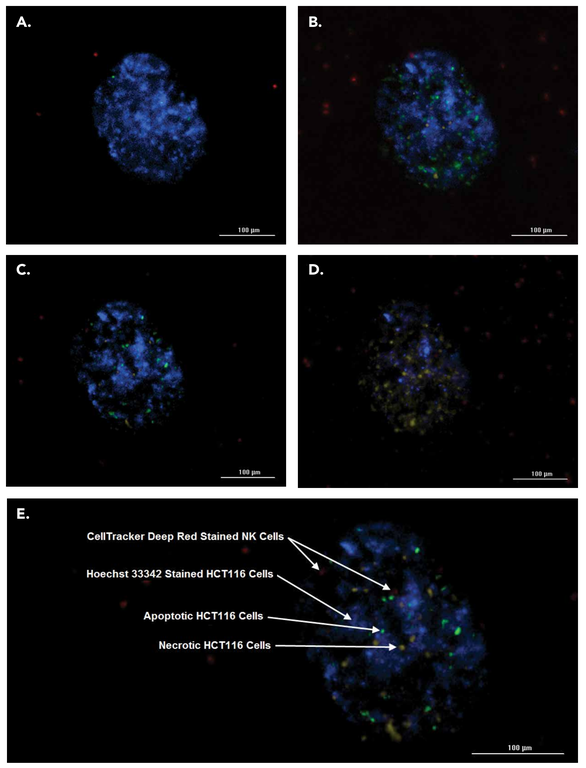

Figure 3. NK cell induced apoptosis and necrosis within HCT116 tumoroids. Final projected overlaid 4x images following treatment with a 20:1 ratio of IL-2 stimulated NK to 3D cultured HCT116 tumoroid cells (E:T) following **(A)** 0; **(B)** 12; **(C)** 24; or **(D)** 120 hour incubations. **(E)** Projected image showing blue Hoechst 33342 stained HCT116 tumoroid cells, green pSIVA-IANDB stained apoptotic cells, orange propidium iodide stained necrotic cells, and red CellTracker Deep Red labeled NK cells.

By using overlaid final projections of the z-stacked images, NK cell interactions with HCT116 tumoroids, in addition to apoptotic and necrotic cell induction, could be kinetically tracked for each test condition.

3D NK Cell Apoptosis and Necrosis Induction Analysis

Using the z-stacked images, Gen5 software automatically pre-processed the samples to remove excess background signal and prepare the image for quantitative analysis. Two separate cellular analysis steps were conducted to place object masks around areas within the image meeting primary analysis criteria in either the GFP or PI imaging channel. Minimum and maximum object sizes and threshold fluorescence values were set such that only apoptotic or necrotic areas within target tumoroids were identified (Figure 4A-B).

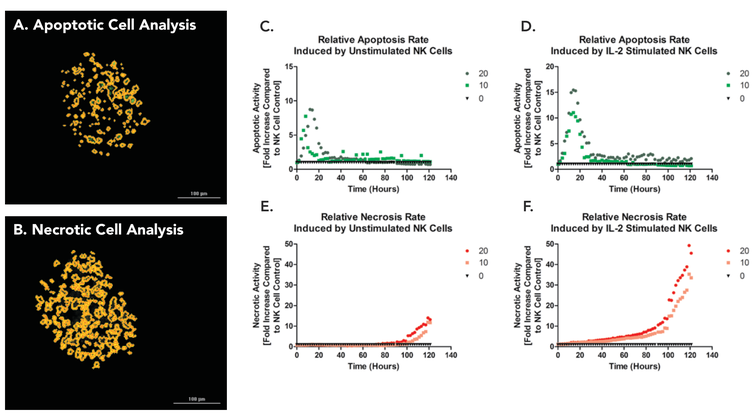

Figure 4. 3D NK CMC analysis. Gen5 placed object masks around **(A)** apoptotic or **(B)** necrotic cells during individual analysis of images captured from 3D test plates. Fold stimulation calculated of induced apoptotic activity from interaction with **(C)** unstimulated or **(D)** IL-2 stimulated NK cells; and induced necrotic activity from interaction with **(E)** unstimulated or **(F)** IL-2 stimulated NK cells at each time point.

