Immune Profile of Activated Natural Killer Cells

Brian Astry, Sol Degese, Jim Rivard, Hemant Mishra, Li Peng, Kevin Flynn, Christine Goetz, Damani Bryant and Megan Larson | Bio-Techne, 614 McKinley Place NE, Minneapolis, MN 55413

ABSTRACT

Natural killer cells (NK cells) are innate lymphoid cells that function as both cytotoxic effectors and regulators of immune responses. NK cells are activated following the detection of abnormalities in target cells such as the loss of MHC class I expression, up-regulation of stress-induced ligands that occurs in response to infection or cancer, and activation of antibody-dependent cell-mediated cytotoxicity (ADCC) through IgG Fc receptor ligation. We analyzed the activity of ADCC of NK cells on human epidermal growth factor receptor 2 (HER2) positive SKOV-3 cells with an anti-HER2 antibody, Trastuzumab. Using the R&D Systems® FlowX Human NK Cell Killing Flow Cytometry Kit and the R&D Systems® Human XL Cytokine Luminex® Performance Assay 44-plex, NK cells isolated and expanded from four separate donors were analyzed for Cytotoxicity, Granzyme B, Cytokine and Chemokine production. When cocultured with the target cell, the NK Cells produced statistically significant more Granzyme B, Interferon-gamma (IFNγ), Granulocyte-macrophage colony-stimulating factor (GM-CSF), and several pro-inflammatory chemokines. This increase in pro-inflammatory markers correlated with increased expression of hLAMP-1/CD107a on the NK cell surface – a sign of NK cell activation – and a decrease in target cell viability. Together, this data shows an in vitro method developed by R&D Systems® to study NK cell activity and cytotoxicity. When run in parallel with Flow Cytometry cell surface analysis, R&D Systems® Luminex® immunoassays provides a clear picture of the NK cell effector function.

INTRODUCTION

Ovarian cancer is responsible for more than 100,000 deaths annually. The tyrosine kinase receptor, human epidermal growth factor receptor 2 (HER2) is a known prognostic biomarker for some histological subtypes of ovarian cancer1. Although the monoclonal HER 2 antibody Trastuzumab, has been used to successfully treat HER2 receptor positive breast cancer, it failed to demonstrate the same therapeutic efficacy in ovarian cancer2. In light of this data, it is important to understand mechanisms of antibody-dependent cell-mediated cytotoxicity (ADCC) in ovarian cancer. Natural Killer (NK) cells are of great interest due to their ability to evoke ADCC via Fc-gamma receptor IIIa- antibody ligation3. NK cells are a well characterized subset of innate lymphocytes with anti-tumor activities4. NK cell cytotoxicity is mediated, in part by natural cytotoxicity receptors such as NKp30, NKp46, and NKG2D. Later stages of the NK cell killing process typically involves the formation of a pore between the NK cell and the target cell by the glycoprotein perforin. Next, the serine protease Granzyme B is released by the NK cell into the target cell to trigger apoptosis via caspase-dependent and caspase-independent mechanisms. NK cells also secrete chemotactic, proinflammatory, and immunosuppressive cytokines4. These cytokines can attract and/or stimulate other immune cells such as T cells, DC cells, macrophages, and neutrophils. To investigate NK mediated ADCC, we isolated NK cells from donor peripheral blood mononuclear cells (PBMCs) and tested their cytotoxic activity on the HER2 positive SKOV-3 human ovarian cell cancer line.

METHODS

Cell Culture
Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood using a Ficoll gradient. PBMCs were expanded for 14 days with plate-bound Human NKp46/NCR1 Antibody (Catalog # MAB1850; 10 ug/mL) in ExCellerate NK Expansion Media (Catalog # CCM032) supplemented with recombinant human cytokines IL-2 (Catalog # 202-IL; 27 ng/mL), IL-12 (Catalog # 219-IL; 10 ng/mL), IL-18 (Catalog # 9124-IL; 10 ng/mL), and IL-21 (Catalog # 8879-IL; 10 ng/mL). Media and 2x cytokine were added every 2-3 days up to Day 14.

