APPLICATION NOTE

Unravel the Complexity of Single Natural Killer Cell Cytotoxicity and ADCC Mediation

APPLICATION SPOTLIGHT

- Directly measure natural killer (NK) cell cytotoxicity at the single-cell level
- Investigate NK cell killing mechanisms and responses to chemical treatment
- Identify NK cell populations that elicit the most robust antibody-dependent cellular cytotoxicity (ADCC) in response to therapeutic monoclonal antibodies

INTRODUCTION

Immunotherapies have transformed how we approach cancer treatment. Most immuno-oncology treatments aim to activate T cells that target tumor cells; however, an increasing number of natural killer (NK) cell-based therapies are emerging[1]. NK cells, part of the innate immune system, can recognize and eradicate cancer cells. They are heterogeneous lymphocytes, both in phenotype and function, whose cytotoxicity is mediated by the balance between activating and inhibitory receptors, as well as interactions with regulatory ligands on target cells[2]. This interplay is complicated further by the fact that the expression of NK cell receptors and target cell ligands changes over time, affecting treatment response. Decoding this relationship at the single-cell level is important when developing NK cell-based therapies to deliver the most promising results.

NK cells also play a role in antibody-dependent cellular cytotoxicity (ADCC), one of the mechanisms used by monoclonal antibodies (mAb) to target antigens such as EGFR, HER2, and CD20[3]. Yet, the contribution of NK cells to the efficacy of these therapies is poorly understood. Many factors can impact the strength of the NK cell response, including the glycosylation state of therapeutic antibodies, which can influence and even improve the cytotoxic activity of NK cells[4].

These relationships illustrate the complexity of NK cell behavior and the need for technologies that allow us to characterize NK cell function at the single-cell level. Current experimental methods, however, rely on bulk measurements of heterogenous samples that often contain contaminating cell types, because NK cells are rare and their lineage markers are poorly defined. This is especially true for methods that require recovery time after enrichment since growth media containing cytokines like IL-2 and IL-15 preferentially cause T cells to proliferate. This results in coarse measurements that reflect the average cytotoxicity of mixed samples, which hampers the development of more efficacious therapies.

In this application note, we demonstrate how NK cell function can be assessed at the single-cell level using the Opto™ Cell Therapy Development workflow on the Berkeley Lights Platform to characterize both cytotoxicity and ADCC mediation in the presence of target cells. The ability to use fluorescent imaging and opto-electropositioning (OEP™) to selectively analyze NK cell populations of interest, incubate them with target cells and/or known cancer therapeutics in a single experiment, followed by downstream genomic analysis will empower scientists to accelerate the development of NK cell-based therapies.

MATERIALS AND METHODS

Isolating NK cells

We enriched NK cells from healthy donor peripheral blood using the EasySep Human NK Cell Isolation Kit (STEMCELL Technologies). We rested enriched cells overnight in media containing Advanced RPMI 1640, 1X Glutamax, 10% human serum, and 50 µM β-Mercaptoethanol (Thermo Fisher Scientific) supplemented with 100 U/mL IL-2 and 100 ng/mL IL-15 (R&D Systems).

Cytotoxicity and secretion assays on the Beacon® optofluidic system

We harvested and stained NK cells with Brilliant Violet 421-conjugated anti-CD3, PE-conjugated anti-CD56, and Brilliant Violet 421-conjugated Annexin V antibodies (BioLegend). For experiments in which K562 cells were used as target cells, we co-cultured CFSE+ Annexin V- K562 cells with NK cells. We tracked target cell killing by co-culturing cells in NanoPen™ chambers (pens) on an OptoSelect™ chip and perfusing the chip with media containing 5 µM NucView 530 Caspase-3 Substrate (Biotium). The pens were imaged every 30 minutes in FITC and PE. For experiments using Concanamycin A, we treated NK cells with 200 nM Concanamycin A for 4 hours before antibody staining.

For ADCC experiments with Raji cells as targets, we incubated CFSE-labeled Raji cells with 40 ng/mL anti-human CD20 IgG1 non-fucosylated mAb or anti-human CD20 IgG1 fucosylated mAb (InvivoGen) for 30 minutes prior to Annexin V staining. Fucosylated antibody- and non-fucosylated antibody-treated CFSE+ Annexin V- Raji cells were loaded into separate regions of the chip and incubated with NK cells as above. Data were analyzed using Assay Analyzer 2.1 and Image Analyzer 2.1 software.

