Technical Note

Development of a kinetic antibody-dependent cellular cytotoxicity assay

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ABSTRACT

Antibody-dependent cellular cytotoxicity (ADCC) is an important mechanism of action (MOA) of monoclonal antibody (mAb) therapeutics. Target cells opsonized with therapeutic antibody bind and activate FcγR-bearing immune effector cells, resulting in target cell lysis. A key step in mAb drug development is the characterization of ADCC activity for its potential to inform mAb efficacy and safety. A number of *in vitro* assays are commonly used to assess ADCC. Most are endpoint assays that measure a surrogate marker of cell lysis. Newer imaging technologies allow direct measurement of ADCC-mediated cell lysis over time. In this study, we detail the development and characterization of a kinetic ADCC assay applicable to multiple target and effector cell types. This kinetic assay shows comparable sensitivity to an endpoint fluorescence release ADCC assay, while offering the advantages of a simpler set up and shorter assay time. Our results demonstrate that kinetic ADCC activity is a valid alternative assay format for measuring *in vitro* ADCC of mAbs.

1. Introduction

Therapeutic antibodies, primarily IgG-based monoclonal antibodies (mAbs) have become standard treatment for a variety of diseases (Schnueriger et al., 2011). Anti-tumor therapeutic mAbs can kill target cells in multiple ways, but one of the predominant mechanisms is antibody-dependent cellular cytotoxicity (ADCC) (Jiang et al., 2011). MAbs opsonize cells by binding to their cognate antigen on the target cell surface. The Fc region of the antibody is then able to bind and crosslink FcγR on the surface of immune effector cells, leading to immune activation. NK cells, which express FcγRIIIA, are the predominant immune effector cell involved in ADCC (Seidel et al., 2013). Crosslinking of FcγRIIIA on NK cells by opsonized mAb leads to the release of perforin and granzyme, resulting in target cell lysis. Since all IgG mAbs have the potential to induce ADCC which can impact both efficacy (MOA) and safety, preclinical assessment of ADCC activity is an essential step in mAb drug development.

Commonly used *in vitro* ADCC assays include chromium-51 (^{51}Cr) release assays, lactate dehydrogenase (LDH) absorbance assays, bioluminescent lucerin/luciferase reporter assays, and calcein acetyloxymethyl (AM) release assays (Chung et al., 2014). The chromium release assay was the standard technique for many years, but in recent years many laboratories have switched to non-radioactive fluorescence assays, notably the calcein AM release assay which has been shown to generate results comparable to the ^{51}Cr release assays while offering the advantages in speed and sensitivity (Chung et al., 2017). These conventional methods all have one thing in common – they provide an endpoint readout of ADCC activity.

The advent of new live-cell imaging systems, which automatically detect and count individual cells, has enabled the development of a kinetic ADCC assay. Target cells labelled with a fluorescent marker can be imaged and counted. When NK effector cells and mAb are added, the ADCC reaction takes place and opsonized target cells are lysed by effector NK cells. As a result, the number of target cells decreases, and this decrease in cell number can be quantitated and plotted against mAb concentration to determine the kinetics of ADCC activity. In contrast to endpoint assays that are based on a surrogate marker of ADCC activity such as calcein AM release, the live-cell imaging approach directly measures ADCC activity in real time, which could prove advantageous.

In this study, we detail the development of a kinetic ADCC assay. The assay was validated using a panel of therapeutic mAbs with different glycosylation profiles as well as target cells with varied levels of target receptor expression. The kinetic ADCC assay was tested in parallel with the endpoint calcein AM release assay and the sensitivity, assay time, and overall ease of use were compared.

