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Validation of an ADCC assay using human primary natural killer cells to evaluate biotherapeutic products bearing an Fc region

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ABSTRACT

The development of biotherapeutics requires continuous improvement in analytical methodologies for the assessment of their quality attributes. A subset of biotherapeutics is designed to interact with specific antigens that are exposed on the membranes of target cells or circulating in a soluble form, and effector functions are achieved via recognition of their Fc region by effector cells that induce mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, ADCC induction is a critical quality attribute (CQA) that must be evaluated to ensure biotherapeutic efficacy. Induction of ADCC can be evaluated by employing effector cells from different sources, such as peripheral blood mononuclear cells (PBMC) and genetically modified cell lines (e.g., transfected NKs or Jurkat cells), and different approaches can be used for detection and results interpretation depending on the type of effector cells used. In this regard, validation of the assays is relevant to ensure the reliability of the results according to the intended purpose. Herein, we show the standardization and validation of ADCC assays to test the potency of three biotherapeutic proteins using primary NK cells obtained from fresh blood as effector cells and detecting cell death by flow cytometry. The advantage of using primary NKs instead of modified cells is that the response is closer to that occurring in vivo since cytotoxicity is evaluated in a direct manner. Our results indicate that in all cases, the assays exhibited a characteristic sigmoidal dose/response curve complying with accurate, precise and specific parameters. Thereby, the validated ADCC assay is an appropriate alternative to evaluate the biological activities of these type of biotherapeutics.

1. Introduction

Monoclonal antibodies and other engineered proteins bearing Fc regions have been successfully used in the treatment of chronic and degenerative diseases. Despite the fact that each of these therapeutic molecules have different targets, they share common mechanisms of action, including the induction of complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) (Golay and Introna, 2012; Pescovitz, 2006).

ADCC plays a key role in the clinical efficacy of many immunotherapies (Kohrt et al., 2012) ADCC requires the recognition and binding of antibodies/fusion proteins to specific epitopes on the surface of target cells. Thereafter, Fc-bearing molecules are recognized by the Fc receptors (FcR) of effector cells, particularly natural killer (NK) cells, which are triggered to induce death of the target cells.

To increase the ADCC activity of therapeutic antibodies, the pharmaceutical industry has structurally improved the Fc region. For example, the insertion of point mutations or modifications into the glycosylation profiles of Fc regions has been shown to increase their affinity towards FcRs on different effector cells. Defucosylation of N297

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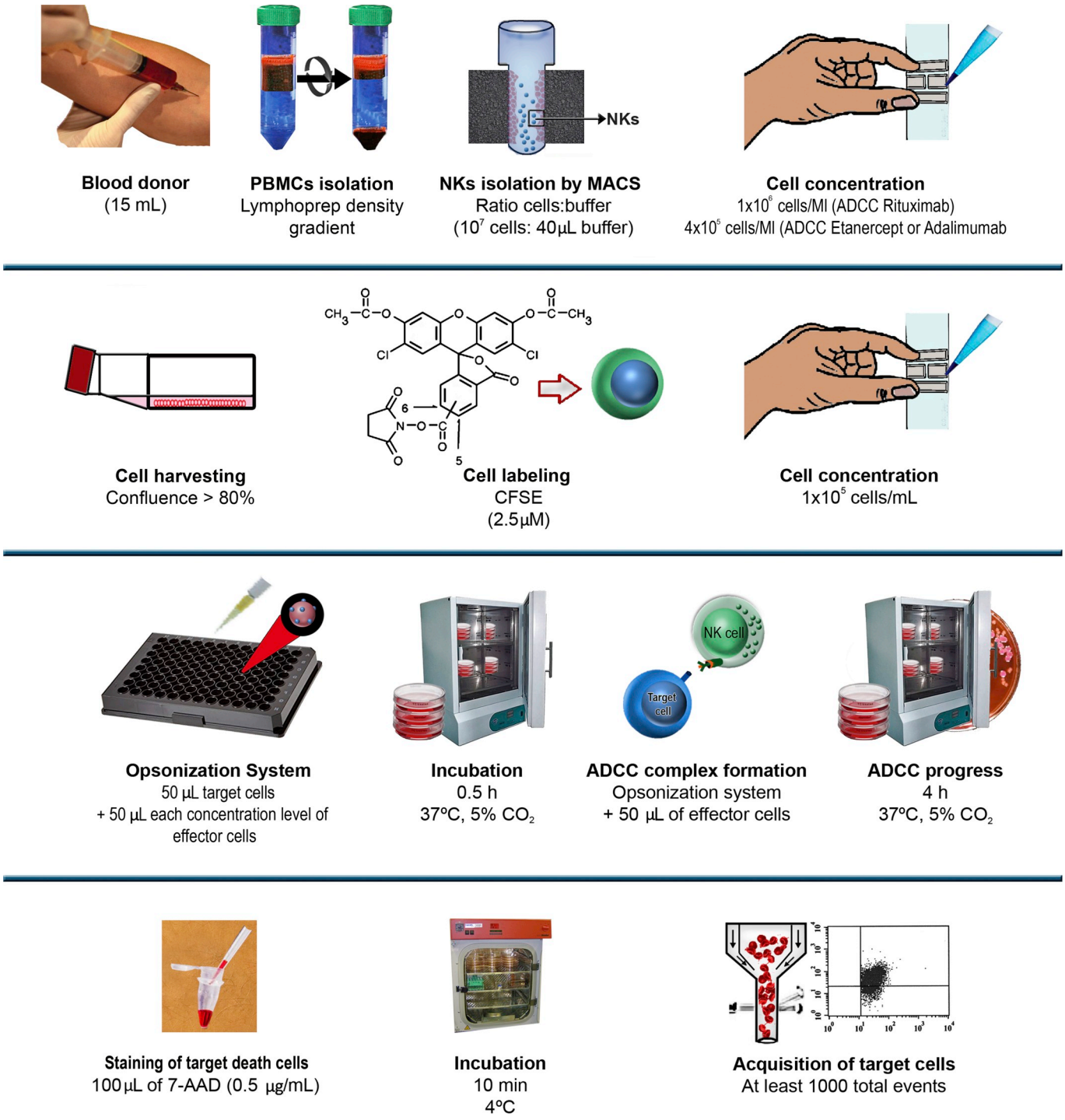

