Application Note

Immuno-Oncology

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Semi-Automation of a Nonradioactive Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay

Part II: Comparisons with freshly isolated and cryopreserved human natural killer cells

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Abstract

This companion application note to Part I: Automation of a Bioluminescent ADCC Procedure with a Microplate Pipetting System, examines the effect of using different effector cell:target cell ratios and compares the results between freshly isolated human Natural Killer (NK) cells and commercially available cryopreserved human NK cells.

Introduction

In vitro antibody-dependent cell-mediated cytotoxicity (ADCC) assays are common tools for immunotherapeutic drug discovery and biosimilar development. Commonly, the search for a new immunotherapeutic or biosimilar drug uses a cancer cell line to model targeting proliferating cancer cells. In Part I of this companion application note, the use of Daudi cells (human Burkitt's lymphoma cell line) as a model for Rituxan-based biosimilar development was described. It demonstrated the utility of using a bioluminescent assay (aCella-TOX) to quantify the ability of rituximab to recruit freshly isolated human NK cells to induce the Daudi cells to undergo apoptosis.

The isolation of primary effector cells, such as human NK cells from peripheral blood, is a laborious, expensive process. Furthermore, donor-to-donor differences can lead to variations in ADCC assay performance. This application note examines the effect of using NK cells from different donors, reducing effector cell:target cell (E:T) ratios (20:1 to 10:1 NK cells to Daudi cells) and also the use of cryopreserved, commercially available NK cells, to relieve some of the issues associated with the isolation process. The use of cryopreserved NK cells tends to provide an off-the-shelf solution for users of ADCC assays by obviating the need for NK cell isolation.

Materials and methods

Materials

Daudi cells (human Burkitt's lymphoma cell line) were obtained from ATCC (part number CCL-213) and used as target cells in the ADCC assay. Rituximab, the monoclonal antibody in the drug Rituxan, and aCella-TOX (part number CLATOX 100-3) were provided by Cell Technology, Inc. Human primary NK cells were freshly isolated by Cell Technology for all experiments. NK cells from two individual donors were obtained and tested separately. Cryopreserved NK cells (part number PB012F) were provided by STEMCELL Technologies. These cells were thawed and used to manufacturer's instructions.

Daudi Cell Propagation Medium consisted of RPMI 1640 (Life Technologies, part number 11875), FBS, 10% (Life Technologies, part number 10437), NEAA, 1X (Life Technologies, part number 11140), and Pen-Strep-Glutamine, 1x (Life Technologies, part number 10378). ADCC Assay Medium consisted of the same components with the exception that Ultra-Low IgG FBS, 10% (Life Technologies, part number 16250), was substituted for the original FBS.

Instrumentation

The Agilent BioTek Precision microplate pipetting system combines an eight-channel pipetting head and an eight-channel bulk reagent dispenser in one instrument. The instrument was used to dispense all assay components, including target (Daudi) cells and complement, serially titrate antibody across a 96-well polypropylene plate, transfer samples from plate to plate, and dispense the aCella-TOX assay components.

The Agilent BioTek Synergy H4 hybrid multimode microplate reader combines filter- and monochromator-based detection systems in the same unit. A dedicated luminescence detection system was used to quantify the luminescent signal from each assay well. The plates were read in kinetic mode, using integrated Agilent BioTek Gen5 data analysis software, to capture the luminescent signal every 5 minutes.

Only semi-automated assays conducted with an Agilent BioTek Precision microplate pipetting system were performed.

Automated ADCC assay procedure

Daudi target cells, at a concentration of 2×10^5 cells/mL in 25 µL were added to the 96-well assay plate. An 8-point titration curve was then created of the test antibody using serial 1:5 dilutions beginning at 1 µg/mL. 25 µL of each antibody dilution was added to the plate to start the reaction. The cells were allowed to opsonize for 15 minutes at 37 °C. NK effector cells, at a concentration of 4×10^6 cells/mL or 2×10^6 cells/mL, were then added (in 25 µL) to give an E:T ratio of 20:1 or 10:1, respectively for both freshly isolated and cryopreserved NK cells. The plate was centrifuged for one minute, and incubated at 37 °C for 1.75 hours. The plate was removed from the 37 °C incubator and allowed to cool to room temperature for 5 to 10 minutes. The target cells in the maximum lysis control wells were lysed by adding 10 µL of the lysis buffer, and the plate was incubated for an additional 5 minutes. 125 µL of ADCC assay medium was then added to each well to bring the volume to 200 µL. The plates were centrifuged for one minute. 50 µL of enzyme assay diluent was then transferred to the appropriate wells of an opaque white luminescence plate. Then, 50 µL of each reaction supernatant was transferred to wells containing the assay diluent. Then, 100 µL of 2x enzyme assay reagent (containing G3P), followed by 50 µL of 1x detection reagent was added to each diluted supernatant. The plates were immediately read using an Agilent BioTek Synergy H4 at 5-minute intervals. The RLUs were graphed and the data reduced by four-parameter fits for analysis.

Percent total cytotoxicity calculation

The luminescent signal from the wells containing media and other assay components was subtracted from all other wells to correct for background interference. Average nonlysed target cell-only control well signal was then subtracted from all sample wells. Percent total cytotoxicity was then calculated by dividing adjusted sample well signal by the average maximum lysis signal, and multiplying the result by 100.

