Ex Vivo Natural Killer Cell Assay Using 3D Bioprinted Cervical Cancer Tumor Models

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Abstract

Natural Killer (NK) cells, a type of innate immune cell, play a critical role in eliminating transformed or cancerous cells from the body by exerting cytotoxic effects. In recent years, several immune-stimulatory molecules and biologics have been tested to improve the tumor-lytic effects of NK cells. Three-dimensional (3D) cell culture techniques have evolved to better recapitulate the *in vivo* architecture and physiological relevance to evaluate such drugs and biologics *in vitro*. In this proof of concept study, we developed a 3D bioprinted tumor model of cervical cancer to demonstrate NK cell cytotoxic activities *ex vivo*. Green fluorescent protein (GFP)-tagged human cervical cancer cells SiHa and CaSki were 3D bioprinted with collagen I and co-cultured with human peripheral blood-derived NK cells for 96 hours. NK cell cytotoxic effects on the tumor cells were evaluated by fluorescent imaging, which showed a higher degree of tumor killing in the presence of an increasing concentration of NK cells. The method described herein is scalable, compatible with high-content imaging, and easily translatable to other tumor models.

Introduction

Natural Killer (NK) cells are a type of cytotoxic effector cells of the innate immune system that use an array of predetermined germline-encoded receptors to sense pathogens and transformed cells, including tumor cells. Unlike cytotoxic T lymphocytes, NK cells do not require tumor antigen-specific activation, rather they search for a "missing self" receptor, such as an MHC class I molecule, on the surface of tumor cells. The cytolytic activity of NK cells is regulated by the relative balance of signals received from cell surface receptors that deliver either activating or inhibitory signals. Under normal physiological conditions, MHC class I molecules on the surface of a healthy cell interact with inhibitory receptors on NK cells, while, at the same time, fewer activating ligands interact with activating receptors on the NK cells (Figure 1). However, the inhibitory signal outweighs the activation signal, resulting in self-tolerance (Long, 2013). Tumor cells tend to downregulate MHC class I molecules to escape T cell–mediated killing and thus become prone to NK cell-mediated cytotoxicity (Garrido, 2016). Natural Killer cells are traditionally characterized by the presence of the CD56 cell surface marker and the absence of CD3. In addition, CD16 is important for NK cell function because CD16 induces cytokine expression and cytotoxic effector activity.

The infiltration of NK cells into solid tumors has been associated with better prognoses in many cancer indications, including non-small cell lung cancer, colorectal cancer and cervical cancer (Burke, 2010). The cellular and extracellular matrix (ECM) composition of these tumor tissues is thought to play a critical role in the cytotoxic and infiltration functionality of NK cells that are often challenging to study using *in vitro* models.

Several 3D tumor models, including spheroids, hanging droplets and microfluidic chip models have been studied for NK cell cytotoxicity assays. For example, Giannattasio (2015) reported a spheroid model of cervical cancer to study *ex vivo* NK cell-killing efficacy. He (2014) used a rotary cell culture system to generate large numbers of uniform spheroids for NK cell-killing assays. While these models mimic certain aspects of the *in vivo* settings and some level of throughputness, many of them fail to consider the critical role of the ECM in tumor biology.

3D bioprinting is an emerging technology that enables researchers to automate fabrication of tumor constructs for the screening of anti-cancer drugs or immune-stimulatory agents. The technique dispenses a cell-laden ECM material, or bioink, blended with tumor cells that is subsequently cured chemically or thermally to provide mechanical strength to the printed structure.

Herein, we explore the potential of 3D bioprinting technology for NK cell cytotoxicity assays to aid in the evaluation of *ex vivo* NK cell cytotoxicity. Green fluorescent protein (GFP)-tagged human cervical cancer cells SiHa and CaSki were 3D bioprinted with collagen I and co-cultured with human peripheral blood-derived NK cells. NK cell cytotoxic effects on the tumor cells were evaluated using fluorescent imaging and showed a higher degree of tumor killing in the presence of increasing concentrations of NK cells. The method described here is scalable, compatible with high-content imaging, and easily translatable to other tumor models.