Fig. 1. Customized process for an ADCC assay using primary NK cells isolated from human blood. Schematic diagram that represent the four critical steps in the ADCC assay: 1) NK isolation by negative selection, 2) target cell preparation, 3) ADCC complex formation, and 4) cell death detection by flow cytometry.

and the point mutations S239D, I332E and/or A330L in the CH2 domain of the Fc region increases the activation of Fc γRIIIa (CD16) and therefore induces better cytotoxic responses (Abès and Teillaud, 2010; Ho et al., 2016; Hristodorov et al., 2013; Kubota et al., 2009). To evaluate the efficacy of these biotherapeutics, several ADCC methodologies have been developed, but not all are suitable for all laboratories.

For instance, ADCC assays using radiolabeled (^{51}Cr) target cells coincubated with PBMC or NK cells as effectors have been used to detect and quantify cytotoxicity (Sung et al., 2018). This method presents disadvantages, such as the high spontaneous radioisotope release and hazards associated with the use of radioactivity (Derby et al., 2001; Sung et al., 2018). Reporter-based ADCC assays (e.g., the T Cell Activation Bioassay, NFAT, which uses genetically engineered Jurkat T cells) are widely used (Cheng et al., 2014; Tada et al., 2014); however, these assays evaluate the signal transduction through Fc receptors in the effector cells rather than assess the cytotoxic effects on target cells (Cheng et al., 2014; Gurjar et al., 2017). Hence, more accessible and affordable alternatives, such as flow cytometry-based methodologies (e.g., based on the uptake of 7-amino-actinomycin D (7-AAD) or propidium iodide) have been successfully implemented in some laboratories (Salinas-Jazmín et al., 2014; Yamashita et al., 2016).

