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Assessment of NK Cell-Mediated Killing and Phenotypic Analysis Using Advanced Flow Cytometry and an Optimized Multiplexed Assay.

Julie Lovchik and Mark Carter
Sartorius, 5700 Pasadena Ave. NE, Albuquerque, NM 87113

Abstract

In this study, a novel multiplexed assay was developed that simultaneously measures Natural Killer (NK) cell phenotype and function in relation to tumor target killing. NK cells are an essential part of the innate immune system, interface with the adaptive immune system, and play a critical role in tumor immune surveillance and anti-tumor responses. Although chimeric antigen receptor (CAR)-modified T cells have proven to be highly effective as an anti-cancer therapeutic, there are potential problems associated with their use, particularly the risk of inducing graft-versus-host disease (GVHD) and aberrant cytokine release. In contrast to T cells, NK cells are potent cytotoxic effector cells that do not require MHC restriction and have a low risk of inducing GVHD. Recent advances in the development of CAR-modified NK cells have shown great promise for clinical utilization in cancer immunotherapy with capability for both direct tumor killing as well as for use in combination therapy with monoclonal antibodies to boost NK cell antibody-dependent cell-mediated cytotoxicity (ADCC). A critical process in the development of new NK cell-mediated therapeutics is the ability to expediently assay, screen, and analyze data for NK cell activation and tumor killing. To address this need, a novel multiplexed assay was developed that measures tumor target killing, target-dependent expression of activation markers (CD69 and CD25), and the quantitation of secreted Granzyme B, IFNg, and TNFa in a single well of a microtiter plate. To validate the assay, a direct NK cell-tumor cell killing model was used. Purified human NK cells were mixed with target cells at different ratios. Tumor cell killing, NK cell activation status, and quantification of secreted proteins were measured at 4 and 24 hours after co-culture. Assay data acquisition was performed on the iQue® 3 platform for advanced flow cytometry. Data analysis and visualization was done using the integrated iQue Forecyt® software package. Tumor cell killing, along with detection of Granzyme B release, was evident by 4 hr following co-culture, and both were increased with the increase in effector to target ratio (E:T). Killing was further enhanced when NK cells were stimulated with IL-2 + IL-15. As expected, an increase in CD69 and CD25 expression was also observed following cytokine stimulation and further increased upon co-culture of NK cells with tumor targets, along with detection of secreted IFNg and TNFa. Further validation of the assay using an ADCC model was also performed. These data show that a miniaturized, multiplexed NK cell killing assay, combined with high throughput flow cytometry, is a powerful tool to rapidly screen CAR-NK cell effector functions and other NK cell based therapeutics.

Introduction

Natural Killer (NK) cells are an essential part of the innate immune system and play a crucial role in anti-tumor responses. Currently, several types of NK cell-related immunotherapeutics are being developed for the treatment of cancers. These include the *in vitro* expansion and/or activation of allogeneic NK cells for adoptive transfer into cancer patients, tumor-specific antibodies (mAbs, BIKES, TRIKES) to induce NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) against tumor cells, and production of CAR-NK cells. A critical process in evaluating the efficacy of new NK cell-mediated therapeutics is the ability to screen for NK cell activation and tumor cell killing. Here we introduce a novel multiplexed assay that simultaneously measures target cell killing, expression of NK cell activation markers, and quantitation of secreted effector proteins and cytokines in a single well of a microtiter plate using a mixed cells and beads assay format and the iQue® platform.

Experimental approach

1. Workflow

Direct Tumor Killing Model: NK cell cytotoxic activity was assessed in a direct tumor cell killing model utilizing the K562 human chronic myelogenous leukemia cell line as the tumor target cells. The K562 cells were first stained with a fluorescent encoder dye, and then combined (20K/well) with PBMCs or enriched (negatively selected) human NK cells (Astarte Biologics) that had been incubated for 16-18 h in either media alone (Non-activated NK cells) or media containing 200 u/mL of IL-2 + 100 ng/mL of IL-15 (Activated NK cells) at Effector:Target (E:T) ratios of 1:1 or 5:1, and incubated at 37 °C, 5% CO_2.

ADCC Model: Raji tumor cells (Human B lymphoblast-like cells; Burkitt's lymphoma) were first stained with a fluorescent encoder dye, and then incubated (20K/well) for 30 min at RT with doses of anti-CD20 mAbs (InvivoGen) between 0.00012- 10 ug/mL. PBMC effector cells were then added at an E:T of 10:1 and incubated at 37 °C, 5% CO_2. Effectors and target cells were also cultured alone as controls, as well as Raji + PBMCs without mAb to determine direct tumor cell killing , and Raji + mAb without effectors to assess direct mAb effects.

