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Donor and Antibody Diversity in NK Cell-Mediated Antibody Dependent Cellular Cytotoxicity (ADCC) Detected Using an Optimized Multiplexed Assay and Advanced Flow Cytometry

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Introduction

Several types of NK cell-related immunotherapeutics are being developed for the treatment of cancers, including tumor-specific monoclonal antibodies (mAbs) to induce NK cell-mediated ADCC against tumor cells. For example, mAbs targeting CD20, such as rituximab and obinutuzumab, have long been used in the treatment of various B cell malignancies. Rituximab is a type I, chimeric (mouse variable region + human IgG1 Fc region) anti-CD20 mAb, whereas obinutuzumab is a type II, fully humanized IgG1 mAb that binds a partially overlapping, but unique epitope on CD20 compared to rituximab, and was glycoengineered to reduce fucosylation in the Fc region which improved its binding affinity for FcγRIIIa (CD16a) resulting in enhanced ADCC (1-3).

Assessing NK cell-mediated ADCC is critical in determining the potential clinical efficacy of newly developed tumor-specific mAbs. However, donor cell variability can affect the degree of NK cell-mediated ADCC. Thus, in addition to assessing cytolytic activity, it is necessary to also characterize the number and activation state of the donor NK cells utilized. Traditional cytotoxicity assays are time-intensive and require additional downstream assays in order to characterize donor effector cells. In the current study, anti-hCD20 mAbs similar to rituximab and obinutuzumab were used to test whether a multiplexed, high throughput assay that simultaneously measures target cell killing, expression of NK cell phenotypic markers, and effector protein secretion, could be utilized to discern differences in ADCC potency between mAbs, as well as provide insight into their potential treatment efficacy across donors.

References:
1. Salles G., *et al., Adv Ther.* 2017;*34*(10), 2232-2273
2. Tobinai K, *et al., Adv Ther.* 2017;34(2):324-356.
3. Mössner E, *et al., Blood.* 2010;115(22):4393-402.

Experimental approach

1. Workflow

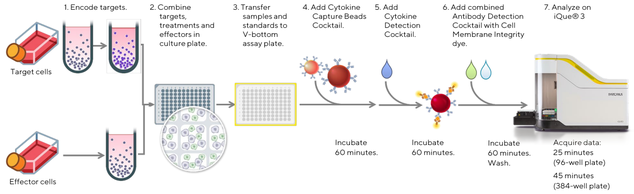

Figure 1: Schematic diagram of the workflow for the analysis of NK cell cytotoxic function, along with assessment of the NK cell activation state and cytokine/effector protein release using the iQue® Human NK Cell Killing Kit. Effector cells (Cellero; human PBMCs or enriched, negatively selected human NK cells) were thawed and allowed to recover in media (Corning; RPMI 1640 medium with 10% fetal bovine serum for enriched NK cells, or RPMI 1640 containing 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% Pen/Strep for PBMCs) for 16-18 h overnight. On the assay day, a frozen aliquot of CD20+ Raji tumor target cells (ATCC, Burkitt's lymphoma cell line) previously stained with a fluorescent encoder dye, was thawed, counted, and plated in triplicate in a 96 well plate at 20K/well for 30 min at room temperature in the presence of individual anti-CD20 mAbs at concentrations ranging between 0- 10 µg/mL. The anti-hCD20 mAbs (InvivoGen) included hcd20-mab1 (Rtx-G1) composed of a human IgG1 Fc region and variable region of rituximab, hcd20ga-mab1 (Ob-G1) which was a fully humanized mAb with the variable region of obinutuzumab and a native human IgG1 Fc region, and hcd20ga-mab13 (Ob-G1nF) with the variable region of obinutuzumab and a non-fucosylated human IgG1 Fc region. Another anti-hCD20 mAb, hcd20ga-mab7 (Ob-A2), was used as a negative control. It also possessed the variable region of obinutuzumab, but had a human IgA2 constant region, and did not bind FcγRIIIa (CD16a). Next, effector cells were added at an Effector to Target (E:T) ratio of 10:1 (PBMCs) or 1:1 (enriched NK cells). In parallel, effector cells were cultured in triplicate in media alone, or co-cultured with targets alone to evaluate direct tumor cell killing without antibody. Direct antibody-mediated killing of tumor cells was also assessed by culturing Raji cells with anti-CD20 mAbs in the absence of effector cells. Following co-culture for 4h at 37°C, 5% CO_2, 10 µL samples were assayed using the multiplexed iQue® Human NK Cell Killing Kit plus iQue® Human NK Companion Kits, and data acquisition was performed on the iQue® platform (VBR configuration) for advanced flow cytometry.