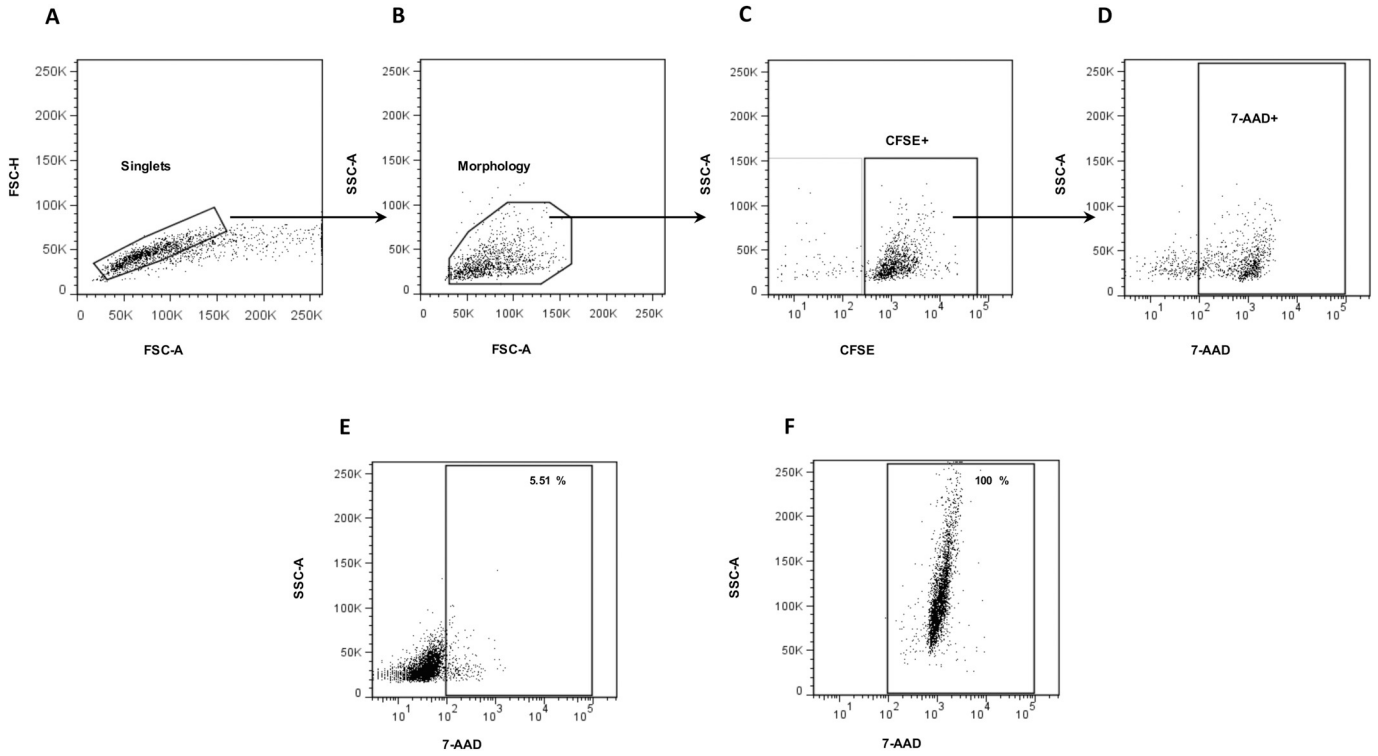

Fig. 2. Flow cytometric analysis of the death of target cells by ADCC. Daudi cells were harvested and cultured with rituximab and primary NK cells for the ADCC assay as described in the methods section. Representative dot plots for selecting target cells are shown. Cells were gated based on singlet events (A) and morphology (B) by the size (FSC) and granularity (SSC) parameters. Target cells were gated as CFSE-positive (C), and the subpopulation of dead cells was identified by 7-AAD staining (D). A representative dot plot of basal dead (without treatment) (E) and control dead cells, in which death was induced by 70% ethanol for 4 h (F).

Currently, several methodologies exist for evaluating ADCC activity; however, few are validated (Parekh et al., 2012; Rossignol et al., 2017; Yamashita et al., 2016). Thus, the standardization and validation of ADCC assays is fundamental since each variation in the methodology might impact the sensitivity and specificity of the assay (Cheng et al., 2014; Sung et al., 2018; Tada et al., 2014; Yamashita et al., 2016). Validation ensures the quality and consistency of results that could be implemented in several laboratories, including those in the pharmaceutical industry (Lee et al., 2018).

In this work, we present the development and validation of ADCC assays to test the efficacy and potency of biopharmaceutical products using primary NK cells obtained from fresh blood as effector cells. Unlike genetically engineered effector cells, primary NK cells represent the natural variability of a system in vitro, which is closer to the physiological conditions present when an Fc-bearing biopharmaceutical product is administered. The validation exercise reported herein includes assessment of the biological activities of the monoclonal antibodies rituximab and adalimumab as well as that of the fusion protein etanercept. The validation exercise was conducted according to the USP ⟨1033⟩ Biological Assay Validation Chapter and the Q2(R1) ICH Validation Guideline (2005, 2013).

2. Materials and methods

2.1. Standardization

The aim of the standardization was to identify the critical steps of the assay that allow the establishment of experimental conditions and parameters to minimize the variability associated with the use of primary NK cells.

2.1.1. Preparation of cells

First, peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood by density gradient centrifugation using the reagent Lymphoprep® (Axis Shield, Dundee, Scotland) according to the manufacturer's instructions. PBMCs were resuspended in Magnetic Activated Cell Sorting MACS® buffer solution (Miltenyi Biotec, Bergisch Gladbach, Germany) and then counted by the trypan blue exclusion method. NK cells were isolated by negative selection using MACS® microbeads (Cat no: 130-092-657) according to the manufacturer's instructions; thereafter, the isolated NK cells were resuspended in RPMI medium (ATCC, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA). To test the performance of the assay, we used NK cells from three donors.

Two cell lines were used as targets to test the potency of the biotherapeutics. Daudi Burkit's lymphoma cells (ATCC) were cultured in complete RPMI medium supplemented with 10% FBS (hereinafter referred to as medium 1) at 37 °C and 5% CO_2 to test rituximab. Engineered CHO-K1 cells expressing membrane-bound TNF-α (mTNF-α cells, PROMEGA, Madison, WI, USA) were cultured in complete F12 Hams medium (ATCC) supplemented with 10% FBS and 500 µg/mL neomycin (hereinafter referred to as medium 2) at 37 °C and 5% CO_2 to test adalimumab and etanercept. The cells were harvested at 80% confluence, CHO-K1 cells were Trypsinized (Trypsin, GIBCO Waltham, MA, USA), and their viability was determined by the trypan blue exclusion method. For detection, the target cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously reported (Salinas-Jazmín et al., 2014).

2.1.2. Formation of the ADCC complex

Serial independent dilutions of the biotherapeutics were prepared and incubated during 30 min with the target cells in 96-well plates containing culture medium (1 or 2, depending on the biotherapeutic). Then, the effector cells were dispensed to the wells and cocultured with the target cells during 4 h.

