

Title:

Quantitating ADCC against adherent cells: impedance-based detection is superior to release, membrane permeability, or caspase activation assays in resolving antibody dose response.

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Running headline (45 chars. incl. space)

Impedance-based cell analyzer for ADCC

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Abstract

Monoclonal antibody-based immunotherapeutics will dominate Pharma's next generation of blockbuster drugs, and Fc-associated functions, including antibody dependent cellular cytotoxicity (ADCC) are among the highly desired activities mediated by these antibodies. Therefore, quantitative evaluation of ADCC is required during drug development.

Our objective was to find the most suitable and reliable non-radioactive method for quantitative analysis of in vitro ADCC against adherent cells, which often serve as models for solid tumors. The test system was comprised the HER2 positive JIMT-1 cells targeted by the specific therapeutic antibodies trastuzumab (Herceptin[®]) and pertuzumab (Perjeta[®]). These cells are resistant to the direct biological effects of these antibodies, and therefore allow the isolated assessment of ADCC.

We compared fluorescein diacetate (FDA) and carboxyfluorescein diacetate succinimidyl ester (CFSE) release as a fluorescent alternative to ⁵¹Cr release; propidium iodide (PI) uptake revealing increased membrane permeability; the PanToxiLux assay measuring ADCC induced pro-apoptotic protease activity in flow cytometry; and an impedance-based real time cell adhesion test.

We found that release assays are compromised by high spontaneous release of the label. PI uptake could not differentiate well between spontaneous NK activity and specific ADCC. The PanToxiLux assay, besides allowing for shorter assay times, offers improvement over the previous approaches in distinguishing spontaneous and antibody mediated NK action, but, probably owed to the prolonged detached state of adherent target cells, only at highly saturating antibody concentrations.

In the case of adherent target cells, impedance-based cell analysis attains functional information exclusively on the target cells without having to label them for distinguishing from effectors or assay readout. It also allows continuous monitoring for days, and specifically detects target cell detachment, as the final functional consequence of ADCC. The sensitivity of this method even allows for quantitating the additivity and saturability of ADCC as a function of antibody concentration.

We conclude that impedance-based assays are the most sensitive for quantitatively assessing in vitro ADCC on adherent target cells.

Key terms

Antibody Dependent Cellular Cytotoxicity, non-radioactive ADCC assay, adherent cell, impedance-based cell analyzer, PanToxiLux, PI/CFSE, cytometric ADCC assay

Introduction:

Immunotherapy of cancer and other diseases has become one of the most promising and popular research areas in medicine (1). One focus of these therapeutic approaches is how we can modify or improve the patient's cellular immune system to cope with a specific disease.

Monoclonal antibody-based immunotherapeutics will dominate Pharma's next generation of blockbuster drugs (2). The evaluation of the biological activity of mAbs is required during most drug development (3), and the European Medicines Agency and the U.S. Food and Drug Administration have each drafted guidelines (4,5) for the development of biosimilar products; extensive structural and functional characterization of the proposed product is recommended.

When antibody therapy using humanized monoclonal antibodies against tumors was first coined, parent antibodies were selected based on their *in vitro* antitumor effect. Consecutively, it became apparent that in addition to direct cell biological effects, antibody dependent cell-mediated cytotoxicity (ADCC), and other Fc fragment-associated functions such as complement-dependent cytotoxicity (CDC) and complement activation are equally, if not more important in the antitumor therapeutic effect of these antibodies, when applied in the clinical setting.

Thus, one important scenario is the quantitative assessment of the ADCC-promoting ability of newly developed therapeutic antibodies. The other emphatic area is the use of antibody combinations, where additive, synergistic, or antagonistic interactions need to be explored quantitatively.

The objective of the present study was to find the most suitable and reliable non-radioactive method for quantitative analysis of *in vitro* antibody-dependent cellular cytotoxicity (ADCC) against adherent target cells, which often serve as models for solid tumors. Therefore, the ^{51}Cr release assay was not included in the comparison. While this latter was originally regarded as reproducible and easy-to-perform, several of its drawbacks have been revealed lately. It turned out that it is semi-quantitative, only moderately sensitive, prone to poor labeling, and there is high spontaneous release from some target cell lines (6). In addition, the ^{51}Cr release assay raises the obvious biohazard and disposal issues, and all these contraindications have led to attempts for its replacement with non-radioactive assays (6-8).