Area within each object mask was automatically calculated from all images captured during the entire incubation period. Object area values from wells containing spheroids treated with NK cells were compared to negative control object areas at individual time points to determine the level of apoptotic or necrotic activity within the tumoroids induced by stimulated or unstimulated NK and HCT116 target cell interaction (Figures 4C-F). As seen in Figures 4C and 4D, apoptotic fold stimulation peaks within the first 24-36 hours following NK cell addition, as the number of apoptotic cells increases in treated tumoroids relative to untreated tumoroids. Signal then diminishes as cells become increasingly necrotic and PS internalizes, releasing the probe. Membrane integrity then becomes compromised, allowing PI to enter and bind to DNA; and signal increases appropriately (Figures 4E-F). This pattern of initial cellular apoptosis followed by secondary necrosis agrees with previously published literature[5].

From the data, it is also apparent that the 20:1 NK cell ratio increases the level of induced target HCT116 tumoroid cell cytotoxicity compared to the lower 10:1 ratio, demonstrating the cumulative effect of NK cells on cancer tumoroids. Finally, stimulation by IL-2 was also shown to positively influence NK induced cytotoxicity compared to resting unactivated NK cells at both ratios, again agreeing with previous literature findings[6].

2D NK Cell Mediated Cytotoxicity Imaging

Kinetic montage images were also captured from test plates containing 2D cultured HCT116 and NK cells or HCT116 cells alone during the same automated monitoring procedure (Figure 5).

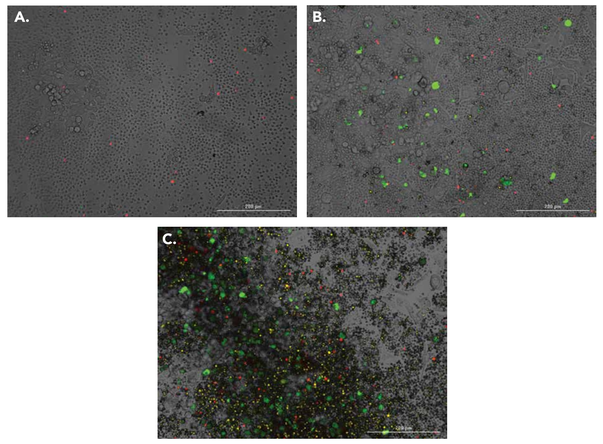

Figure 5. NK cell induced apoptosis and necrosis within 2D HCT116 cells. Final stitched images following treatment with a 20:1 ratio of IL-2 stimulated NK to 2D cultured HCT116 cells following **(A)** 0; **(B)** 24; or **(C)** 120 hour incubations. Brightfield: HCT116 and NK cells; green: pSIVA-IANBD stained apoptotic cells; yellow: propidium iodide stained necrotic cells; red: CellTracker Deep Red labeled NK cells.

Following image pre-processing and stitching, individual cell analyses were once again carried out on the final complete images using the GFP and PI channels to determine the number of apoptotic or necrotic cells per image, respectively (Figures 6A-B). Cell numbers from positive control wells were compared to those from negative controls without NK cells to determine fold stimulation following NK interactions with 2D cultured target cells.

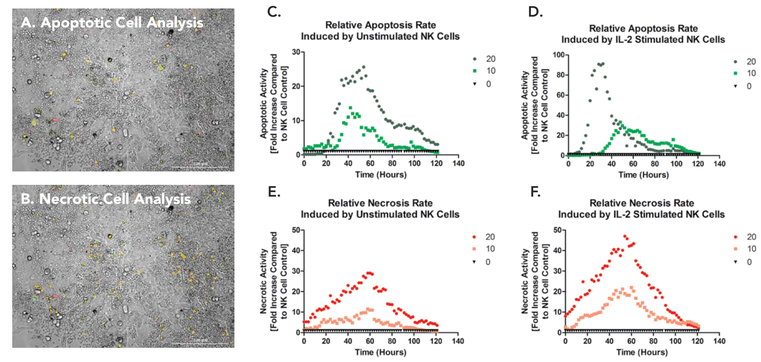

Figure 6. 2D NK CMC analysis. Gen5 placed object masks around **(A)** apoptotic or **(B)** necrotic cells during individual analysis of images captured from 2D test plates. Fold stimulation calculated of induced apoptotic activity from interaction with **(C)** unstimulated or **(D)** IL-2 stimulated NK cells; and induced necrotic activity from interaction with **(E)** unstimulated or **(F)** IL-2 stimulated NK cells at each time point.