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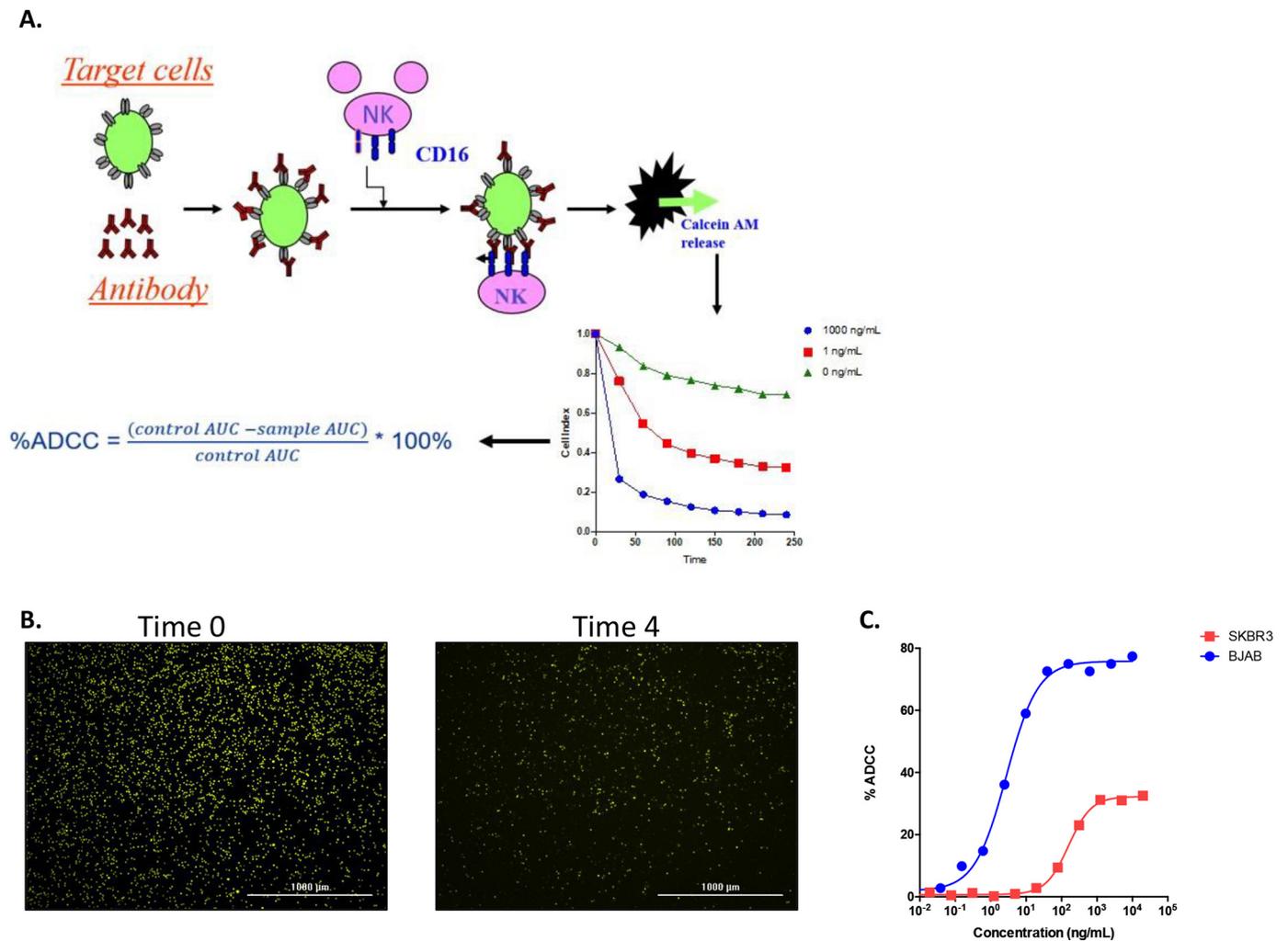
Fig. 1. Format of the kinetic ADCC assay. A. Calcein AM-labelled target cells opsonized with a dose range of test antibody are incubated with NK-92_158V effector cells. Over time, target cells are lysed leading to a decrease in the number of fluorescent cells, and plotted as the cellular index. The cell index for each dose of antibody is converted to AUC and % ADCC activity over time is calculated. B. Representative images from wells containing calcein AM-labelled target cells opsonized with 1 μg/mL ocrelizumab and NK effector cells are shown. Time 0 corresponds to the start of the reaction. Time 4 corresponds to the 4 h timepoint at the end of the reaction. C. Shown is the % ADCC activity of rituximab against CD20-expressing BJAB cells, and of trastuzumab against HER2-expressing SKBR3 cells. Data shown is representative of three independent experiments.