Materials and methods

Cell and cell line preparation

Human cervical cancer cell lines SiHa (ATCC HTB-35) and CaSki (ATCC CRL-1550) were obtained from ATCC. SiHa and CaSki cells were cultured in EMEM and RPMI-1640 media, respectively, supplemented with 10% heat inactivated FBS, 1% Pen/Strep solution. SiHa and CaSki cells were transduced with GFP-encoding lentiviruses as previously described (Kercher, 2020). Stable GFP-expressing cells were sorted using FACSaria (BD Biosciences) cell sorter 72 hours following transduction. Primary human NK cells (2W-501, Lonza) were collected from the peripheral blood of a heathy donor, negatively selected, and received as cryopreserved. The NK cells were thawed and cultured in LGM-3 lymphocyte growth medium (Lonza, CC-3211) in the presence of 500 U/mL of human recombinant IL-2 (PeproTech, 200-02). All the cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Bioink preparation and bioprinting

Collagen I (Coll-1, CELLINK) was neutralized and mixed with 1 x 10^6 SiHa or CaSki cell per mL of collagen as per the manufacturer's instruction. All components, including syringes, needles and tips were kept on ice until

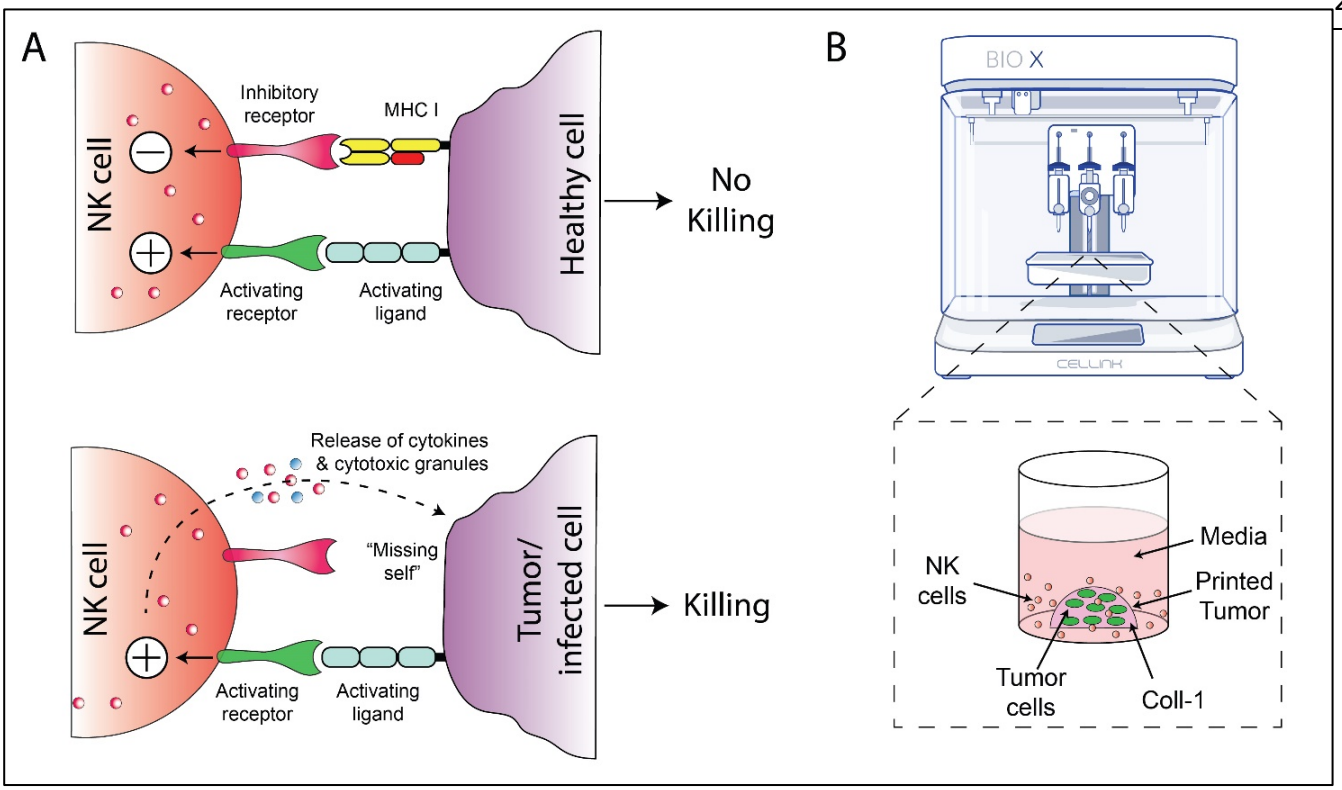

Figure 1. *3D bioprinted NK cell cytotoxicity assay.* ***(A)*** *Mechanisms of NK cell-mediated cytotoxicity: NK cells search for a "missing self" receptor, such as an MHC class I molecule, on the surface of tumor cells. The cytolytic activity of NK cells is regulated by the relative balance of signals received from cell surface receptors that deliver either activating or inhibitory signals.* ***(B)*** *3D bioprinting of cervical tumors. SiHa and CaSki cells were bioprinted using collagen and the droplet printing function of the BIO X. The collagen droplets (tumor models) were thermally gelated and maintained in growth media for 4 days before introducing the co-culture with NK cells.*