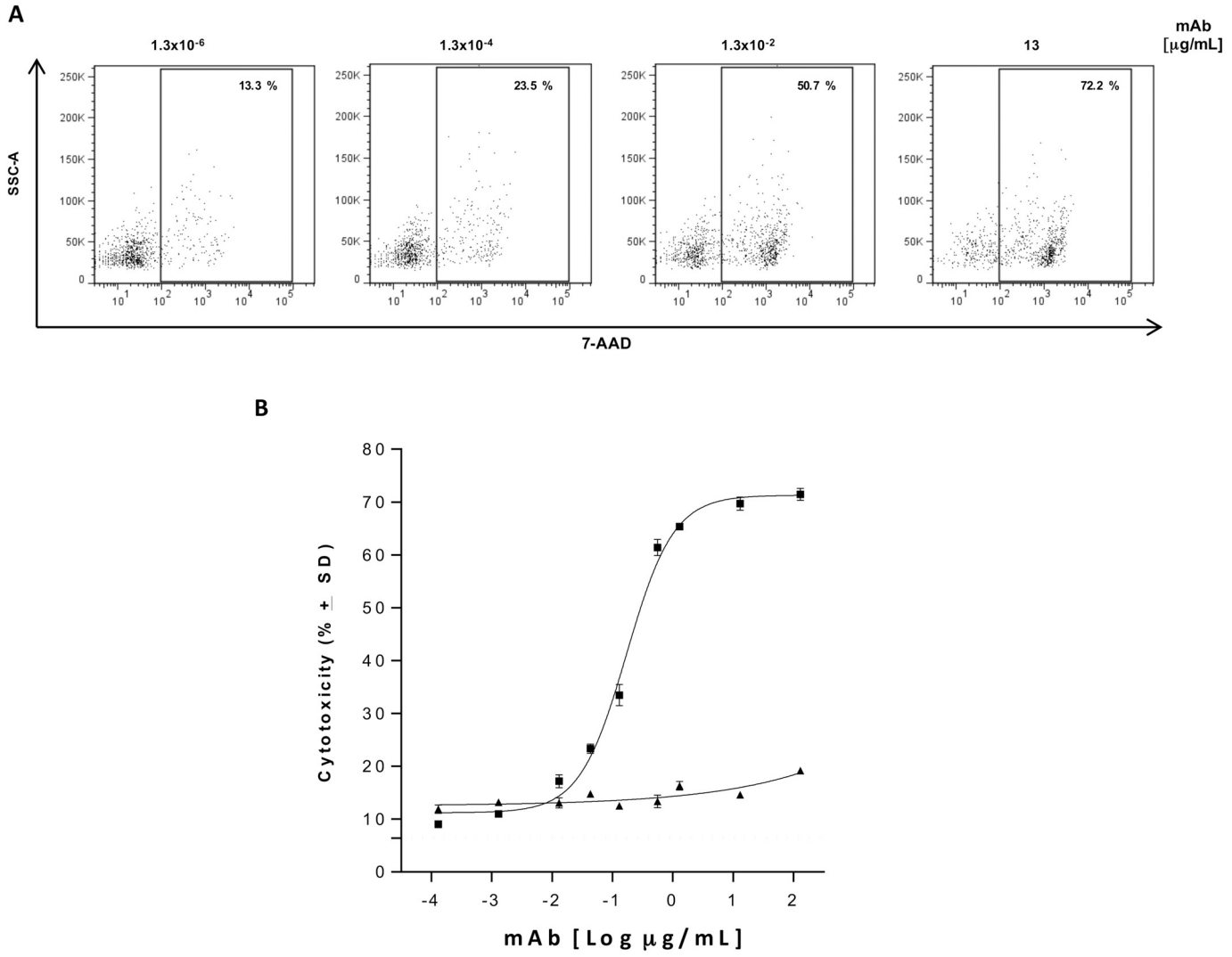

Fig. 3. Dose-response curve for the ADCC assay. Daudi target cells were incubated with primary NK cells at a 1:4 ratio with increasing concentrations of rituximab for 4 h. A) Representative dot plots of $CFSE^+/7AAD^+$ cells (dead target cells) according to increasing rituximab (mAb) concentrations are shown. B) Graph shows the four-parameter logistic model fit of the log of the rituximab concentration versus cytotoxicity ($n = 3$, $r^2 = 0.98$). The dotted line shows the response of basal death control (without treatment), and the triangles indicate the response with deglycosylated rituximab.

Table 1
Repeatability and intermediate precision.

Concentration level	Acceptance criteria	Repeatability	Intermediate precision		
		% CV	Log EC_{50}		% CV
130	CV ≤ 20%	5.0	Day 1	−1.36	7.4%
13		7.2			
1.3		2.0			
0.56		4.7			
0.13		6.4	Day 2	−1.56	
0.043		1.6			
0.013		7.5			
0.0013		5.6			
0.00013		4.2	Day 3	−1.52	
0.000013		8.7			
0		4.7			

2.1.3. Detection of cell death by flow cytometry

To identify nonviable cells, staining with 7-actinomycin D dye (7-AAD, BD, San José, CA, USA) was performed as previously reported (Salinas-Jazmín et al., 2014). The harvested cells were transferred to polystyrene tubes and analyzed on a FACS Aria III Flow cytometer (La Jolla, California, BD Bioscience). The FSC-A and FSC-H parameters were used to achieve accurate single-cell selection. The target cell population was gated from the FSC-A vs. SSC-A plot and selected as $CFSE^+$ (at least 1000 events). The proportion of cell death was determined based on the percentage of $CFSE^+/7\text{-}AAD^+$ cells.