2. Materials and methods

2.1. Cell lines and test antibodies

B lymphoma cell lines including BJAB, Rec-1, and SUDHL-8, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Corning, Tewksbury, MA) supplemented with 10% fetal bovine serum (FBS: Hyclone, Logan, UT), 25 mM HEPES (Corning, Tewksbury, MA), 1% Glutamax (Gibco, Carlsbad, CA), and 1% penicillin/streptomycin (Gibco). SKBR-3, a human epithelial cell line derived from adenocarcinoma, was obtained from the American Type Culture Collection. SKBR-3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% Glutamax, and 1% penicillin/streptomycin. NK-92_158V, an engineered NK cell line that expresses human CD16a (FCRγIIIa), was obtained from Roche (Schnueriger et al., 2011). NK-92_158V were grown in minimal essential medium (MEM) alpha medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 10% horse serum (Invitrogen, Carlsbad, CA) 0.2 mM myo-inositol (Sigma Aldrich, St. Louis, MO), 0.02 mM folic acid (Sigma Aldrich), 2 μg/mL puromycin (Sigma Aldrich), and 150 U hIL-2/mL (Roche, Indianapolis, IN). After culture and expansion, NK-92_158V

cells were frozen in FBS with 10% DMSO and used in a thaw-and-use format.

Rituximab and ocrelizumab are engineered mAbs that bind to human CD20 with different affinities and specificities (Klein et al., 2013). Rituximab is a chimeric IgG$_1$ antibody, while ocrelizumab is a humanized IgG$_1$ antibody, produced by engineered Chinese hamster ovary (CHO) cell lines at Genentech (South San Francisco, CA). Trastuzumab is a humanized IgG$_1$ antibody that binds to human HER2 and is manufactured from engineered CHO cell lines at Genentech.