NK cells and the Single Cell Cytotoxicity Assay

We enriched NK cells from PBMCs using negative selection with antibody-coated magnetic beads. Purity of the resulting cells can vary, with contaminating T cells, NKT cells, and monocytes representing up to 40% of the NK cell-enriched fraction. To address this, we stained our enriched cells with antibodies against both CD56, a classic NK cell phenotypic marker, and CD3, a marker expressed on both T cells and NKT cells. We also stained with an antibody against Annexin V to identify dead cells. We used the Target Penning and Selection operation within Cell Analysis Suite (CAS™) software to selectively load CD56+ CD3- Annexin V- cells into NanoPen chambers. NK cells were incubated with CFSE-labeled K562 (green) cells, a myelogenous leukemia cell line that expresses activating NK ligands and is sensitive to NK-mediated cytotoxicity. Cells were co-cultured for 10 hours; fluorescent time-lapse imaging was performed during culture and apoptosis was monitored using a caspase-3 substrate that fluoresces when caspase-3 is activated (red) (Figure 1).

RESULTS AND DISCUSSION

Characterizing NK cell killing behavior with the Cytotoxicity Assay

NK cells kill their targets via two primary mechanisms: perforin/granzyme stored in lytic granules or by the FasL/FasR mechanism. To identify the dominant mechanism of action, we pre-treated NK cells with Concanamycin A (CMA), a small molecule that specifically disrupts the perforin/granzyme pathway without interfering with the FasL/FasR mechanism[5]. After pre-treatment, we loaded cells into NanoPen chambers on an OptoSelect chip and tracked the cumulative percentage of pens with K562 caspase-3 activity over time to assess the kinetics of NK-mediated killing.

We found that 38% of pens showed target cell death in the presence of single untreated NK cells (Figure 2A).