Herein, we evaluate several non-radioactive assays with two particular therapeutic antibodies targeting the HER2 oncoprotein. The overexpression of this cell surface protein can be demonstrated in many tumor types, and correlates with poor clinical outcome. Trastuzumab (Herceptin[®]) and pertuzumab (Perjeta[®]) are both humanized monoclonal antibodies in clinical use (9,10) targeting distinct extracellular regions of HER2 (11,12). Their mechanisms of action include direct biological effects as well (13), but antibody-dependent cellular cytotoxicity (ADCC) appears to be of the greatest clinical relevance (3,14-16), and recently it was established that antibody doses exceeding the highest approved clinical doses do not saturate the ADCC

response (14). This emphasizes the need for quantitatively analyzing the dose dependence of ADCC for antibodies under development or evaluation.

As target system, we have chosen the JIMT-1, a HER2-positive human breast cancer cell line, established from pleural metastasis (17). JIMT-1 cells, even though they express the target HER2 molecule, have proved to be in vitro resistant to the direct cell biological effects of both trastuzumab and pertuzumab. However, both in vitro, and when freshly inoculated into SCID mice, JIMT-1 tumors are sensitive to ADCC mediated by these antibodies (3,14-16). Therefore, their use allows the isolated observation and quantification of ADCC without being disturbed by the direct biological effects of these antibodies.

The principle driving the choice of ADCC assays to be evaluated was to choose at least one assay from each major group of widely applied assays, and to choose the most frequently suggested ones when such could be identified. So we set out to analyze the following groups.

(a) Since ADCC imposes the damage of target cell membrane, assays quantitating the release of a preloaded or native substance upon damage are the abundant. Of these, we have tested the applicability of fluorescein diacetate (FDA) as well as of carboxyfluorescein diacetate succinimidyl ester (CFSE). (b) The complementary assay to release-based methods are uptake-based ones. Of these, measurement of propidium iodide (PI) uptake is a gold standard for assessing increased membrane permeability during cell death. We have tested this method, supplemented with CFSE labeling of the target cells to differentiate them from effector cells. (c) Since ADCC induces the activation of caspases by depositing active granzyme B into the target cells (18), a group of assays testing the activity of these enzymes in target cells using specific substrates has recently gained grounds (19). Of these, we have chosen the PanToxiLux assay (OncoImmunin) that can be supplemented with the fluorescent identification of target vs. effector cells and the exclusion of target cells that are dead or dying before adding the effector cells (20). (d) Complex impedance variations caused by cells attached to electrodes can be related to cell coverage, establishment of cell-to-cell junctions, and cell-substrate interactions (21), thus being useful in proliferation, viability and spreading (22), wound healing (23) and cytotoxicity (24-27) assays. Thus, we have chosen to evaluate impedance-based cell analysis as well, since it is inherently suitable for quantitating ADCC induced cell death in adherent cultures, which often serve as models for solid tumors.

Materials and Methods

All reagents were purchased from Sigma-Aldrich (Budapest, Hungary) unless otherwise indicated.

Cell lines:

Target cells: **JIMT-1** cells were cultured in 1:1 ratio of Ham's F-12 (N6760) and DMEM supplemented with 20% FCS, 300 U/L insulin (I9278), glutamine and antibiotics and split every 3-4 days.

Effector cells: the **CD16.176V.NK-92 cell line**, here abbreviated NK (from Dr. Kerry S. Campbell, the Fox Chase Cancer Center, Philadelphia, PA) was used. The cell line was derived from a human NK-like phenotype non-Hodgkin's lymphoma (28), and has been transduced to express a high affinity variant (176V) of the FcγRIIIA receptor (CD16) (29,30). The cell line was cultured in special NK medium: α -MEM containing 10% FCS and 10% horse serum (Hyclone), supplemented with glutamine, non-essential amino acids, Na-pyruvate, antibiotics and IL-2 at 100 IU/ml (Proleukin, Novartis).