NK cell induction of apoptotic fold stimulation follows the same pattern for 2D cultured cells (Figures 6C-D) as that seen for 3D cultured cells (Figures 4C-D), peaking earlier than necrotic induction. Greater NK cell ratios, in addition to IL-2 stimulation, again exert a greater toxic effect. However, apoptotic induction is greater in 2D cultured cells, and also left shifted when using IL-2 stimulated NK cells at a 20:1 ratio. This may be due to the fact that the activated cells can easily bind to and exert their cytotoxic effect on 2D cultured target cells. This phenomenon can also explain the rapid increase in 2D cultured HCT116 necrotic cells (Figures 6E-F) compared to 3D cultured tumoroid cells (Figures 4E-F). In 3D, NK cells must penetrate the ECM and have initial access only to cells on the outside of the tumoroid, similar to *in vivo* situations, which is not the case for 2D cells cultured evenly on the bottom of a microplate well.

A second difference seen in the fold stimulation results between 3D and 2D cultured cells is the decrease in fold values after approximately 60 hours of incubation. In 3D, fold stimulation values continue to increase, whereas in 2D, fold values steadily decrease. By looking at the images in Figure 7 captured of untreated 3D and 2D cultured HCT116 cells, respectively, after 120 hours of incubation, it can be seen that 3D cultured cells remain healthy, with little apoptotic or necrotic activity within the tumoroid (Figure 7A). A high percentage of 2D cultured cells, in comparison, have rounded up and are necrotic (Figure 7B). This confirms that use of 2D cell culture may not be suitable for long-term CMC assays, as increasing levels of uninduced necrosis can skew final conclusions for test molecules.

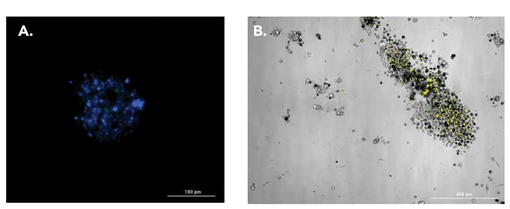

Figure 7. Untreated 3D and 2D HCT116 cells. Images captured of untreated **(A)** 3D, and **(B)** 2D cultured HCT116 cells following a 120 hour incubation. Brightfield: 2D cultured HCT116 cells; green: pSIVA-IANBD stained apoptotic cells; yellow: propidium iodide stained necrotic cells.

Conclusions

1. The RAFT 3D cell culture system provides an ideal method to prepare and study co-cultures of tumoroids and immune cells.
2. Peripheral blood CD56+ CD16+ natural killer cells can be used with 3D and 2D cultured target cells to assess potential CMC activity.
3. IL-2 activation increases the cytotoxic effect of NK cells on 3D and 2D cultured target HCT116 cells.
4. The BioSpa 8 and Cytation 5 can be combined to provide an automated method, with proper environmental conditions, to perform long-term CMC assays.
5. The combination of appropriate 3D cell models, assay methodology, and walk away automation provide a robust process to generate accurate *in vitro* NK cell mediated cytotoxicity results.

1. Ohtani, H. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human colorectal cancer. *Cancer Immun.* **2007**, *7*(4), 1-9. 2. Bruno, A.; Ferlazzo, G.; Albini, A.; Noonan, DM. A think tank of TINK/TANKs: tumor-infiltrating/tumor-associated natural killer cells in tumor progression and angiogenesis. *J Natl Cancer Inst.* **2014**, *106*(8), 1-13. 3. Eckl, J.; Buchner, A.; Prinz, PU.; Riesenberg, R.; Siegert, SI.; Kammerer, R.; Nelson, PJ.; Noessner, E. Transcript signature predicts tissue NK cell content and defines renal cell carcinoma subgroups independent of TNM staging. *J Mol Med (Berl).* **2012,** *90*(1), 55-66. 4. Hirschhaeuser, F.; Menne, H.; Dittgeld, C.; West, J.; Mueller-Klieser, W.; Kunz-Schughart, LA. Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol.* **2010,** *148*(1), 3-15. 5. Blom, WM.; De Bont, HJ.; Meijerman, I.; Kuppen, PJ, Mulder, GJ.; Nagelkerke, JF. Interleukin-2-activated natural killer cells can induce both apoptosis and necrosis in rat hepatocytes. *Hepatology.* **1999,** *29*(3), 785-792. 6. Lehmann, C.; Zeis, M.; Uharek, L.; Activation of natural killer cells with interleukin 2 (IL-2) and IL-12 increases perforin binding and subsequent lysis of tumour cells. *Br J Haematol.* **2001,** *114*(3), 660-665.