Natural Killer Cell Phenotyping
Using the R&D Systems® FlowX Human NK Cell Phenotyping Flow Cytometry Kit (Catalog # FMC033), the expanded NK cells were washed with 1x PBS, then Fc receptors were blocked using total IgG (1 µg IgG/106 cells) for 10 minutes at room temperature. Viability staining was also carried out during the blocking step (Biotium live-or-dye 405/545). The NK cell positive marker antibodies (hCD56 Alexa Fluor® 647 antibody, hNKp30 Alexa Fluor® 488 antibody, hNKG2D Alexa Fluor® 700 antibody, hNKp46 PE antibody, and hCD16 PerCP antibody) were added to the blocked cells and incubated for 45 minutes at room temperature. hCD3ε Alexa Fluor® 405 antibody was also added to the blocked cells to exclude T cells. The stained cells were washed and resuspended in flow staining buffer and run on a Flow Cytometer. Data was analyzed using FlowJo™ (Becton Dickinson) analysis software.

Natural Killer Cell Killing Assay
At day 14, the expanded NK cells were labeled with Janelia Fluor NK cell dye and the target cells (SKOV-3 ovarian cancer cells) were labeled with Mito Mark target cell dye. NK and SKOV-3 cells were then co-incubated for 2 hours at 37oC with an Effector (NK) to Target (SKOV-3) ratio of 0.5:1 (1 = 0.25 x 106 cells) in the presence of 5 ug/mL anti-HER2 (Trastuzumab). LAMP-1/CD107a PE (or Ms IgG2b PE) was included in the cell culture media for the entirety of the killing assay for detection of LAMP-1/CD107a proteins. After the 2-hour killing assay, cells were harvested, blocked and viability staining with Biotium Live-or-Dye™ 405/545 was carried out. Cells were then washed in flow staining buffer and stained with hCD3ε Alexa Fluor® 405 antibody and hCD56 Alexa Fluor® 700 antibody. Cells were washed in 1x PBS, fixed in 1% formaldehyde for 15 minutes, washed in 1x HBSS, and then washed a final time in flow staining buffer. Cell Count Particles were added (Novus Biologicals NBP3-00495), samples were run on a Flow Cytometer, and data was analyzed using FlowJo™ (Becton Dickinson) analysis software.

Luminex
The NK killing assay cell culture samples were run using R&D Systems® Human XL Cytokine Luminex® Performance Panel (Bio-Techne Catalog # FCSTM18). This is a 44-analyte multiplex assay utilizing the Luminex® MagPlex Microspheres. Cell culture supernatant samples were diluted to 1:2 and 1:200 with the assay diluent. The assay was run according to kit instructions and tested on a Luminex® MAGPIX®.