All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and were routinely checked for the absence of mycoplasma contamination.

FDA and CFSE labeling

Being actively converted by living cells into the green fluorescent compound fluorescein, FDA is routinely used in the assessment cell viability. Carboxyfluorescein diacetate succinimidyl ester (CFSE) is also a cell-permeant, fluorescein-based tracer, which is retained in the living cell not only by its polar nature, but also by covalent attachment to proteins, thus allowing longer term labeling than CDA. For release assays using these two tracers, freshly detached JIMT-1 cells were suspended in sterile PBS at 1 million cells/ml and labeled with 10 μ g/ml FDA, or 10 μ g/ml CFSE (mixed isomers, ThermoFisher Scientific) for 30 minutes at 37°C. Cells were washed 3 times with indicator free DMEM at 37°C for 1, 5 and 10 mins for cell number calibration and 10, 20 and 30 mins for flow cytometric ADCC experiments.

For assessing the time course of spontaneous release, JIMT-1 cells were seeded in 24-well plates, at 100,000 cells/well. 24 hours later cells were labeled with FDA or CFSE at 10 μ g/ml concentration in sterile PBS for 30 mins at 37°C, followed by washing three times with indicator free DMEM.

FDA and CFSE spontaneous release assays

For examining spontaneous dye release as a function of cell number, labeled JIMT-1 cells were seeded in 96-well plates at numbers indicated in 200 μ l indicator free DMEM. Cells were incubated for 24 hours at 37°C, then a 100 μ l sample was taken from each well after gentle mixing of the medium and placed into wells of another 96-well plate for fluorescence measurement.

To assess the amount of remaining dye, the medium was changed on the cells and cells were lysed with 0.1% Triton X-100.

For assessing the time course of spontaneous release, after labeling and washing of JIMT-1 cells attached in 24-well plates (100,000 cells/well), 1 ml indicator free DMEM was placed on the cells. 100 μ l samples from supernatant were taken from each well at given time points and placed in wells of a 96-well plate for fluorescence measurement. Consecutive readings were corrected for the cumulative loss of volume owed to previous samplings.

Fluorescence was read out with a Synergy HT Multi-Detection Microplate Reader, (Bio-Tek, Winooski, VT, USA), with optics at the bottom using 485/20 nm excitation and 538/20 nm emission filters, and sensitivity set to 50. Fluorescence intensity was background corrected by subtracting that of the unlabeled medium.

Flow cytometric ADCC assay based on PI uptake

PI reveals cell death due to increased membrane permeability. To distinguish targets from effectors, freshly detached JIMT-1 cells were labeled with CFSE as described above, suspended in indicator free DMEM, and 100,000 cells in 200 μ l were placed in sorter tubes. 200,000 NK cells suspended in 300 μ l NK medium (without IL-2 to prevent general non-specific activation) were added to the target cells. Trastuzumab was added at 10 μ g/ml final concentration where indicated. During incubation at 37°C, samples were protected from light. 100 to 200 μ l samples were taken from each tube at given time points for up to 5 hours. PI was added to the samples 5 minutes before measurement at a final concentration of 5 μ g/ml. Flow cytometry measurements were performed on a BD FACSCalibur flow cytometer; 20,000 events were collected. Both CFSE and PI were excited with a 488 nm laser, CFSE signal detected in the FL1 (530/30), and PI in the FL3 (670LP) channel to minimize spillage of the CFSE signal into the PI signal and thus obviate the need for compensation. Data analysis was performed with FCS Express 4 (De Novo Software, Glendale, CA, USA).