2.2. Validation

The validation exercise was designed according to the Q2(R1) ICH guideline and the USP < 1033 > Biological Assay Validation Chapter considering fit to the four parameter logistic model (4PL): specificity, precision, accuracy and system suitability.

2.2.1. Four-parameter logistic model fitting

The cytotoxicity sigmoidal dose-response curve was obtained from independent triplicates. The curve was considered to fit if it contained at least two points at each asymptote and three points in the slope. Fitting was tested with a four-parameter logistic model using Graph Pad Prism 6.0 software (La Jolla, CA).

2.2.2. Specificity

Specificity was assessed within the expected sigmoidal behavior in

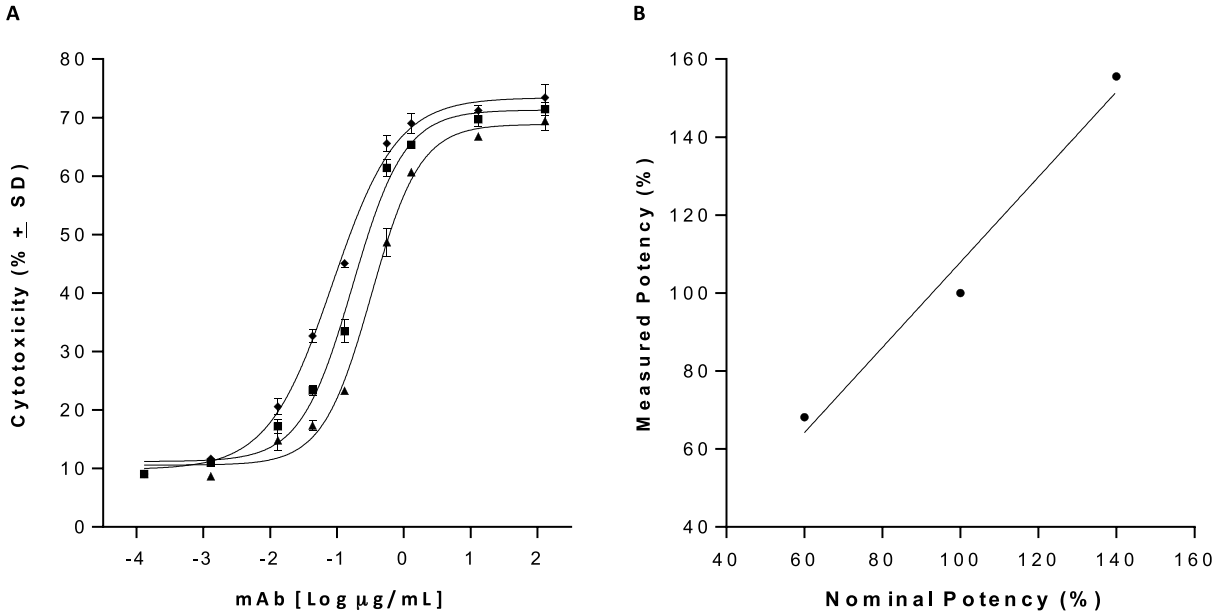

Fig. 4. Accuracy (dilutional linearity). The ADCC assay was performed for rituximab using Daudi cells. Graphs show A) the dilutional linearity of rituximab concentration-response curves (range: 60–140%: up triangles 60% (r^2 = 0.99), square 100% (r^2 = 0.98), diamonds 140% (r^2 = 0.99)) and B) the relationship between nominal and measured potency (EC50, r^2 = 0.97, slope: 1.14).

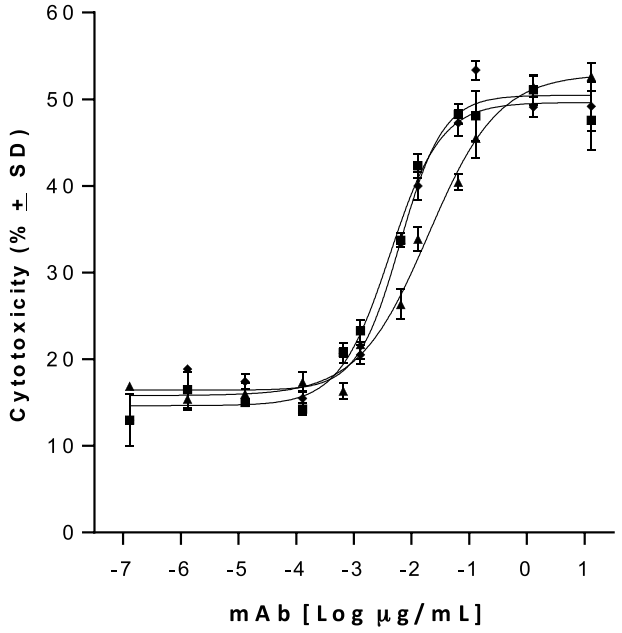

Fig. 5. Cytotoxic response of rituximab in Daudi target cells using primary NK cells from different donors. Primary NK cells, freshly isolated from three blood donors, were used as effector cells in the ADCC assay. The dose-response curve of each donor is represented by triangles (r^2 = 0.9719), square (r^2 = 0.9629) and circles (r^2 = 0.9701).