ready for use. A temperature-controlled printhead (TCPH) was set to 8°C, and the printbed was set to 10°C. Three dimensional (3D) SiHa or CaSki tumors were bioprinted by dispensing ~8 µL of cell-collagen suspension using the droplet function on the BIO X (software version 1.8) in 96-well plates (n=3) (see Figure 1B). A total volume of 1 mL cell-collagen suspension was sufficient to print tumor droplets in a 96-well plate. Following the printing, the 96-well plate was transferred to a 37°C humidified incubator for 20 minutes to allow collagen polymerization. Next, 200 µL of EMEM (SiHa) or RPMI-1640 (CaSki) media was added to each well. Media was refreshed every 2 days. The bioprinted constructs were grown for 4 days before co-culturing with NK cells on Day 5. On Day 4, a few samples were stained for actin using Alexa flour 647-conjugated phalloidin.

3D co-culture assay

On Day 5, printed tumors were washed and co-cultured with NK cells at different effector to target (E:T) ratios (0:20) for 72 hours in 200 µL of LGM-3 growth medium in the presence of IL-2. For positive control, tumors were incubated with etoposide or TNFα to induce cell death by apoptosis. The negative control wells did not receive any NK cells or apoptotic inducers.

Imaging and statistics

At the end of the assay, printed tumors were stained with propidium iodide (PI) for 10 minutes prior to imaging. PI was washed with DPBS twice to remove loosely adhered/dead NK cells. Loss of GFP fluorescence and elevation of PI signals were used as a readout of tumor cell death as described previously (Kercher, 2020). Imaging was performed using an epifluorescent microscope connected to a monochrome camera. Fluorescence intensity was measured using ImageJ software (National Institutes of Health) and graphs were translated and prepared using GraphPad Prism 8. The results were statistically measured in Prism using Student's t-test, and the data were expressed as the mean ±SEM.

Results and discussion

Tumor cell growth and bioprinted tumor formation

The droplet function on the BIO X was able to automate the dispensing of consistent collagen-tumor droplets in 96-well plates. Uniformity in droplet shape and well placement aided in the overall microscopy and analysis workflow. 3D bioprinted tumor droplets were imaged for GFP fluorescence and stained with PI to determine cell viability. The results illustrated in Figure 2 indicate that the cervical cancer cells were viable in the printed