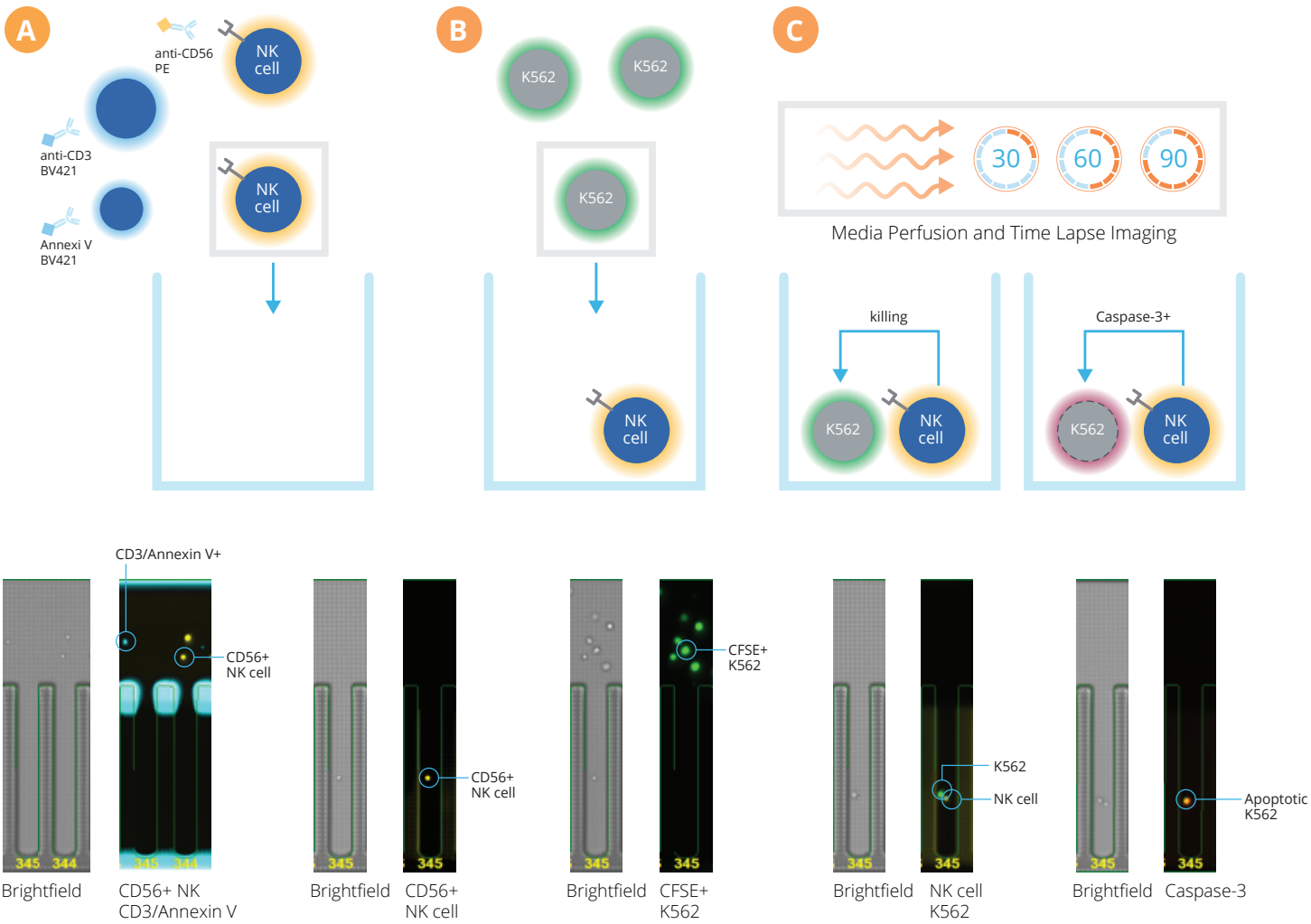

Figure 1. Direct measurement of NK-mediated cell killing using the Cytotoxicity Assay. A. Images from representative NanoPen chambers show selective loading of CD56+CD3-Annexin V- NK cells (yellow). **B.** CFSE+ K562 target cells (green) are then loaded into the NanoPen chamber and cultured with media containing a fluorescent reporter of caspase-3 activity. **C.** Fluorescent images are captured every 30 minutes to identify PE+ apoptotic K562 cells (red).

K562 death decreased to 25% when target cells were co-cultured with CMA-treated NK cells. These pens also showed slower killing kinetics than pens with untreated NK cells (Figure 2B). This demonstrates that the perforin/granzyme pathway was the dominant mechanism. Additionally, determining the killing behavior of these single NK cells was uniquely possible on the Berkeley Lights Platform, where selectively loading cells is central to each workflow.

Investigating the contribution of single NK cells to ADCC

Rituximab, a chimeric IgG1κ antibody against CD20, has dramatically improved the outcome of most B-lymphoproliferative disorders[4]. However, positive responses are not universal and resistance can develop. Obinutuzumab, a new generation anti-CD20 antibody therapeutic, was designed to overcome several hypothesized mechanisms of resistance[4]. This Type II antibody was glycoengineered to have non-fucosylated sugars on the Fc portion of the antibody and has been shown to have superior activity in preclinical trials[4]. Individual variability of ADCC responses to therapeutic