Deglycosylated ocrelizumab was produced by incubating 1 mg of ocrelizumab with 275 units of PNGase F (New England Biolabs, Ipswich, MA) at 37 °C for 24 h and further purified through a protein A column. Afucosylated ocrelizumab was produced from a CHO cell line deficient in FUT8. The Man-5 glycoform of ocrelizumab was produced by addition of kifunensine (Cayman Chemical Company, Ann Arbor, MI) to the cell culture media at 5 mg/L as an α-mannosidase inhibitor to prevent removal of extra mannose molecules in the endogenous glycosylation pathway. The purified glycoforms at 10 mg/mL were then incubated with 20 mU/mL α-mannosidase I from *Aspergillus saitoi* (Prozyme, Hayward, CA) at 37 °C for 24 h to produce Man5 glycoforms *in vitro*.
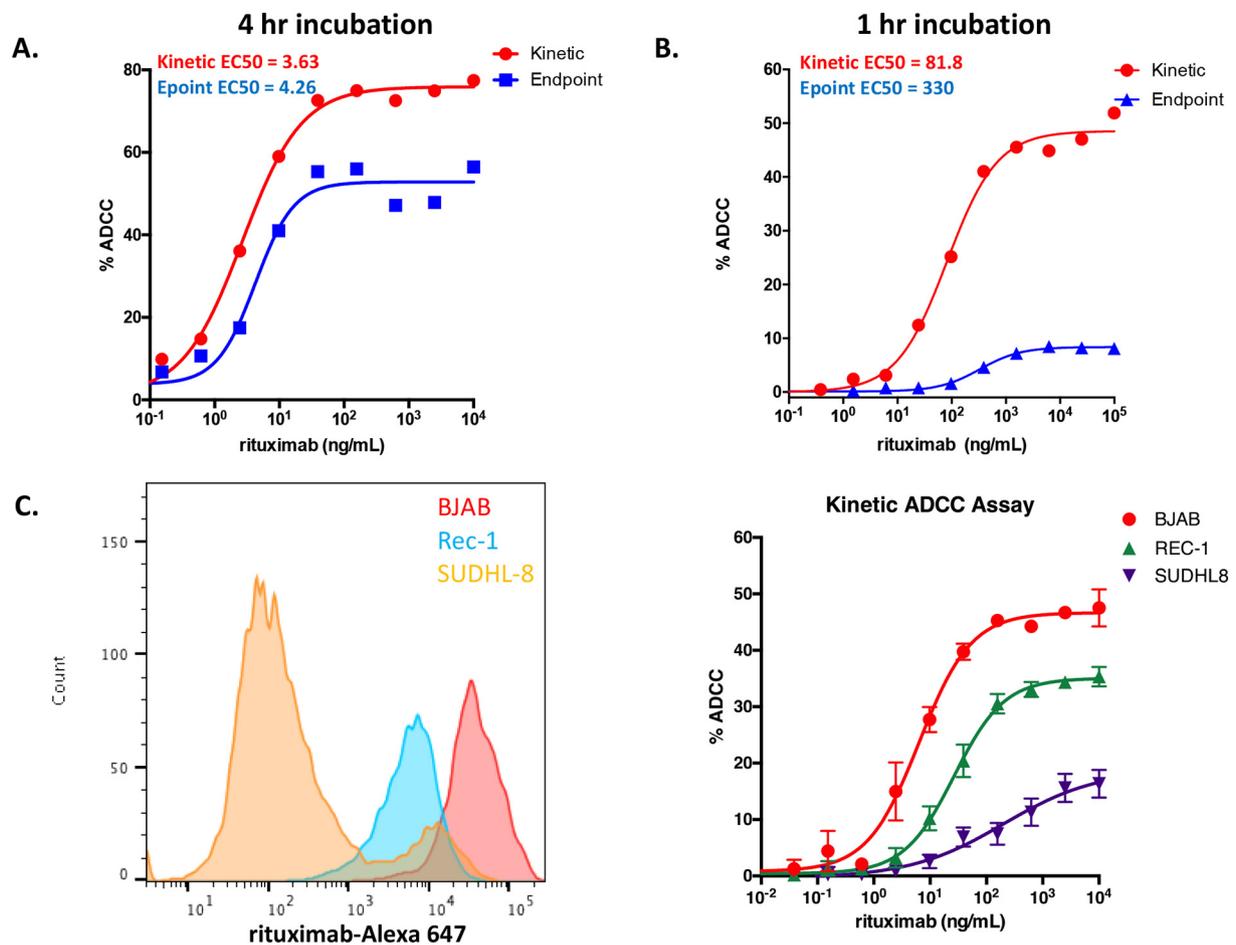
Fig. 2. Direct comparison of kinetic and endpoint ADCC assays. A. Calcein AM-labelled BJAB cells opsonized with the indicated concentrations of rituximab were incubated with NK-92_158V effector cells for 4 h. % ADCC activity was measured and calculated as described based on calcein AM in supernatant for the endpoint assay and number of calcein AM positive cells over time in the kinetic assay. B. % ADCC activity after a 1-h incubation, as described for A. C. BJAB, REC-1, and SUDHL-8 cells were stained with Alexa-Fluor-labelled rituximab and antibody binding was analyzed on a flow cytometer. The B-cell lines were tested in the 1 h format kinetic ADCC assay. % ADCC activity was calculated and plotted against the dose response of rituximab. Data shown was pooled from three separate experiments, error bars represent SEM.

2.2. CD20 expression

B lymphoma cell lines including BJAB, Rec-1, and SUDHL-8, were stained for CD20 expression using rituximab-labelled Alexa 647(rituximab-Alexa 647). Briefly, cells were resuspended in FACs buffer (PBS, 2% FBS, 0.02% sodium azide) at 1 million cells/mL and added at 100 μL/well in a 96-well U-bottom plate. Rituximab-Alexa 647 was diluted in FACs buffer to 40 ng/mL and added to the cells in duplicate and incubated for 1 h at 4 °C. After incubation, cells were washed with cold FACs buffer and fixed in 4% paraformaldehyde before analyzing on a FACSCanto 10 IVD flow cytometer (BD Biosciences, San Jose, CA). The fluorescence for Alexa Fluor 647 was analyzed with FlowJo software (Treestar, Ashland, OR).