Results and discussion

Figure 1 depicts the comparative performance of using different donors of freshly isolated NK cell and E:T ratios (20:1 and 10:1). It is apparent that for Donor 1, reducing the amount of NK cells by a factor of 2 has no discernable effect on the extent of cytotoxicity as the high doses of rituximab (i.e. >100 ng/mL) leads to complete (100%) Daudi cell toxicity. There is a small right shift in EC_{50} in using this lower amount of NK cells by nearly a factor of 2, however, but this may well not be pharmacologically relevant. A different situation is seen for the second donor of NK cells. High doses of rituximab do not provide complete Daudi cell toxicity using either 20:1 or 10:1 E:T ratios. Furthermore, reducing the NK cell amount lowers the extent of Daudi cell toxicity by approximately one third. The EC_{50} results for Donor 2 at both E:T ratios are significantly different from Donor 1 and remain the same for both ratios. It is apparent for both donors that higher E:T ratios tend to produce higher background toxicity (rituximab dosing <1 ng/mL).

As NK cells are primary cells, they can only be cultured for so long before they undergo senescence. Thus, pooling of donor samples has limited utility for reducing donor-to-donor variability in ADCC assays. The ability to cryopreserve NK cells, however, should assist in pooling a large number of donor samples and thus reduce variability in the ADCC assay. Figure 2 depicts the use of cryopreserved cells at both 20:1 and 10:1 E:T ratios. It is apparent that % total cytotoxicity responses are similar to Donor 2, where high doses of rituximab produce 60% and 40% total cytotoxicity for 20:1 and 10:1, respectively, but EC_{50}s are more consistent with Donor 1.

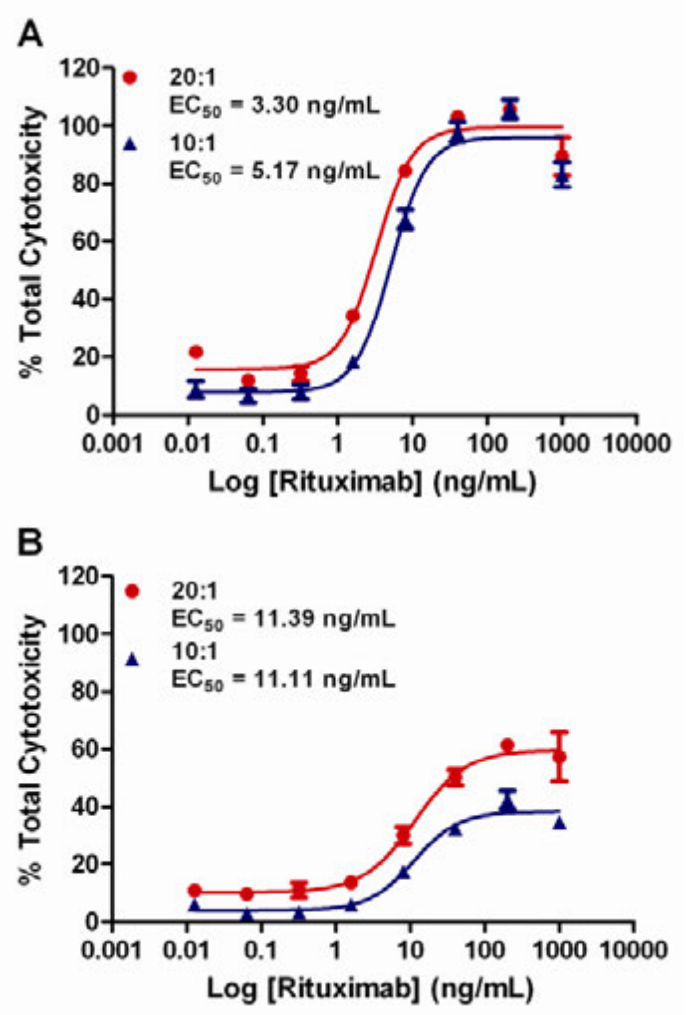

Figure 1. Comparative performance in 20:1 and 10:1 E:T ratios in rituximab dose-response ADCC assay for Donor 1 (A) and Donor 2 (B).

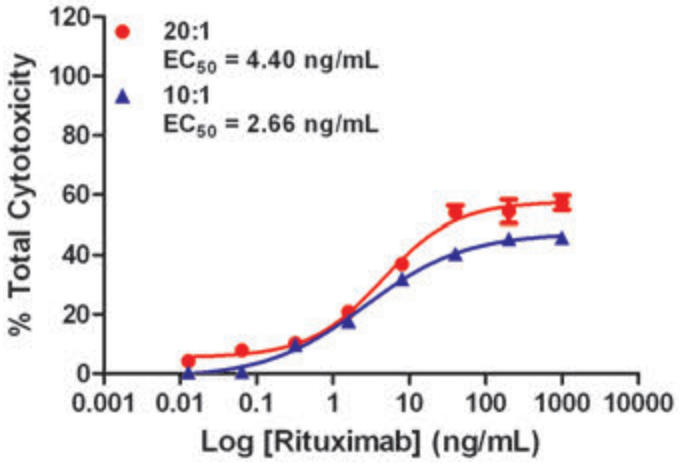

Figure 2. Comparative performance in 20:1 and 10:1 E:T ratios in rituximab dose-response ADCC assay using cryopreserved NK cells.

Conclusion

It has been shown that there can be differences in ADCC assay performance produced between donors of freshly isolated NK cells and from differing E:T ratios. This has some implication for immunotherapeutic drug discovery. It would be prudent for investigators to determine up front the range of donor-to-donor variability for the target cell they use in their ADCC model, to obtain consistent data between testing one antibody against another. The use of cryopreserved NK cells can relieve this problem to some extent, but one would also assume there would be lot-to-lot variability from the manufacturer, as well.

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