2. Simultaneous endpoint measurement in a single well.

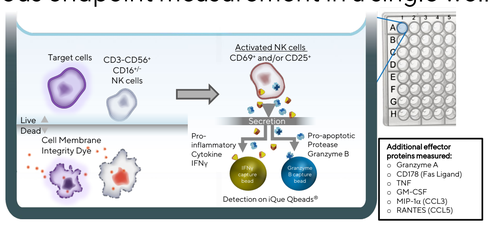

Figure 2. Illustration of iQue® Human NK Cell Killing Kit assay principles. Target cells are distinguished from effector cells by staining with a fluorescent encoder dye, and tumor cell killing is then determined by staining with a fluorescent cell membrane integrity dye. NK cells are identified using 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 iQue Qbeads® in a sandwich immunoassay format in the same well. Additional compatible cytokines and effector proteins are measured by incorporating other iQue® Human NK Cell Companion Kits into the assay.

Results

1. The level of ADCC is mAb and donor-dependent

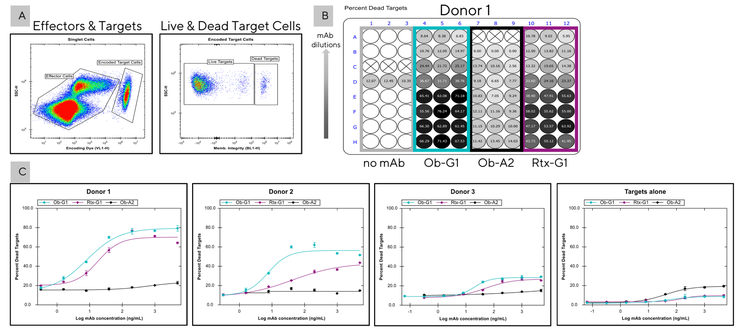

Figure 3. Comparison of the ADCC potency of two anti-hCD20 mAbs among three different donors. PBMCs (200K/well) from three separate donors were co-cultured in triplicate with encoded Raji tumor cells (20K/well) in the presence of different anti-hCD20 mAbs (Ob-G1, Rtx-G1, or Ob-A2, as a negative control). After 4h, 10 µL samples were transferred to assay plates and analyzed using the iQue® Human NK Cell Killing Kit plus iQue® Human NK Cell Companion Kits. (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) Percent dead targets displayed as a plate heat-map for Donor 1 over serial dilutions of 3 mAbs starting at 10 µg/mL. (C) EC_{50} curves showing percent tumor cell killing by effector cells from 3 donors tested in a second experiment with 3 mAbs over five-fold serial dilutions starting at 5 µg/mL. Raji cells were also incubated with the mAbs alone to evaluate direct Ab-mediated cytotoxicity (Targets alone).

Table 1. Comparison of mAb EC_{50} in relation to donor NK cell phenotype and FcγRIIIa genotype

Donor ID	FcRγIIIa 158 genotype	% NK cells (CD3-CD56+)	% CD16+ NK Cells	Direct NK cell Killing (%)	mAb EC_{50} (ng/mL)	
					Rtx-G1	Ob-G1
Donor 1	V/V	14.6	81	7	18	8
Donor 2	V/F	8	82	3	43	8
Donor 3	F/F	14.6	44	5	50	20

2. Effector protein secretion levels correspond with mAb potency.

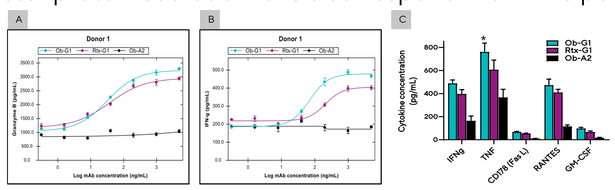

Figure 4: Effector proteins and cytokines secreted during the PBMC- Raji tumor cell co-culture described in Fig. 3C. Secretion levels of (A) Granzyme B and (B) IFNγ by PBMCs from Donor 1 cultured with Raji tumor cell in the presence of anti-hCD20 mAbs at concentrations ranging between 0 – 5 µg/mL. (C) Summary of cytokines produced by Donor 1 PBMCs co-cultured with Raji cells and 3 different mAbs at 1 µg/mL dose. Bar graph data presented as Mean +/- 1 SD, 3 replicates/mAb dose. (*) Significantly higher levels of cytokines produced in co-culture using Ob-G1 compared to Rtx-G1 anti-CD20 mAb, $p < 0.05$.

3. Donor ADCC differences are reflected in effector protein levels.

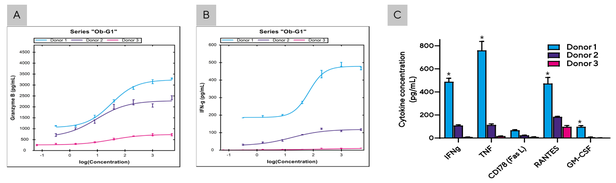

Figure 5: Secretion levels of (A) Granzyme B and (B) IFNγ by PBMCs from all 3 donors cultured with Raji tumor cells as described in Fig. 3C at Ob-G1 concentrations ranging between 0 – 5 µg/mL. (C) Summary of cytokines produced by PBMCs from 3 donors co-cultured with Raji cells in the presence of Ob-G1 mAb at 1 µg/mL. Bar graph data presented as Mean +/- 1 SD, 3 replicates/mAb dose. (*) Significantly higher levels of cytokines produced by Donor 1 compared to Donor 2 or 3; $p < 0.05$.