Fluorogenic ADCC assay specific for apoptosis using the PanToxiLux kit

Fluorogenic ADCC assay kits aim to detect lethal hits long before target cells disappear owed to fragmentation. By lethal hit we understand any complex process initiated by the immune cell that eventually will lead to the death of the target cell. Granzyme B is a major component of the granules of cytotoxic lymphocytes and has received the most attention as a cytotoxic mediator because its protease activity is quite similar to caspases (18). Although no granzyme B activity is measurable in CTL or NK effector cells, this activity rapidly becomes detectable throughout the cytoplasm of the target cell after effector-target engagement. Detection of granzyme B and caspase 3 activity in the target cells can provide a unique readout of a potentially lethal injury delivered by cytotoxic immune cells (31). We have used the PanToxiLuxTM kit (OncoImmunit Inc., Gaithersburg, MD) to detect granzyme B and caspase activity in the target cells. Two additional intracellular dyes, NFL1 and TFL4 can be used in conjunction with the kit. The former labels cells that have died prior to the start of experiment and allows their exclusion from analysis, while the latter labels all target cells and can be used to discriminate target cells from effector cells.

Target cells were suspended in PBS containing NFL1 and TFL4 intracellular dyes, incubated for 30 mins at 37°C and washed twice, according to product manual. Coincubation of target and effector cells was done according to product manual in 50 μ l PanToxiLux substrate containing the ADCC-mediating antibodies at given concentrations and incubated for 40 mins at 37°C. 30,000 events from each sample resuspended in 200 μ l wash buffer were measured with a BD FACS Aria III, with excitation and emission set as follows: NFL1 (ex: 405 nm, em: 450/20),

TFL4 (ex: 633 nm, em: 660/20), PanToxiLux substrate (ex: 445 nm, em: 530/30). Gating strategy is provided as a supplementary figure along with MIFlowCyt specifications.

Impedance-based real-time cell analyzer

The ECIS Z0 impedance-based cell analyzer system from Applied Bioscience (Troy, NY) measures simultaneously complex impedance spectra (Z, R, C) of adherent cells growing in wells on gold electrodes over a broad range of AC frequencies between 62.5 Hz to 64000 Hz. The measured impedance depends on ionic concentration in the well and whether cells are attached to the electrodes. Cells attached to the electrodes will act as insulators and thereby alter the local ionic environment at the electrode/solution interface, leading to an increase in impedance. It is a non-invasive instrument capable of monitoring cell behavior in real-time without the use of labels.

JIMT-1 cells were seeded in 8-well chambers (Ibidi, 8W10E) in JIMT-1 medium, at a concentration of 100,000 cells/well that provides a confluent cell layer on the electrodes after spreading (24 hours). Effector/target ratio was set at 2.5:1, NK effector cells and/or antibodies at defined concentration were administered in NK medium after impedance of the target cells has reached plateau (24 hours). Wells that did not receive NK cells as treatment were also administered the same volume of cell-free NK medium. The impedance spectrum of cells adhered to the electrodes assessed was measured ~ every 3 minutes in each well. The resistive component of impedance at 4000 Hz showed the greatest sensitivity and was chosen for further analysis. Impedance values were normalized to the value of the same well measured at the start of treatment. The plates and the measuring device was kept in a 5% CO₂ humidified incubator at 37°C. Plates were only briefly removed from the incubator to administer treatment.

Statistical evaluation of data

Experimental data are presented as means \pm SD or SEM as indicated at the figures. Statistical analysis was performed using GraphPad Prism (version 7.0). To analyze the effect of ADCC, survival of the target cells in various treatments was compared using one-way ANOVA followed by Tukey's multiple comparison test at $\alpha=0.05$.

Results and Discussion

FDA and CFSE labels suffer spontaneous release which can confound specific ADCC dependent release

In ADCC assays, it is indispensable to discriminate target cells from effector cells. Labeling all living target cells with FDA or CFSE prior to mixing them with NK cells serves this purpose well. Importantly, the same label released from the target cells by cytolysis during ADCC can be quantitated in the supernatant.

Given the time needed to implement ADCC resulting in measurable membrane damage, first we tested the spontaneous release of the label from the target cells. The supernatant contained a well detectable amount of fluorescent dye due to spontaneous release (Figure 1a), which correlated with the number of cells releasing the dye. This is problematic, since this spontaneous release can interfere with the reliable estimation of release owed to ADCC-based cell lysis.

CFSE is expected to be better retained within the cells as it covalently binds to cellular proteins. We found that the supernatant of CFSE-labeled cells showed lower fluorescent intensity than that of the FDA stained (Figure 1a), but this spontaneous release could still be the cause of concern.