2.3. Endpoint ADCC assay

Target B cell lines were kept in log growth phase by subculturing at 2×10^5 cells/mL, and split to 0.5×10^5 cells/mL the day before labeling. SKBR3 cells were subcultured at a 1:3 ratio. Calcein AM (Molecular Probes, Eugene, OR) was dissolved in pluronic F127/DMSO (Molecular Probes) at 1.4 mM before use. Target cells were labelled in assay medium by adding Calcein AM solution 10 μL/mL of cells up to 1×10^6 cells/mL. Cells were incubated at 37 °C for 30 min. After two washes with assay medium and centrifugation at 1400 rpm for 5 min, cells were adjusted to 0.4×10^6 cells/mL and dispensed in 50-μL

aliquots into a tissue cultured treated 96-well flat-bottom plate (Corning, Corning, NY).

Test antibodies were diluted in ADCC assay media (RPMI 1640 without phenol red medium (Life Technologies, Carlsbad, CA) supplemented with1% bovine serum albumin (Sigma Aldrich) starting at 1000 ng/mL. The final column remained as an antibody-independent cellular cytotoxicity (AICC) control. An equal volume of antibody dilutions (50 μL) was added to each well containing target cells, followed by incubation at RT for 30 min to allow antibody binding.

NK-92_158V cells were thawed immediately before use in the assay, counted, resuspended at 1×10^6 cells/mL and added to the assay plates (100 μL) containing the opsonized WIL2-S cells to achieve an E:T ratio of 5:1. To bring the cells in proximity to one another, the plate was spun down at 700 rpm for 1 min.

The assay plate was incubated for 1–4 h at 37 °C, 5% CO_2. Following incubation, the plate was spun down for 3 min at 1500 rpm and 100 μL of supernatant was transferred to a new 96-well flat bottom plate. Absorbance was measured on a SpectraMax® I3 microplate reader (Molecular Devices; Sunnyvale, CA), excitation at 485 nm and emission at 520 nm, with cut off set at 515 nm. Spontaneous release was defined as the calcein-AM signal from wells containing only the target cells, and maximum release was defined as the signal from wells containing target cells lysed with 2% Triton™ X-100 (Genentech). Antibody-independent cellular cytotoxicity (AICC) was measured in wells containing target and effector cells without the addition of antibody. Samples and
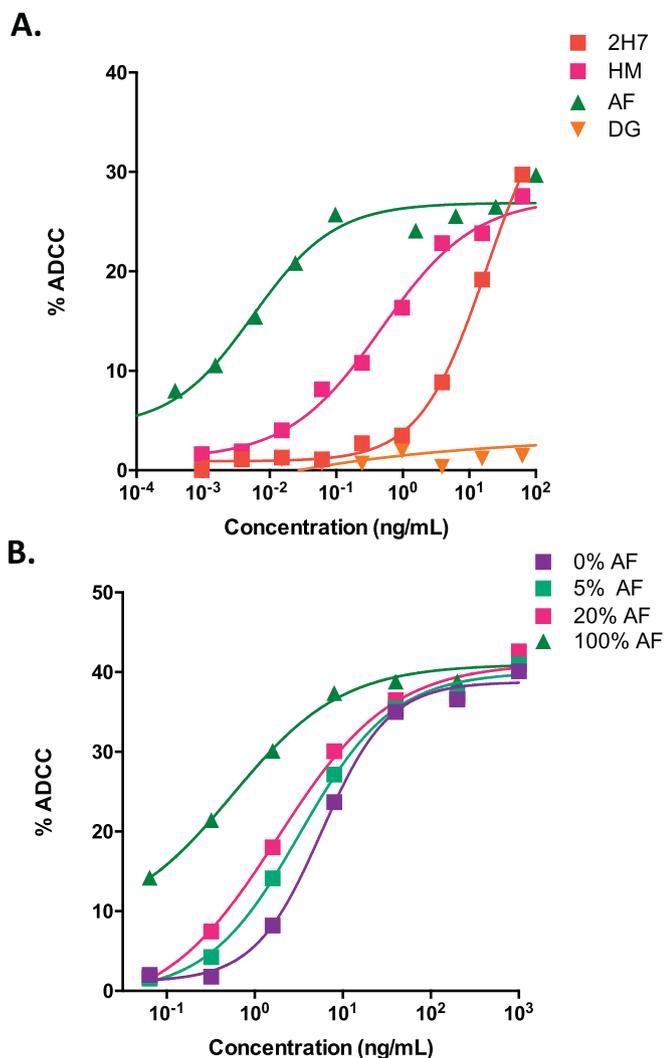
A.