4. ADCC and cytokine production were enhanced using a non-fucosylated anti-CD20 mAb with either PBMCs or enriched NK cells as effector cells.

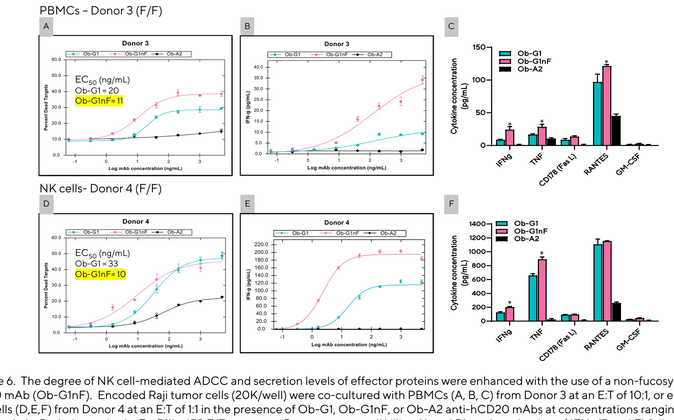

Figure 6. The degree of NK cell-mediated ADCC and secretion levels of effector proteins were enhanced with the use of a non-fucosylated anti-CD20 mAb (Ob-G1nF). Encoded Raji tumor cells (20K/well) were co-cultured with PBMCs (A, B, C) from Donor 3 at an E:T of 10:1, or enriched NK cells (D,E,F) from Donor 4 at an E:T of 1:1 in the presence of Ob-G1, Ob-G1nF, or Ob-A2 anti-hCD20 mAbs at concentrations ranging between 0 - 5 µg/mL. Both donors had a FcγRIIIa-158 F/F genotype. Percent tumor cell killing (A and D), and production of IFNγ (B and E) following co-culture for 4h. (C and F) Summary of cytokines produced in the presence of 3 different mAbs at 1 µg/mL dose. Bar graph data presented as Mean +/- 1 SD, 3 replicates/mAb dose. (*) Significantly higher levels of cytokines produced in co-culture when using anti-CD20 mAb Ob-G1nF compared to Ob-G1, $p < 0.05$.

Conclusion

Use of the iQue® Human NK Cell Killing Kit allowed for the simultaneous measurement of NK-mediated ADCC, expression of NK cell phenotypic markers, and quantification of IFNγ and Granzyme B with the flexibility to quantitate up to six additional effector proteins and cytokines all in the same well using the iQue® Human NK Cell Companion Kits. The results of this study showed that the Ob-G1 anti-hCD20 mAb demonstrated greater ADCC potency as compared to Rtx-G1 for all donors tested. Further enhancement in ADCC and cytokine secretion was seen with the use of the non-fucosylated anti-mAb, Ob-G1nF, using either PBMCs or enriched NK cells. No increase in ADCC above baseline levels was seen when the negative control anti-hCD20 mAb, Ob-A2, was included in the co-cultures, and only very low levels of direct killing of Raji tumor cells was observed using the anti-hCD20 mAbs alone. Differences in the level of tumor cell killing was also observed between donors, with PBMCs from Donor 1 with a FcγRIIIa-158 V/V genotype, exhibiting greater ADCC than Donor 2 (FcγRIIIa-158 V/F) or Donor 3 (FcγRIIIa-158 F/F) using either the Ob-G1 or Rtx-G1 anti-hCD20 mAbs. In addition, the secretion levels of several effector proteins and cytokines reflected the level of ADCC induced by the different mAbs tested, as well as the differences in ADCC observed between donors.

Overall, this study demonstrates that use of the iQue® Human NK Cell Killing Kit plus iQue® Human NK Cell Companion Kits enables rapid assessment of the potency of different mAbs in ADCC mediated by NK cells, and can effectively distinguish differences in mAb efficacy between donors. In addition, the simultaneous characterization of NK-cell surface marker expression and cytokine secretion may reveal potential mechanistic differences related to the variation in tumor cell killing observed between donors. The assay requires only 10 µl of sample and measures multiple endpoints from the same effector + target co-culture with a short assay time of only 3.5 hours. Furthermore, the results could be immediately visualized with the use of the iQue® platform and iQue Forecyt® software. Thus, the multiplexed iQue® Human NK Cell Killing Kit collapses traditional workflows into a single assay platform which allows for streamlined and rapid data acquisition in the development and testing of new NK cell-related cancer immunotherapies.

Key Advantages

- Collapses traditional workflows into one rapid, miniaturized, easy to use, multiplex assay requiring minimal sample manipulation that can be performed in either 96 or 384-well plates.
- Simultaneously quantifies NK cell surface protein & cytokine expression in relation to target cell killing.
- Requires low sample volumes, thereby conserving precious samples.
- Use of the iQue® platform with the integrated iQue Forecyt® Software allows for real time data analysis and novel visualization tools.