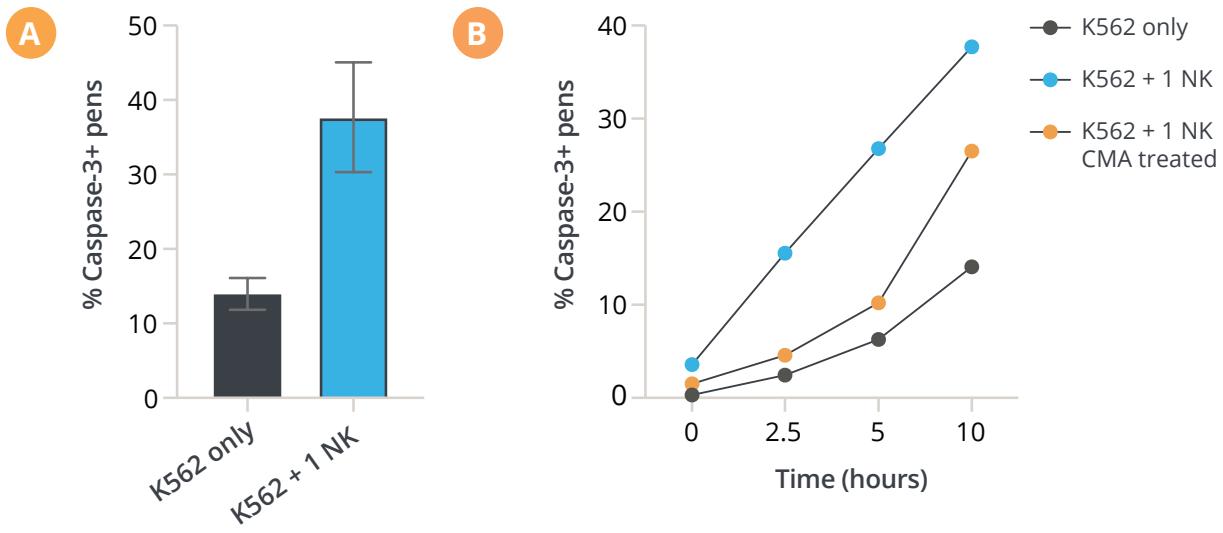

Figure 2. Mapping single NK cell killing kinetics using the Cytotoxicity Assay. A. The percentage of NanoPen chambers containing apoptotic K562 target cells increased from 14% to 38% when the target cell was co-cultured with an NK cell. **B.** A time-course of NanoPen chambers containing a K562 cell, K562 and single NK cell, or K562 and single NK cell pre-treated with CMA tracks the kinetics of NK-mediated killing. Pre-treating the NK cell with CMA resulted in slower killing kinetics and decreased overall killing compared to NK cells without CMA pre-treatment.

antibody treatment has been shown to be significantly related to the relative contribution of NK cell subsets[6]. To investigate the heterogeneity of NK cell-mediated ADCC, we performed the Cytotoxicity Assay on single NK cells co-cultured with target cells incubated with either of two anti-CD20 antibodies that both share the variable region of Obinutuzumab; one is fucosylated (FUT+) while the other is non-fucosylated (FUT–).

We enriched NK cells from PBMCs and selectively loaded single CD56+ CD3- Annexin V- cells into NanoPen chambers. We chose the lymphoblast-like Raji cell line as target cells as they are semi-resistant to NK cell killing; they express an HLA class I inhibitory KIR ligand and lack a stimulatory NK receptor ligand[7]. Raji cells were incubated with either fucosylated anti-CD20 IgG1 antibody (FUT+) or non-fucosylated anti-CD20 IgG1 antibody (FUT–) for 30 minutes before they were incubated with NK cells on the chip. Annexin V- Raji cells were incubated with NK cells for 15 hours. Fluorescent time-lapse imaging monitored cytotoxicity (caspase-3 activation) in the target cells.

In negative controls (either NanoPen chambers containing one pre-treated Raji cell only or pens containing one un-treated Raji cell and one NK cell), the percentage of dying cells after 15 hours was 13% (Figure 3A).

By contrast, NanoPen chambers containing an NK cell and pre-treated Raji cells exhibited higher rates of Raji cell death after 15 hours. Furthermore, the fucosylated antibody condition exhibited only 24% cell death while the non-fucosylated antibody condition exhibited 32% cell death. Kinetic analyses of Raji cell death show that the non-fucosylated, new generation antibody yielded the fastest rates of ADCC (Figure 3B).

Due to the heterogeneous nature of NK cells, we chose to further investigate the kinetics of individual NK cells' response to the fucosylated anti-CD20 antibody. We found single NK cells that killed their cognate Raji over the course of 3–4 hours (slow ADCC, Figure 3C) as well as NK cells that induced apoptosis in their cognate Raji within 1 hour (fast ADCC, Figure 3D). Importantly, these NK cells remained viable in the NanoPen chambers after the conclusion of the assay (data not shown). Individual NK cells responding to antibody treatments in significant or unexpected ways can be recovered alive for downstream processing and even undergo genomic analysis to investigate the molecular mechanisms underlying the heterogeneity of their activity.