To examine the dynamics of spontaneous release, its time dependence was measured as well (Figure 1b). FDA and CFSE release showed a saturation curve as a function of time, reaching the endpoint of the release after 2 and 3 hours, respectively. By 24 hours, no further label could be released from the cells (Figure 1c): fluorescence in the freshly changed medium was not significantly increased during a four-hour follow up period. Consecutive lysis of the cells with 0.1% Triton X-100 releasing all remaining intracellular fluorescent staining caused some increase in the case of CFSE labeled cells but no increase with FDA labeled cells. This supports the expectation that CFSE is retained better and for a longer period in the cell, but the absolute amount of the retained fluorescent dye that can be released by lysis is negligible, only ~5% of the amount released spontaneously over the first 4 hours. This does not provide sufficient signal for detecting minute changes, and, importantly, the consequent ADCC reading would also be confounded with continuing spontaneous release.

Regarding the FDA and CFSE release assays, we can conclude that the rate and timing of spontaneous release does not allow the quantitation of dye release caused specifically by ADCC, which occurs on the same time scale. Therefore this assay has not been further analyzed in terms of quantitative performance.

PI uptake combined with CFSE as a target cell marker detects decreased cell viability, but cannot differentiate between background NK activity and specific ADCC

In spite of spontaneous release, the intracellular fluorescence of the more stable CFSE label still can be sufficient for discriminating labeled target cells from non-labeled effector cells by flow cytometry even 4 hours after labeling. In fact, detection and quantification of dead and live CFSE-labeled target cells using flow cytometry was shown to be more sensitive than release assays (6). The spectral separation also allows the separate observation of PI uptake by target cells with damaged cell membrane. The lowest spectral overlap was attained by using the FL1 and FL3 channels for CFSE and PI, respectively. After gating for intact cells in the FSC-SSC dot plot, CFSE signal discriminating NK cells from JIMT-1 target cells and PI signal discriminating PI positive dead cells from PI negative live cells resulted in 4 subgroups clearly distinguishable from each other (Figure 2a). Survival ratio of target cells was calculated and normalized to that of NK treated samples of the same experiment. This method of normalization was used throughout for comparing the various assays. In this PI uptake assay, NK cells significantly decreased the survival of the JIMT-1 target cells compared to untreated control even without antibody. However, adding trastuzumab antibody did not cause further significant changes in survival. (Figure 2b).

There can be at least two reasons why this method cannot distinguish between each of the control, NK only, and NK + Tr groups. On the one hand, PI positivity may not be specific enough; a temporary perturbation caused by NK cells could be reflected in a greater PI positive fraction of target cells than those that actually have suffered a lethal hit. On the other hand, since most cellular models from solid tumors (including the JIMT-1 cells) are adherent, their viability and sensitivity to NK cells could be different in the longer term (several hours) under suspension culture conditions.

Thus, the conclusion about the PI/CFSE assay is that it is probably not specific enough and is heavily influenced by the adherent target cells having to be trypsinized. Therefore, in our next experiments, we have explored assays that are (a) more specific in quantitating lethal hits and (b) are also adapted to adherent cells in addition to being specific.

Flow-cytometric measurement of caspase activity using PanToxiLux improves the specificity of detecting lethal hits in ADCC as compared to PI uptake

In an attempt to improve the specific detection of lethal hits, the PanToxiLux kit was chosen as a representative of the methods that detect activated granzyme B and/or caspases. The additional TFL4 label on target cells proved to be as effective in discriminating them from NK cells as the CFSE staining, while NFL1 labeling allowed the discrimination of target cells that were already dead before ADCC started (Supplementary Figure for MIFlowCyt). NK cells even without the antibody significantly ($p = 0.013$) increased the proportion of lethal hits compared to control, similarly to the PI-based test ($p = 0.012$, Table 1). In addition, the further increase in the ratio of lethal hits upon supplementing NK cells with the antibody was also significant (for 10 $\mu\text{g/ml}$ trastuzumab, $p < 0.0001$ as opposed to $p = 0.516$ measured with PI, Table 1).