the evaluated concentration range established for each biotherapeutic molecule. We prepared negative controls containing all the components of the assay except the biotherapeutic component (basal cell death). In experiments with rituximab, a deglycosylated form was included. Deglycosylation of rituximab was performed using PNGase F amidase (New England Biolabs, USA) and confirmed using an UPLC-ESI-Q-TOF Vion spectrometer (Waters Corporation, Milford Massachusetts, USA).

2.2.3. Precision

Repeatability was determined by the coefficient of variation (% CV) from three independent replicates within the established concentration range from the 4PL fitting. Reproducibility was defined as the CV of the log EC50 value obtained from the dose-response curve of assays performed on three different days (intermediate precision) with NK cells obtained from three different donors (one for each day) sampled on the same day of the assay.

2.2.4. Accuracy

Accuracy for rituximab was determined by dilutional linearity at 60, 100 and 140% from the dose-response curve. In the cases of adalimumab and etanercept, recovery was determined at a single point.

2.2.5. Robustness

We evaluated the performance of the methodology using NK cells from different donors under the same conditions.

2.2.6. System suitability

System suitability was established according to the parameters obtained from the 4PL and precision.

3. Results and discussion

3.1. Standardization

Development of the assay allowed the establishment of four critical steps: i) NK cell isolation, ii) target cell preparation, iii) ADCC complex formation, and iv) cell death detection (Fig. 1). In addition, the specific parameters and conditions necessary to ensure appropriate execution of the assay were established to obtain reliable results.

The concentration of NK cells was adjusted to 4×10^5 cells/mL for rituximab and to 1×10^6 cells/mL for adalimumab and etanercept.

3.1.1. Preparation of cells

The negative selection strategy used allowed us to obtain primary NK cells that remained unstimulated until execution of the assay. This strategy was key for standardization because it facilitated the in vitro assessment of activation due to recognition of the Fc portion and

Table 2
Validation parameters for rituximab.

Characteristic	Parameter	Acceptance criteria	Results
4PL model fitting	Curve fitting	Fitting of the positive sample to the 4PL: $r^2 > 0.90$	0.98
Specificity	Fitting of the positive sample to the 4PL model	$r^2 > 0.90$	0.98
	Negative sample do not fit 4PL	Curve profile	Did not fit
Precision	Repeatability: Coefficient of variation (%CV) among independent triplicates at each concentration level of the dose-response curve	≤20.0%	0.4–12.6%
	Reproducibility: Intermediate precision Coefficient of variation (%CV) of $LogEC_{50}$ among assays at different days	≤20 0.0%	7.4%
Accuracy	Correspondence between the nominal potency and the measured potency obtained from dilutional linearity	$r^2 \geq 0.90$	0.97
	Slope of relative potency vs Nominal potency	0.70–1.30	1.14
System suitability	Ratio between maximum response / minimum response of the positive sample	> 2.0	6.5
	Differential dose-response among samples within a concentration range	The dose-response curve fitting to 4PL in the range of the 1×10^{-7}–1×10^{3} µg/mL	$r^2 = 0.98$ of fitting to 4PL in the range of 1.3×10^{-6}–1×10^{2} µg/mL
	Precision	≤20.0%	7.4%