B.

Fig. 3. Glycosylation variants tested in kinetic ADCC assay. Data shown are representative of three independent experiments. A. Ocrelizumab (2H7) variants with high mannose (HM), afucosylation (AF), or deglcyosylation (DG) were tested on CD20-expressing target cells during a 2-h kinetic ADCC assay. B. Ocrelizumab with increasing levels of afucosylation (AF) were tested on CD20-expressing B cells during a 2-h kinetic ADCC assay.

controls were tested at least in duplicate, and ADCC was calculated using mean values as follows:

$$\%ADCC = 100 \times (\text{mean sample release} - \text{mean AICC})$$
$$/(\text{mean max release} - \text{mean spontaneous release})$$

ADCC activity curves were generated by plotting the mean ADCC values from duplicates of mAb sample dilutions against the concentration of mAb and dose-response curves were fitted to a four-parameter model using GraphPad Prism (La Jolla, CA).

2.4. Kinetic ADCC assay

The assay plate was set up as described above using a 96-well black clear-bottom plate (Greiner, Monroe, NC). Cells were concentrated at the bottom of the plate by a brief centrifugation step and placed inside the Lionheart imaging system prewarmed to the cell culture conditions of 37 °C and 5% CO_2 (BioTek Instruments, Winooski, VT). Images were taken every 30 min for 4 h using the 2.5× objective. The total number of cells (NK and target) were captured on the bright-field channel, and calcein AM-labelled target cells were captured on the YFP channel. Two

images per well were taken and stitched to cover the center of the well.

Images were processed using Gen5 software (BioTek Instruments). Cellular analysis was performed on the transformed YFP images. The cell size was gated on a range of 10–30 μm and the fluorescence threshold was set to 5500. The analysis yielded cell counts for the number of individual fluorescent cells in each well per time point. Each test antibody concentration had an individual cell count curve that displayed the decrease in target cell number over time. To correct for differences in initial cell number across wells, the cell count for each antibody concentration was normalized to the number of cells at the initial timepoint (t_0). This normalized cell count is referred to as the cellular index (CI), with each test antibody concentration starting with a CI of 1.0. To quantitate the decrease in CI over time, the area under the normalized CI curve was calculated using Prism. The % ADCC was calculated as follows, where "control" refers to the ADCC condition with no antibody:

$$\%ADCC = 100 \times (\text{control AUC} - \text{sample AUC})/\text{control AUC}$$

Similar to the ADCC activity curves generated for the endpoint ADCC assay, the ADCC values were plotted against the concentration of test antibody and dose-response curves generated in Prism.

3. Results

3.1. Development and optimization of the kinetic ADCC assay

Endpoint ADCC assays commonly use fluorescent labels such as calcein AM as a way to measure cell death (Cholujova et al., 2008). As target cells are lysed by effector cells, the fluorescent label is released into the supernatant. At the end of the assay, the supernatant is removed and analyzed on a plate reader. An increase in fluorescent signal indicates cell lysis. ADCC-specific activity is quantified by normalizing the fluorescent signal to the difference between maximum and minimum controls for target cell lysis (Chung et al., 2017).