Following co-culture, 10 µL samples were removed at 4h and 24h to assess tumor cell killing, along with analysis of NK cell phenotypic and activation markers and the quantitation of secreted effector proteins using the NK Cell Mediated Killing Kit and the iQue® 3 equipped with Violet, Blue and Red lasers (VBR).

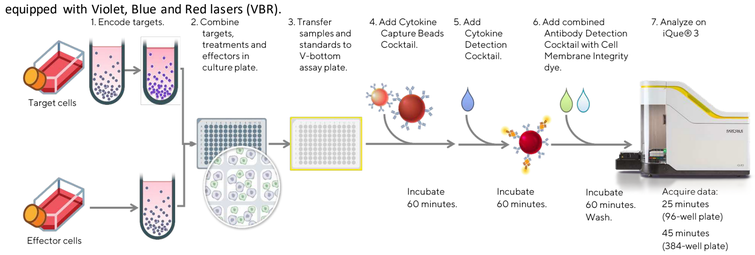

Figure 1: Schematic diagram of the workflow for the analysis of NK cell cytotoxic function, along with assessment of the NK cell activation state and cytokine release using the iQue® Human NK Cell Killing Kit.

2. Simultaneous endpoint measurement in a single well.

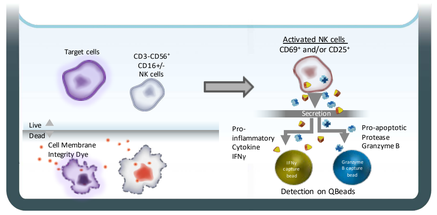

Figure 2: Illustration of iQue® Human NK Cell Killing Kit assay principles. Target cells are distinguished from effector cells by staining with a fluorescent encoder dye, and tumor cell killing is then determined by staining with a fluorescent cell membrane integrity dye. NK cells are identified using CD3, CD56, and CD16. Their activation state is assessed using CD69 and CD25. Production of the pro-inflammatory cytokine, Interferon gamma (IFNγ), and the pro-apoptotic serine protease, Granzyme B, are quantified using a 2-plex QBeads in a sandwich immunoassay format in the same well. Additional compatible cytokines can be assayed with iQue® NK Cell Companion kits, but were not included in this illustration.

Results:

1. Cytokine activation increases NK cell cytotoxic activity.

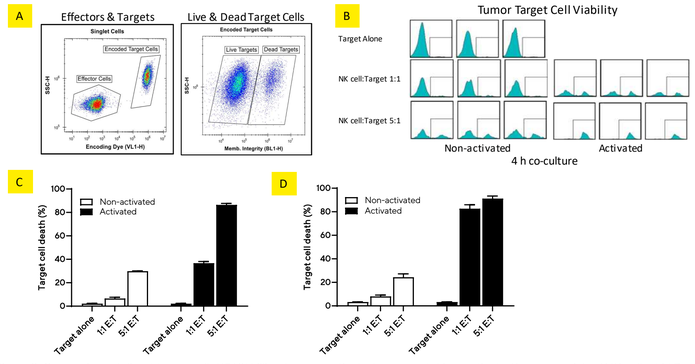

Figure 3. Comparison of direct tumor cell (K562) killing by non-activated versus cytokine-activated human enriched NK cells. (A) Dot plot showing separation of target cells from effector cells with the use of a fluorescent encoder dye. Target cell killing was then determined with the use of a cell membrane integrity dye to distinguish live and dead targets cells. (B) Histogram depicting target cell viability following a 4h co-culture with non-activated or cytokine activated NK cells. (C,D) Percent tumor cell killing after co-culture of tumor cells with non-activated or cytokine activated NK cells for (C) 4h or (D) 24 hr. Co-culture groups were plated in triplicate. Data represents ave/group +/- 1SD.

2. Expression of NK cell activation markers is increased upon interaction with tumor cells.

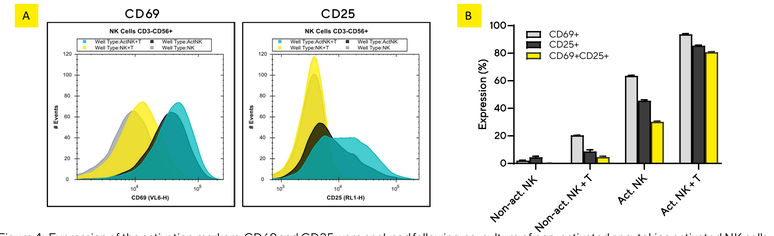

Figure 4: Expression of the activation markers, CD69 and CD25 were analysed following co-culture of non-activated or cytokine activated NK cells with K562 tumor cells (T). (A) Overlay histograms depicting CD69 and CD25 expression on NK cells after 4 h co-culture. (B) Summary of activation marker expression following 24 h of co-culture. (n=3/group, NK, or Non-act. NK = non-activated NK cells, Act. NK = cytokine activated NK cells).