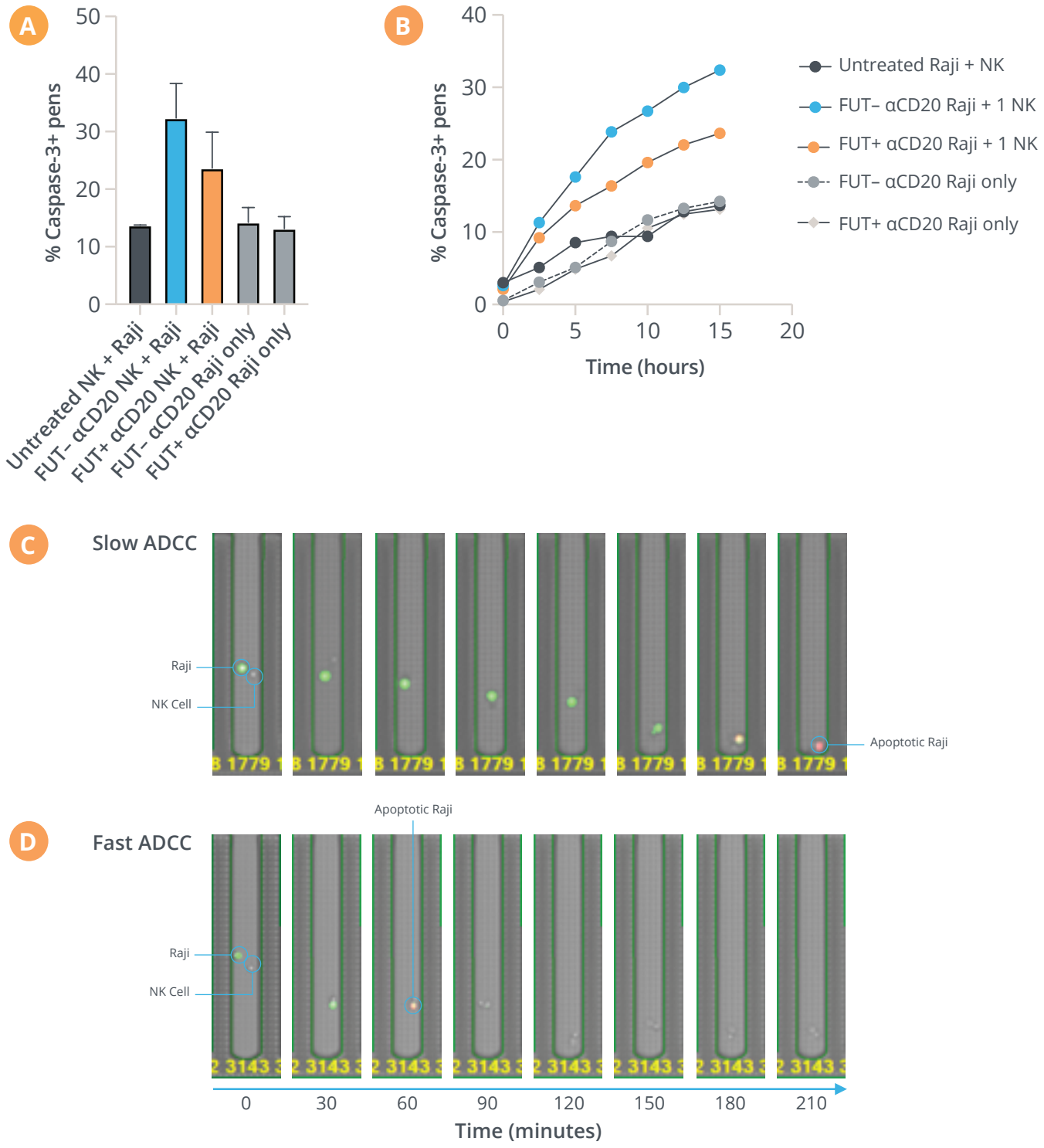

Figure 3. Capturing NK cell-mediated antibody-dependent cellular cytotoxicity using the Cytotoxicity Assay. A. Raji target cell death increased from 13% of pens to either 24% or 32% when the Raji cells were pre-treated with fucosylated or non-fucosylated anti-CD20 antibody, respectively. **B.** Pre-treating the Raji cells with anti-CD20 antibody before they were co-cultured with an NK cell resulted in faster killing kinetics and increased overall killing. This was magnified when non-fucosylated anti-CD20 antibody was used versus fucosylated anti-CD20 antibody. **C-D.** Time-lapse images from representative NanoPen chambers show NK cells (unlabeled) interacting with target cells (green). Cells with activated caspase-3 fluoresce in PE (red). Panel C highlights a NanoPen chamber in which ADCC occurs slowly, while panel D highlights a NanoPen chamber where fast ADCC was observed.

CONCLUSIONS

NK cells are a potent component of the immune system with the potential to increase the efficacy of immunotherapies and mAb therapeutics. Nuanced regulation and population heterogeneity present challenges to fully understanding the role of these cells in various cancer treatments. In this application note, we showed that the Cytotoxicity Assay can measure NK cell-mediated killing of relevant target cells and decode single NK cell killing kinetics in the presence of chemical inhibitors. We also characterized the influence of single NK cells on antibody-dependent cellular cytotoxicity against resistant target cells. Our results are consistent with the increased ADCC-mediated clinical efficacy observed from the new generation, non-fucosylated anti-CD20 antibody[4].

The Cytotoxicity Assay, as part of the Opto Cell Therapy Development workflow, is the first commercially available assay that allows scientists to measure single NK cell killing of target cells that are pre-incubated with antibody therapeutics. NK cells with potent cytotoxicity can be recovered for downstream genomic analysis to identify the exact receptor profiles that elicited the desired response. Additionally, in the future, this assay could be coupled with the Opto™ Plasma B Discovery workflow as a functional assay to screen for antibodies that bind target antigen and initiate strong ADCC-induced NK-mediated killing to develop the most effective, novel therapeutics.

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