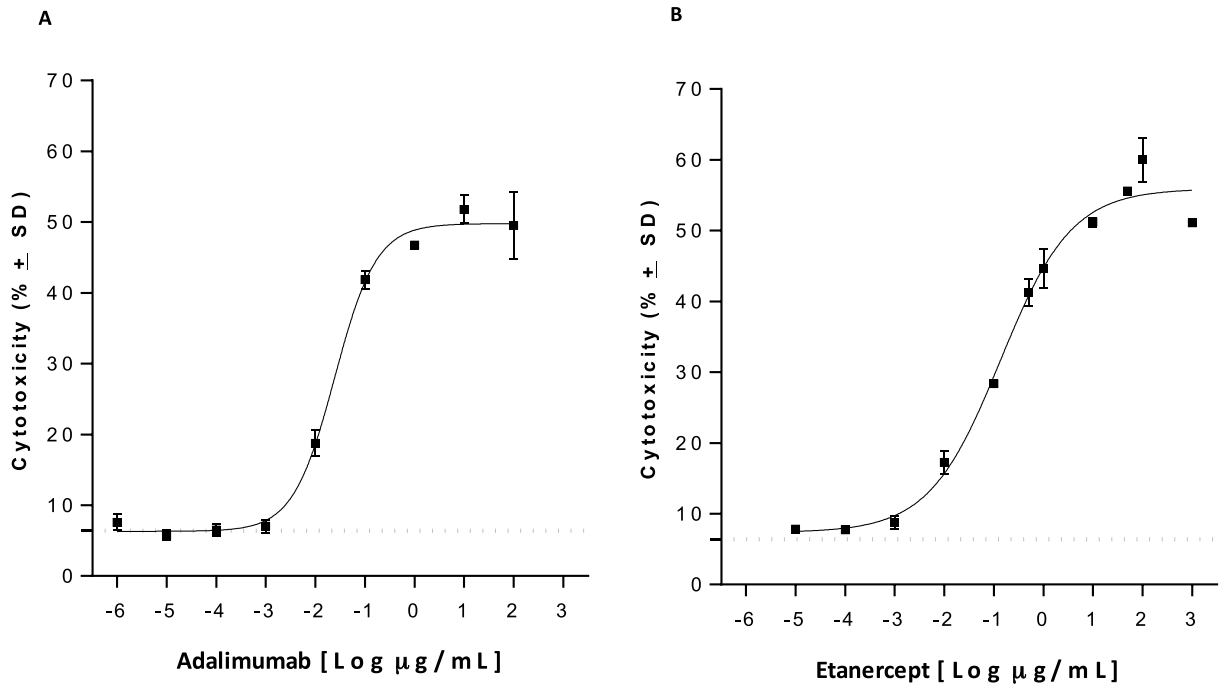

Fig. 6. ADCC assay of Fc-bearing biotherapeutics. Cytotoxic responses of the therapeutic mAb or fusion protein using CHOK1-mTNFα as target cells and primary NK cells as effectors at a ratio of 1:10. The graphs show the dose-response curves obtained from the ADCC assay for A) adalimumab ($r^2 = 0.98$) and B) etanercept ($r^2 = 0.97$). The sigmoidal behavior fits the four-parameter logistic model (4PL).

triggering of the effector functions of NK cells. This condition is closer to how ADCC occurs in vivo and demonstrates that the observed cytotoxicity is due only to the formation of the ADCC complex induced by Fc-bearing biotherapeutics and not to previous activation by cytokines or other modifications of effector cells (Cheng et al., 2014; Gurjar et al., 2017; Sung et al., 2018; Tada et al., 2014; Yamashita et al., 2016).

Regarding target cells, Daudi Burkit's lymphoma cells were resuspended at a concentration of 1×10^5 cells/mL in medium 1, and demonstrated to be appropriate to test the potency of rituximab using the ADCC assay, while CHO-K1 cells at a concentration of 1×10^5 cells/mL resuspended in medium 2 were suitable to test the potency of adalimumab and etanercept.

3.1.2. Formation of ADCC complexes

Serial independent dilutions of the biotherapeutics were prepared in their respective media using the following concentration ranges: rituximab, 1.3×10^{-6}–1.3×10^{2} µg/mL; adalimumab, 1×10^{-5}–1×10^{2} µg/mL; and etanercept, 1×10^{-4}–1×10^{3} µg/mL. We dispensed 50 µL of each reagent at the appropriate concentration into 96-well plates, and a 50 µL (5000 cells) target cell suspension was coincubated with these mixtures for 30 min at 37 °C in a 5% CO_2 atmosphere. Then, 50 µL of the effector cell suspension was dispensed into the wells, and the cocultures were incubated for 4 h at 37 °C in a 5% CO_2 atmosphere (Fig. 1).

3.1.3. Cell death detection by flow cytometry

Use of the dead cell phenotype as a measurement of cytotoxicity is characterized by the loss of size and blebbing of the plasma membrane, which eventually induces cell granularity. Using forward scattering (FSC-A or FSC-H) as a parameter to assess cell size and side scatter (SSC-A) as a parameter for the measurement of internal granularity, we analyzed single events and population homogeneity (Fig. 2A and B).

Table 3
Validation parameters for etanercept and adalimumab.

Characteristic	Parameter	Acceptance criteria	Etanercept results	Adalimumab results
4PL model fitting	Curve fitting	Fitting of positive samples to the 4PL: $r^2 > 0.9$	0.97	0.98
Specificity	Fit to 4PL of positive samples	$r^2 > 0.90$	0.97	0.98
	Negative samples do not fit to 4PL	Curve profile	Did not fit	Did not fit
Precision	Coefficient of variation percentage (%CV) among independent triplicates at each concentration level of the dose-response curve	≤20%	0.5–18.2%	0.4–12.4%
Accuracy	Recovery at a single point	80–125%	99.7%	98.5%
System suitability	Ratio between maximum response / minimum response of biotherapeutic product	> 2	3.1	5.1
	Differential dose-response among samples within a concentration range	The dose-response curve fitting to 4PL in the range of the 1×10^{-7}–1×10^{3} µg/mL	$r^2 = 0.97$ of fitting to 4PL in the range of 1.0×10^{-4}–1×10^{3} µg/mL	$r^2 = 0.98$ of fitting to 4PL in the range of 0.00 - 2×10^{3} µg/mL
	Precision	≤20.0%	0.5–18.2%	0.4–12.4%

Additionally, target cells were discriminated from primary NK cells by efficient CFSE labeling (92.6%) (Fig. 2C). Finally, subpopulations of dead CFSE+ cells were successfully detected by 7-AAD staining (Fig. 2D). Representative dot plots of the basal death control (without treatment) and the control of 100% dead cells are shown in Fig. 2E and F.