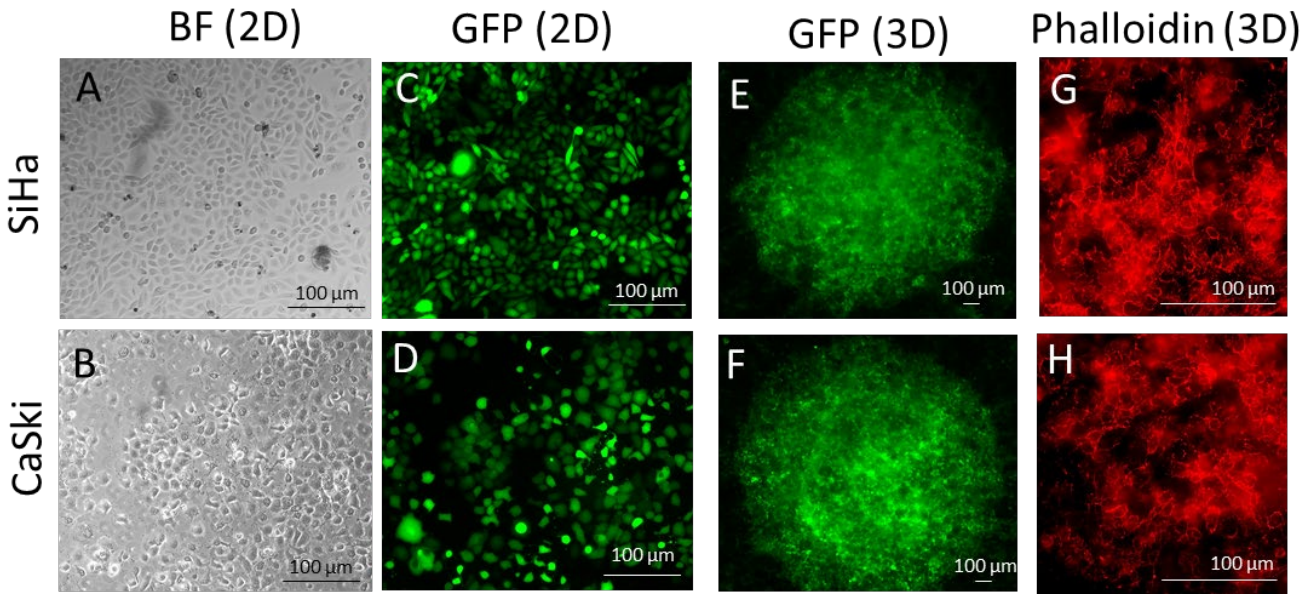

Figure 2. *Human cervical cancer cells SiHa and CaSki were transduced with lentiviruses to express GFP. **(A-D)** SiHa and CaSki cells grown in 2D monolayer and imaged using bright-field (BF) and GFP channels. **(E-F)** GFP-expressing cells were mixed with collagen I and 3D bioprinted in 96-well plates. Images of the 3D constructs were captured on Day 4. **(G-H)** On Day 4, tumor samples were stained for actin using Alexa Flour 647-conjugated phalloidin. Scale bar = 100 µm.*

tumors at Day 4. Phalloidin staining (Figure 2G-H) shows characteristic actin accumulation at the cell periphery within 3D bioprinted tumors.

Tumor-NK co-culture and cytotoxicity analysis

NK cell-mediated cytotoxicity was validated both quantitively and qualitatively. Bright-field images of the co-culture indicate tumor-NK cell interaction (Figure 3A) for both SiHa and CaSki cell types. Following fluorescent imaging and quantification, an NK cell concentration-dependent decrease in tumor cell viability was observed. At 20:1 (E:T) ratio a statistically significant (p=0.0003) reduction (~60%–70%) in tumor viability was observed compared to the no NK control. Representative images of printed tumors SiHa and CaSki in the presence or absence of NK cells are shown in Figure 3, which exhibit qualitative decrease in GFP fluorescence when NK cells were present in the co-culture. Most interestingly, the outer perimeter of the printed tumors showed the highest reduction in GFP fluorescence in the presence of NK cells (Figure 4), suggesting infiltration of NK cells into the collagen matrix of the printed tumor. However, specific staining for NK cells is necessary to confirm the degree of NK cell infiltration.

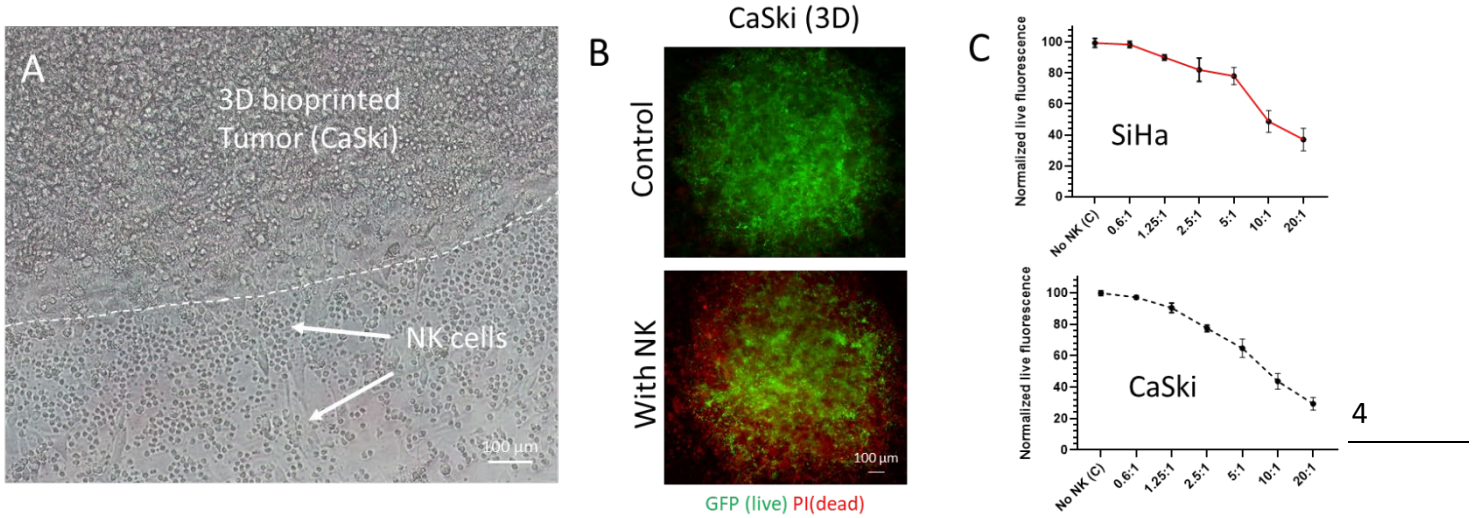

Figure 3. *NK cell cytotoxicity assay in 3D. **(A)** CaSki cells were 3D bioprinted and co-cultured with NK cells. Bright-field image shows infiltrating NK cells at the periphery of the printed tumor. **(B)** At the end of the co-culture, the bioprinted tumors were stained with PI, and images were captured with both GFP and PI channel and merged using ImageJ. **(C)** A range of E:T ratios (0:20) were used for the co-culture. Both the bioprinted tumor models of SiHa (top) and CaSki (bottom) cells show an NK cell-dose dependent decrease in tumor cell viability.*