However, we also learned that this ratio of lethal hits varies widely between experiments in coherence with the variation of background activity of NK cells. Only the sample treated with NK + 10 µg/ml trastuzumab showed a significant difference from the NK-only control, although some other high antibody doses were nearly significantly different as well (Figure 3). Trastuzumab F(ab')₂ served as control showing that in the absence of the Fc region no ADCC is mediated, so the extent of killing is similar to that of NK cells alone (Figure 3).

We can conclude that measurements using the PanToxiLux kit allow to compare the potency of the two antibodies to mediate ADCC. Pertuzumab was less potent than trastuzumab, while their combination showed a similar effect to trastuzumab alone (Figure 3). However, the differentiating ability is decreased by the relatively large variability of data, which could be attributed to the prolonged suspension condition target cells are exposed to. It is reasonable to assume that detachment alone does not heavily influence viability, since PI-positivity of control JIMT-1 cells was ~10%. Rather, detachment influences NK-target interaction by altering both membrane and cytoskeletal organization of the target cells. This is supported by other measurements, where SK-BR-3 mammary carcinoma cells were added to the targets replacing the NK cells, and no significant change in cell death was seen. So, as a final step of our investigation, we set out to characterize ADCC with target cells that are kept adherent.

Real-time, impedance-based cell adhesion assay is specific, and is able to reveal the stoichiometry of ADCC against adherent target cells

For a more robust evaluation of cell impedance data, all time traces were normalized to starting cell index at treatment, and then point by point to the NK-only treated control. Multiple traces from several independent experiments were then pooled (Figure 4a). Dose calibration of the antibodies has revealed that 1 µg/ml and 10 µg/ml used in the other assays were saturating the response and thus the range needed to assess dose-dependence is 1-10 ng/ml.

Using the normalized end-point cell indices, we have compared all treatments with each other similarly to previous experiments (Figure 4b). All doses of NK + antibody treatments were significantly different from the NK-only control at $p = 0.05$ or lower. Differences between similar doses were not significant ($p > 0.05$), e.g. pertuzumab or trastuzumab at 1 ng/ml or their combination at 0.5 + 0.5 ng/ml doses caused a similar degree of ADCC. Furthermore, different antibody doses, such as 10 ng/ml trastuzumab or pertuzumab vs. 1 ng/ml of these antibodies, or 1 ng/ml trastuzumab plus 1 ng/ml pertuzumab vs. 1 ng/ml of either of these antibodies, were statistically different from each other. Being able to distinguish such differences is a key element to any further ADCC experiment conducted for improving or developing antibodies and understanding the pharmacodynamics of ADCC.

PI/CFSE, PanToxiLux and impedance based assays are all suitable for quantitating background NK cell activity

To explore if the assays investigated differ in the measured NK background activity, we have normalized survival data of NK-only treated samples to that of the untreated samples of the same

experiments (Figure 5) and pooled the results of all independent experiments (Table 1). Background NK cell cytotoxicity was identical ($p \geq 0.85$) in all the three ADCC methods. This suggests that background NK cell activity is irrespective of specific molecular targets and can be quantitated by any mainstream in vitro ADCC assay. It also shows that averaging over many experiments cancels the variation among various NK cell populations. However, for a limited number of measurements, it is still better to normalize to the actual NK background activity of each batch.

Concluding remarks

Parallel to the increasing popularity of immunotherapy, there is a need for novel, commonly accepted and standardized experimental methods to fulfill the special requirement of the field. We need to quantitate as precisely as possible the complex actions of the immune system in vitro. It is also desirable to use safer, non-radioactive methods in these investigations. Here we have compared various non-radioactive strategies that are currently available. We have, however, omitted the technically highly complex kits that require proprietary technology and engineered cells (TAG-Lite Assay, ADCC Reporter Bioassay (2)).

ADCC involves the interaction of targets and NK cells, so one primary challenge is to distinguish these during the measurements. In release assays (radioactive and non-radioactive), discrimination of target and effector cells is based on prior labeling of target cells with radioactive or fluorescent materials. Besides the possible effects of this prior labeling on cells, it is the high spontaneous release of these labels that hampers the sensitive and reproducible application of these methods. We have confirmed this for FDA as well as for the more stable CFSE labeling. Heterogeneity of labeling owing to the differential expression and activity of various ABC transporters in the target cell population could pose an additional problem.

