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

Part II: Comparisons with Freshly Isolated and Cryopreserved Human Natural Killer Cells

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In this companion application note to Part I: Automation of a Bioluminescent ADCC Procedure with a Microplate Pipetting System, we examine the effect of using different effector cell:target cell ratios and compare 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, we described the use of Daudi cells (human Burkitt's lymphoma cell line) as a model for Rituxan-based biosimilar development. We 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. In Part II of this application note, we examine 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 (Catalog No. CCL-213) and used as target cells in the ADCC assay. Rituximab, the monoclonal antibody in the drug Rituxan, and aCella™-TOX (Catalog No. 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 (Catalog No. 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, Catalog No. 11875), FBS, 10% (Life Technologies, Catalog No. 10437), NEAA, 1X (Life Technologies, Catalog No. 11140), and Pen-Strep-Glutamine, 1X (Life Technologies, Catalog No. 10378). ADCC Assay Medium consisted of the same components with the exception that Ultra-Low IgG FBS, 10% (Life Technologies, Catalog No. 16250), was substituted for the original FBS.

Instrumentation

The same instrumentation used in Part I was used in Part II. Only semi-automated assays conducted with Precision™ Microplate Pipetting System were performed.

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Automated ADCC Assay Procedure

Daudi target cells, at a concentration of $2x10^5$ 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 $4x10^6$ cells/mL or $2x10^6$ 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 then removed from the 37° C incubator and allowed to cool to room temperature for 5-10 minutes. The target cells in the maximum lysis control wells were then 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. 50 µL of each reaction supernatant was transferred to wells containing the assay diluent. 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 the Synergy™ H4 at 5-minute intervals. The RLUs were graphed and the data reduced by four-parameter fits for analysis.

% Total Cytotoxicity Calculation

% Total Cytotoxicity was calculated as in Part I.

Results and Discussion

Figure 1 below 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 about a 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).

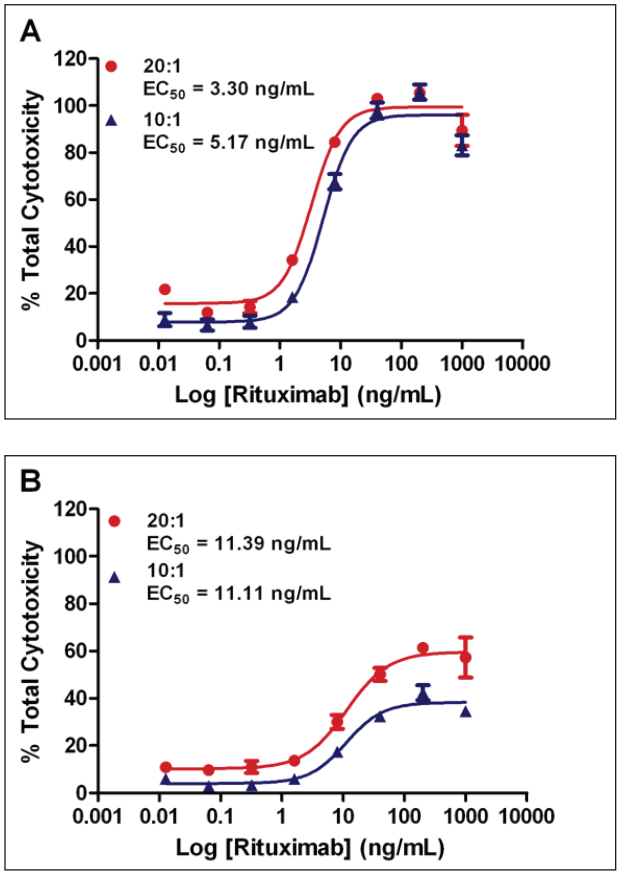

Figure 1. Comparative performance in 20:1 and 10:1 E:T ratios in rituximab dose-response ADCC assay for Donor 1 (A) and Donor 2 (B).

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 below 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.

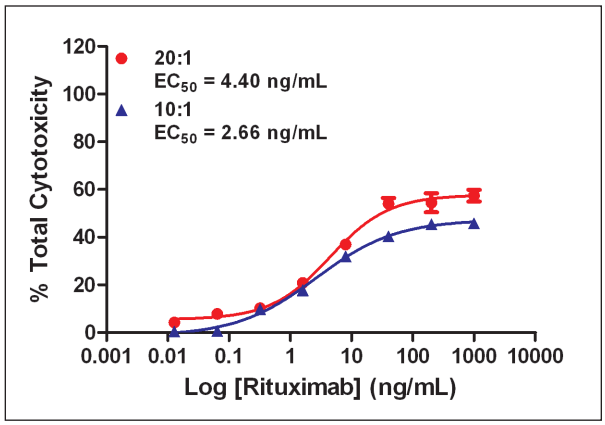

Figure 2. Comparative performance in 20:1 and 10:1 E:T ratios in rituximab dose-response ADCC assay using cryopreserved NK cells.

Conclusions

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, such they can 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, also.