3. Secretion of several effector proteins is dependent on NK cell activation state and tumor cell interaction.

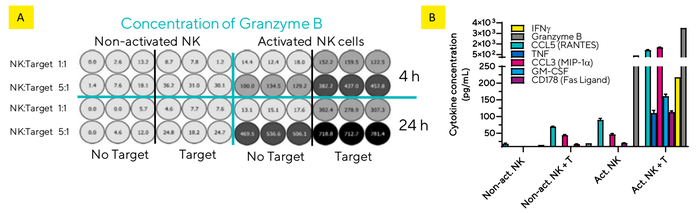

Figure 5. Secretion of effector proteins and cytokine or chemokine production was analyzed following co-culture of non-activated or cytokine activated NK cells with K562 tumor cells. All co-culture groups were plated in triplicate. (A) Plate heat-map showing concentration (pg/mL) of Granzyme B secretion following co-culture for 4 h and 24 h. (B) Summary of the average concentration (+/- 1 SD) of various effector proteins assessed in parallel after NK cell plus K562 tumor cell co-culture for 24 h.

4. NK cell-mediated ADCC and effector protein secretion levels are mAb and donor-dependent

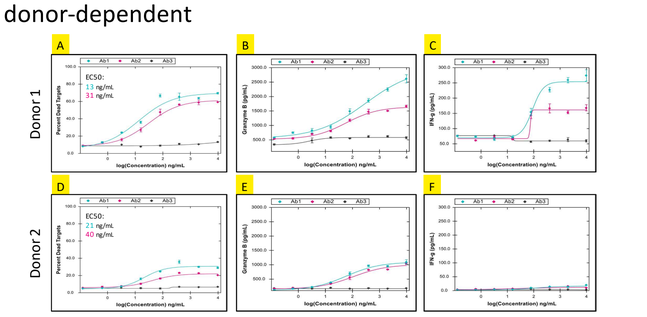

Figure 6: PBMCs from two different donors were incubated with Raji cells at a 10:1 E:T with increasing concentrations of 2 different anti-hCD20 IgG1 mAbs (Ab1 and Ab2), or an anti-CD20 IgA2 mAb (Ab3), as a negative control. After 4 h, target cell killing was assessed (A,D), along with secretion of Granzyme B (B,E) and production of INFγ (C,F). Co-culture groups were plated in triplicate. Data represents target cell count ave/group +/- 1 SD.

Conclusion

Typical immunology research workflows to assess NK cells require multiple assays. The data presented here demonstrates the ability to analyze multiple NK cell function and phenotyping parameters in a single assay. Utilizing a mixed cells and beads assay format, this iQue® Human NK Cell Killing Kit includes the ability to distinguish effector and target cell viability in order to assess NK cell cytolytic function, along with characterization of the NK cell activation state. It also includes the simultaneous measurement of several secreted effector proteins, including the cytokine, IFNγ, and pro-apoptotic protease Granzyme B, along with the flexible option to add additional validated cytokines with iQue® Human NK Cell Companion kits. This assay requires only small sample volumes, thereby conserving precious sample and allowing multiple analyses from the same effector + target co-culture over time. It also requires minimal hands on time with a total assay time of approximately 3.5 hours. Furthermore, the results can be immediately visualized with the use of the iQue® platform and iQue Forecyt® software. Overall, these data show that this miniaturized, multiplexed NK cell killing assay, combined with high throughput flow cytometry, reduces the number of overall tests needed, while providing meaningful data for the characterization of NK cell-mediated tumor cell killing.

Key Advantages:

- Enables biological insights into NK cell mediated tumor cell killing in physiologically relevant models
- Simultaneously quantifies NK cell surface protein & cytokine expression in relation to target cell killing
- Collapses traditional workflows into one miniaturized, multiplex assay with minimal sample manipulation.
- Easy-to-use, low sample volumes required in either 96 well or 384 well plate formats
- Allows real time data analysis and novel visualization tools
- Flexibility to add detection of additional cytokines dependent on user's biological needs when combined with iQue® NK Cell Companion kits including: MIP-1α, RANTES, GM-CSF, CD178 (Fas Ligand), Granzyme A, and TNFα