To develop a kinetic ADCC assay, calcein AM was selected as the target cell label. It is readily internalized by living cells and converted by intracellular esterases into a green fluorescent compound (Papadopoulos et al., 1994). Target cells were labelled with calcein AM and opsonized with test antibodies for 30 min at room temperature. NK-92_158V cells were added to the target cells at a 5:1 effector-to-target ratio, and incubated for 1–4 h in a warmed cell imager. Images were captured using a 2.5× objective. Cells were then counted at each time point using a size gate between 10 and 30 μm and a minimum fluorescent threshold. As target cells were lysed by the NK cells, the number of fluorescent cells in each well decreased over time.

The cellular index (CI) was calculated as the number of cells at a given time point divided by the number of cells at the starting timepoint (t_0). The cell index plotted against assay time showed the decrease in cell number due to ADCC (Fig. 1A). Each mAb concentration tested had its own CI curve. Therefore, to integrate the multiple CI curves, the area under the curve for each cell index was calculated. In the absence of mAb (control AUC), cell lysis was minimal. As the concentration of antibody increased, cellular lysis increased resulting in a steeper CI curve and a smaller AUC value (Fig. 1A). The decrease in number of target cells over time is shown in Fig. 1B. At the start of the reaction (Time 0), the calcein-AM cells were easily detectable. However, by the end of the reaction (Time 4), the number of calcein-AM cells was greatly diminished (Fig. 1B).

As shown in the equation in Fig. 1A, the % kinetic ADCC activity was calculated by subtracting the AUC value of the sample from the control AUC and dividing this number by the control AUC. This %ADCC was plotted against the concentration of the test antibody (Fig. 1C). As a result of calculating the kinetic ADCC activity from the AUC of CI, there was no need for additional min/max lysis controls as in the endpoint assay. Furthermore, since the number of cells is quantitated directly by the cell imager, there were no additional handling steps at the end of

the assay, in contrast to the endpoint assay where cell supernatant is removed and measured for ADCC activity. Rituximab and trastuzumab are well-characterized for inducing ADCC. Rituximab targets CD20-expressing B cells while trastuzumab targets HER2-expressing breast cancer cells. When tested in the kinetic ADCC assay, both rituximab and trastuzumab induced ADCC on their respective target cells (Fig. 1C).

3.2. Comparison of kinetic and endpoint ADCC assays

Endpoint fluorescent release ADCC assays have served as a standard format for measuring the *in vitro* ADCC activity of biologics. Much like in the kinetic assay described above, effector cells are incubated with opsonized target cells for a defined period of time. Intracellular markers in measurable amounts are released from cells generally 3–4 h after addition of effector cells. After incubation, the assay plates are centrifuged and the supernatant removed to read on a fluorescent plate reader. The degree of fluorescence in the supernatant determines the amount of ADCC activity.

Rituximab-induced ADCC with calcein AM-labelled CD20-expressing B cells was used to directly compare endpoint and kinetic ADCC assays. NK-92_158V cells were added at a 5:1 effector-to-target ratio and incubated for 1 or 4 h, as shown in Fig. 2. After a 4-h incubation, the EC_{50} of %ADCC activity measured by the kinetic and endpoint ADCC assays were similar: 3.63 ng/mL and 4.26 ng/mL, respectively (Fig. 2A). Similar results were obtained using human peripheral blood mononuclear cells (PBMC) as effector cells (data not shown). When the incubation time was shortened to 1 h, the results between the two ADCC assays diverged (Fig. 2B). The kinetic assay still showed a robust ADCC activity curve ($EC50 = 81.9$ ng/mL). In contrast, the endpoint ADCC assay showed reduced ADCC activity ($EC50 = 330$ ng/mL). Thus, while the kinetic and endpoint ADCC assays showed similar results at 4 h, the kinetic format produced similar results in a quarter of the time.