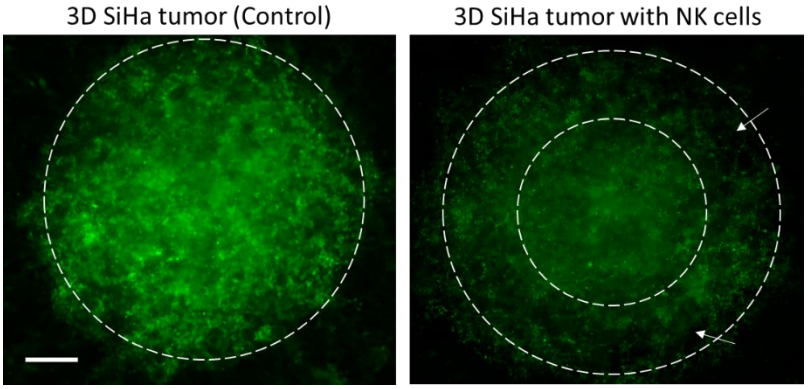

Figure 4. *Tumor cell killing pattern. In the absence of NK cells, SiHa tumor expresses bright GFP fluorescence (left image). However, when co-cultured with NK cells, the overall GFP fluorescence intensity was decreased which was significant in the outer concentric ring of the printed tumor (white dashed line indicated by white arrows) (right image). Scale bar = 500 µm.*

Conclusions and future direction

- Droplet 3D bioprinting technology can be used as an automated process to reproducibly fabricate tumors with human cervical cancer cells SiHa and CaSki, which represent a versatile *in vivo*-like model to facilitate immuno-oncology investigations.
- 3D bioprinted tumor cells remained confined and viable within a collagen matrix for the duration of co-culture study with human NK cells.
- In the cytotoxicity assay, human peripheral blood-derived NK cells exhibited a cell concentration (E:T ratios)-dependent increase in cancer cell killing.
- The NK cell cytotoxicity assay is compatible with high-content imaging workflows and can also be adapted for other tumor models, including PDX organoids, to accelerate the drug screening process and drive clinical translation of personalized medicines.
- Additionally, the multiwall bioprinting format on the BIO X could be used to scale up support for the high-throughput screening of biological agents (such as immune checkpoint inhibitors) and engineered NK cells (CAR-NK).
- Further studies might include quantification of NK cell infiltration into the tumors and cytokine released by tumor or T cells.

References

1. Burke S, Lakshmikanth T, Colucci F, Carbone E. New views on natural killer cell-based immunotherapy for melanoma treatment. *Trends in Immunology*. 2010; 31(9): 339–345. DOI:10.1016/j.it.2010.06.003.
2. Garrido F, Aptsiauri N, Doorduijn EM, et al. The urgent need to recover MHC class I in cancers for effective immuno-therapy. *Current Opinion in Immunology*. 2016; 39: 44–51. DOI:10.1016/j.coi.2015.12.007.
3. Giannattasio A, Weil S, Kloess S, et al. Cytotoxicity and infiltration of human NK cells in in vivo-like tumor spheroids. *BMC Cancer*. 2015; 15(1): 351. DOI:10.1186/s12885-015-1321-y.
4. Kercher EM, Nath S, Rizvi I, Spring BQ. Cancer cell-targeted and activatable photoimmunotherapy spares T cells in a 3D coculture model. *Photochemistry and Photobiology*. 2020; 96(2): 295–300. DOI:10.1111/php.13153.
5. Long EO, Kim HS, Liu D, et al. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annual Review of Immunology*. 2013; 31: 227–258. DOI:10.1146/annurev-immunol-020711-075005.
6. He W, Kuang Y, Xing X, et al. Proteomic comparison of 3D and 2D glioma models reveals increased HLA-E expression in 3D models is associated with resistance to NK cell-mediated cytotoxicity. *Journal of Proteome Research*. 2014; 13(5): 2272–2281. DOI:10.1021/pr500064m.

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