RESULTS

Figure 1A

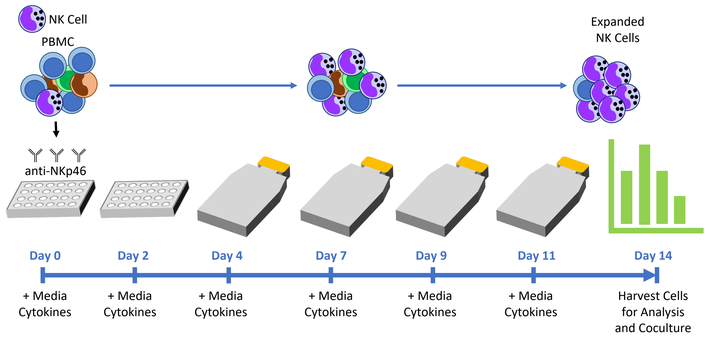

Figure 1B

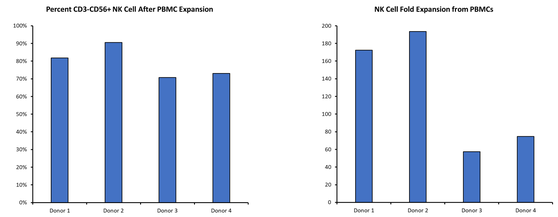

Figure 1C

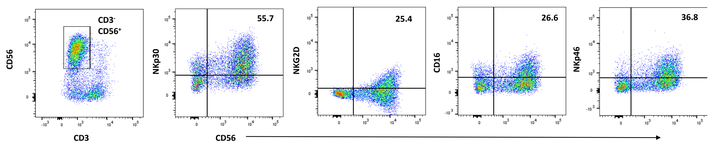

Figure 1. Natural Killer Cell Expansion from Peripheral Blood Mononuclear Cells. (A) Human NK cells were expanded from PBMCs using plate-bound anti-human NKp46 antibody and a cytokine cocktail (IL-2, IL-12, IL-18, and IL-21) in culture for 14 days. **(B)** NK Cell purity after expansion was analyzed based on CD3- and CD56+ expression within the total PBMC population. NK cell fold expansion was determined by the percentage of total PBMCs before and after expansion. **(C)** In the phenotype analysis, CD3-CD56+ NK cells were assessed for expression of NK cell activating cell surface receptors NKp30, NKG2D, NKp46, and CD16. Gating was determined based on unstained cells or isotype controls.

Figure 2A

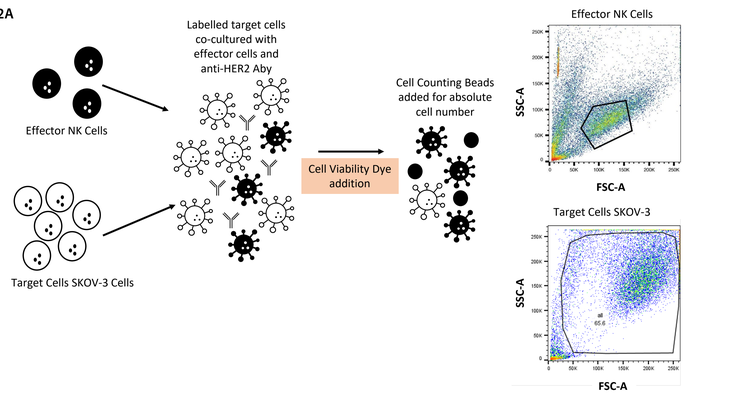

Figure 2A. NK Cell Killing Assay. The expanded NK cells were cocultured with SKOV-3 cells in the presence of anti-human HER2 antibody at a ratio of 0.5:1 (0.125 x 106 NK cells:0.25 x 106 SKOV-3 cells) 2 hours.

Figure 2B

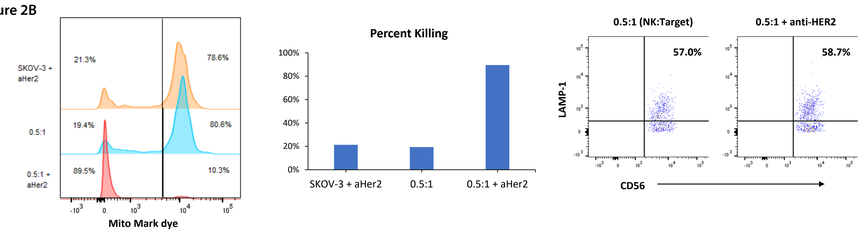

Figure 2B. NK Cell Killing Assay. The percentage of viable cells that were SKOV-3 cells (Mito Mark positive) was normalized to SKOV-3 cells cell culture to determine the percentage of cell death due to NK cell killing. The activation of NK cells was measured using LAMP-1.