If target antigen expression is too low, mAbs may not be able to crosslink FcγR on NK cells and induce detectable ADCC. Therefore, to test the sensitivity of the kinetic ADCC assay, cell lines with different levels of CD20 cell-surface expression were compared. As shown in Fig. 2C, BJAB cells express the highest level of CD20, REC-1 cells express an intermediate amount of CD20, and SUDHL-8 express low levels of CD20. All cells were treated as described previously and analyzed in the kinetic ADCC assay (Fig. 2C). The kinetic ADCC assay showed pronounced differences in ADCC activity dependent upon CD20 expression. Strikingly, while expression of CD20 on SUDHL-8 cells is very low, the kinetic ADCC assay was still able to detect robust ADCC activity. Therefore, the kinetic ADCC assay is sensitive enough to detect ADCC activity on target cells expressing low level of antigen.

3.3. The kinetic ADCC assay is able to differentiate glycosylation variants

A common issue during large scale production of biologics is controlling the extent of glycosylation. Absence of the core fucose in the Fc N-glycan of mAbs can lead to substantially increased binding to FcγRIIIA and enhanced ADCC activity (Chung et al., 2012). Similarly, higher levels of N-linked mannose-5 glycan (Man-5) has been shown to increase ADCC activity (Yu et al., 2012). Levels of mannose and afucosylation can vary across production lots, so it is important that an ADCC assay be able to differentiate these changes. As shown in Fig. 3, control (reference) ocrelizumab was compared to glycosylation variants in the kinetic ADCC assay. In comparison to the high mannose (HM) and afucosylated (AF) variants, reference orelizumab showed decreased ADCC activity, while deglycosylated (DG) ocrelizumab (which is unable to bind to FcγRIIIA) showed minimal ADCC activity (Fig. 3A). Similarly, when ocrelizumab containing varying levels of afucyosylation was tested in the kinetic ADCC assay, there was a fucosylation-dependent decrease in the amount of ADCC activity. Therefore, the kinetic ADCC assay is able to robustly detect varying activity of glycosylation variants, making it an *in vitro* assay suitable for lot-to-lot control testing.

4. Discussion and conclusions

Endpoint calcein AM release from target cells is well-established as a robust and reproducible method to assay *in vitro* ADCC activity of mAbs. However, these assays have several disadvantages: They require a minimum incubation time of 3 h as well as additional handling at the end of the assay to isolate the supernatant and additional plate controls in order to transform the cell lysis signal into a measurement of ADCC activity. In contrast, direct target cell imaging allows quantitation of cell lysis in real time, while avoiding these issues and enabling direct assessment of ADCC.

In this study, we describe a kinetic ADCC assay that measures target cell lysis over time. By converting the cellular index into an AUC analsyis, it is possible to directly calculate ADCC activity over time. This assay can be used with both adherent and suspension target cells. While this particular study used the Biotek Lionheart cell imaging system with Gen5 software, the assay could be transferred to a different cell imaging system, provided that there is an automated cell counting software that can be gated on target cell size and fluorescence.

In a direct comparison of the kinetic ADCC assay and a conventional endpoint fluorescent release assay, the kinetic assay demonstrated similar sensitivity to the endpoint assay (Fig. 2). The kinetic assay required less operator handling time and fewer assay controls (min/max lysis). When target or effector cell availability is limited, the decreased number of assay controls can be advantageous. Furthermore, the kinetic ADCC assay was able to measure similar ADCC activity after only an hour assay time in comparison to the endpoint ADCC assay (Fig. 2). Therefore, while the kinetic ADCC assay has comparable sensitivity to the endpoint assay, it offers distinct advantages of decreased assay time and handling.

A major requirement of any ADCC assay is the ability to distinguish differences in activity due to glycosylation variants. Glycosylation can be difficult to control during manufacturing, so it is essential to be able to detect variations in glycosylation. Reporter gene assays can reveal small differences in glycosylation but usually require overnight assessment to detect upregulation of the reporter gene expression. In contrast, the kinetic ADCC assay is able to differentiate glycosylation variants even with a far shorter incubation time.

Recent advances in cell imaging technology have allowed for the development of a kinetic ADCC assay. This study has demonstrated that the shortened assay time coupled with comparable sensitivity to endpoint ADCC assays makes the kinetic ADCC assay a valid alternative option for measuring *in vitro* ADCC activity of mAbs.

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