3.2. Validation

3.2.1. Four-parameter logistic model fitting

Rituximab concentrations ranging from 1.3×10^{-6} to 13.0 µg/mL showed a positive correlation with the concentration and cytotoxic effect ranging from 13.3 to 72.2% (Fig. 3 A). Our assay fits the four-parameter logistic model (4PL), maintaining a characteristically sigmoidal behavior, with 2 points on each asymptote and five points on the slope (Fig. 3 B). In addition, it is in accordance with previous unvalidated assays using different biological systems that focused on Fc signaling (Lallemand et al., 2017).

3.2.2. Specificity

The assay was demonstrated to be specific for the detection of rituximab ADCC activity, as shown by the characteristic sigmoidal dose-response curve (r^2 of 0.98). Conversely, the signal from basal cell death was below the response of the bottom asymptote from the rituximab curve. The signal intensity from deglycosylated rituximab was on the boundary of the bottom rituximab asymptote (Fig. 3B).

3.2.3. Precision

We observed that the CV values at each concentration level were lower than 20%. Moreover, the CV of the log EC50 obtained from assays performed on three different days was below 20%. The CV values for the repeatability and intermediate precision are shown in Table 1. These results demonstrate that our assay is reproducible and repeatable.

3.2.4. Accuracy

Dilutional linearity proved that the ADCC assay using rituximab was accurate in a range from 60 to 140%, showing dilutional-dependent parallel sigmoidal curves (Fig. 4 A). The plot of nominal versus measured potency exhibited an $r^2 = 0.97$ and a slope of 1.14 (95% CI 0.72–1.21), which complies with the established parameters (Fig. 4B).

3.2.5. Robustness

We observed that the rituximab dose-response curves appeared to be similar among the three different NK donors. This confirms that although NK cells were isolated from different individuals, the assay was robust under our experimental conditions (Fig. 5). Donor selection is key to obtain the ADCC response to evaluate the biological activity induced by biotherapeutics. During the development and standardization of the assays, we observed that the methodology was capable to detect a differential response among donors; this allowed us to selected those individuals who showed a better response consistently. The observed differential response might be related to the presence of the FcγRIIIa-F158F/V mutation, which has been reported, in in vitro studies, that binds to human IgG1 antibodies with higher affinity than FcγRIIIa-158F (Koene et al., 1997; Lallemand et al., 2017; Wu et al., 1997).

3.2.6. System suitability

System suitability showed that the dose-response sigmoidal behavior fit the 4PL model with a 6.5 ratio between the lower and upper asymptotes; additionally, the precision parameter showed a CV < 20% and an $r^2 = 0.98$ (Table 2).

These results of ADCC validation with rituximab comply with the ICH guideline Q2(R1) and USP < 1033 > , confirming that the ADCC assay proposed herein can be used for the intended purpose.

3.3. ADCC assay validation with adalimumab and etanercept

Based on results obtained in the ADCC assay for rituximab, the assay was tested using two other Fc-bearing molecules, the monoclonal antibody adalimumab and the fusion protein etanercept. Similar sigmoidal behaviors were observed, as dose-response curves in concentration ranges from 1×10^{-5} to 1×10^{2} and from 1×10^{-4} to 1×10^{3} μg/mL were observed for adalimumab and etanercept, respectively (Fig. 6).

Validation of the ADCC assays with etanercept and adalimumab showed % CV values for the precision parameter lower than 20%, showing r^2 values of 0.97 and 0.98 for the respective 4PL curve fittings. Thereby, we demonstrated that both assays were specific according to basal death cell and complied with the system suitability (Table 3).

These results demonstrate that our validated ADCC assay based on primary NK cells is useful and is sufficiently robust to assess the biological activity of biotherapeutics that induce ADCC with different degrees of strength. In this case, despite adalimumab and etanercept bear an Fc region and are regarded as anti- TNF-α drugs, there are relevant differences in their mechanism to induce their therapeutic effect. For instance, adalimumab has two recognition sites and is able to neutralize soluble TNF-α, it also binds to TNF-α anchored to membrane to induce ADCC. On the other hand, etanercept has only one recognition site and is designed to neutralize soluble form of TNF-α rather than bind to membrane- TNF-α (Hofmann et al., 2016). it has been reported that etanercept induce a poor ADCC activity (Arora et al., 2009; Nesbitt et al., 2007). Conversely, Mitoma et al., 2008 reported that etanercept could exhibit an ADCC response similar to adalimumab, nevertheless is necessary to make various adjustments in the ratio of effector-to-target cells.

4. Conclusions

ADCC is a critical quality attribute (CQA) that requires evaluation for the determination of Fc-bearing biotherapeutic efficacy. In our investigation, we identified the critical steps and conditions required to minimize variability trough the negative selection of primary NKs, which resulted in a reproducible assay. In addition, the validation exercise demonstrated that the assay is suitable for characterization purposes, stability assessment and batch release analysis of biotherapeutics.

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Conflicts of interest

The authors involved in this research declare no conflict of interest.

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