Figure 3

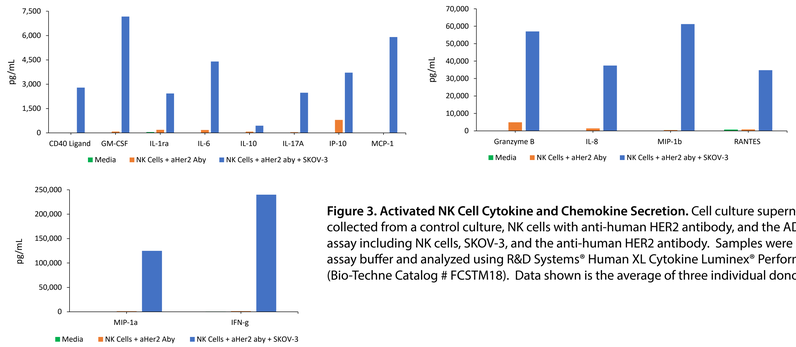

Figure 3. Activated NK Cell Cytokine and Chemokine Secretion. Cell culture supernatants were collected from a control culture, NK cells with anti-human HER2 antibody, and the ADCC killing assay including NK cells, SKOV-3, and the anti-human HER2 antibody. Samples were diluted in assay buffer and analyzed using R&D Systems® Human XL Cytokine Luminex® Performance Panel (Bio-Techne Catalog # FCSTM18). Data shown is the average of three individual donor samples.

Figure 4

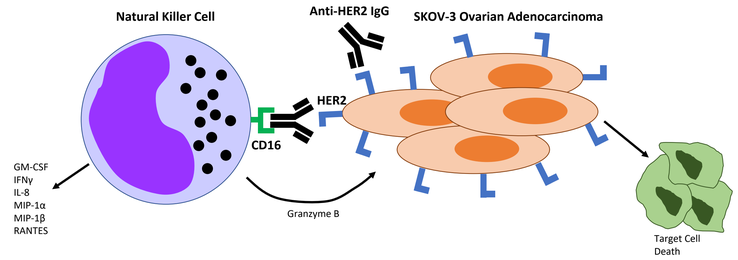

Figure 4. NK Cell Antibody-Dependent Cell-Mediated Cytotoxicity Summary. The novel R&D Systems® Human NK cell killing assay protocol was used to analyze the activation of the ADCC pathway. The activation of the NK cells resulted in measurable cancer cell death and release of pro-inflammatory cytokines, chemokines, and the cytotoxic vesicle protein, Granzyme B.

DISCUSSION

The R&D Systems® Human Natural Killer cell killing assay protocol allows researchers to study the immune profile of ADCC active NK cells. Analyzing secreted proteins with the R&D Systems® Human XL Cytokine Luminex® Performance Panel showed a significant increase in 14 different cytokines and chemokines. These includes pro-inflammatory cytokines GM-CSF, IL-6, IL-8, IL-17A, Interferon-γ and chemokines IP-10/CXCL10, MCP-1, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5. In addition, the cytotoxic enzyme, Granzyme B, and anti-inflammatory cytokines IL-10 and IL-1ra, were also increased with NK cell activation. This increase in inflammatory protein secretion correlated well with the percent killing determined by flow cytometry. Although there was a slight increase in the NK cell activation marker, LAMP-1/CD107a, the increase over the non-ADCC coculture did not correlate with the killing assay. This is likely due to some NK cells becoming activated in the presence of the SKOV-3 ovarian adenocarcinoma cells through non-ADCC mechanisms. However, this activation was not enough to kill the SKOV-3 cells. When utilized with R&D Systems® cell culture and flow cytometry products, the Human XL Cytokine Luminex® Performance Panel can facilitate translational research using NK cells.

REFERENCES

1. Luo, H. et al. (2018) Plos One. 13:1
2. Bookman, M.A. et al. (2003) J. Clin.Oncol. 21:283
3. Lo Nigro, C et al. (2019) Ann. Transl. Med. 7:105
4. Abel, A. M. et al. (2018) Front. Immunol. 9:1869

CONTACT: Megan.Larson@bio-techne.com
bio-techne.com