Regarding other popular release assays, the dissociation-enhanced lanthanide fluorescence immunoassay (DELFI[®]) type time resolved fluorescence assays are not expected to be much different from FDA release in terms of premature/uncontrolled release of the label, even though they offer higher sensitivity. Another popular assay that is based on similar principles is the release of endogenous GAPDH, for which several commercial kits are available. However, it has already been shown that the proportional linear response of GAPDH assays is limited to small ranges of changes in viability and suffers from unsteady light output kinetics (32). These assays are also burdened with unpredictable influence by the actual metabolic state of the target cells, and can be confounded by GAPDH release from the effector cells.

PI uptake, a frequently used indicator of cell death can have a formidable background, and is neither sensitive, nor specific to ADCC induced cell death. Fortunately, several other flow cytometry ADCC assays based on fluorescent substrates, such as PanToxiLux, offer an improvement over sensitivity and specificity, and decrease the amount of time needed to detect

ADCC by detecting activated intracellular processes right at the start of ADCC. However, these assays are set up for flow cytometry, which carries disadvantages in the case of adherent target cells. Most cellular models from solid tumors are adherent, and it is not physiological for adherent cells to stay in suspension, as it can alter viability, sensitivity, and, importantly, the targetability of membrane molecules during ADCC. Fragmentation as a final outcome of cell killing and the simultaneous presence of spontaneous death of detached target cells can also influence assay outcomes.

The use of label-free technologies based on electrical impedance is becoming more and more popular in drug discovery (33), since it allows the continuous monitoring of cellular processes, including attachment, spreading, proliferation, and cell death, including death by ADCC. This makes it possible to attain functional information exclusively on the target cells with no interference by the system used for detection, and minor changes owed to other components of the experimental system which can be corrected for by normalization.

Thus, when using adherent target cells in ADCC, we advise the use of an impedance-based cell analyzer for several reasons:

- (a) adherent target cells may act differently if not attached to the surface. Targets may become more mobile upon detachment, or the antigen masking effect prevalent to adherent states and relevant to the clinical situation (34) can vanish.
 - (b) impedance measurement does not alter target cells unlike loading with substrates
 - (c) effector cells do not affect or alter the impedance readings
 - (d) the assay is specific to the real, complete and functional effect on target cells, as the change in measured impedance is the result of cell detachment or utter change of morphology which can be regarded as the ultimate effect.
 - (e) The extent and the time course of the evoked ADCC can easily be quantitated and different antibodies or antibody concentrations can be compared quantitatively.
- Consequently, this method was of great value for us in understanding the dose response function and stoichiometry of HER2 targeted antibodies alone and in combination (14).

Overall, we can conclude that of the methods tested, impedance-based label free assays provide the greatest sensitivity in quantitating *in vitro* ADCC on adherent target cells. In addition to the ECIS Z0, the xCELLigence Real-Time Cell Analysis Systems (ACEA Biosciences) could be an alternative for conducting such experiments. For non-adherent target cells, such as leukemia cell lines, impedance-based cell analyzers are not applicable, and assays detecting granzyme B and caspase activity in viable target cells are recommended.

Finally, in terms of interpreting any ADCC data, we must emphasize that administering NK cells without any antibody already can have a notable effect on target cells. We consistently refer to this aspecific cytotoxicity as ‘background’ NK cell activity. Based on our experience, it is crucial to take this phenomenon into account in any type of assay by normalizing all specific treatments

to the background activity of the very same set of effector cells. This makes it possible to pool independent experiments with highly different basal NK cell activities.

Figure legends

Figure 1. Fluorescence-based release assays

- a. Spontaneous release of the fluorescent label from FDA or CFSE labeled cells was tested in 96-well plates, with multiple cell densities. Supernatants were collected after 24 hours of incubation. Data points are averages \pm SD from 4 independent experiments, each experiment run on 5 wells.
- b. Time course of spontaneous release was examined right after FDA or CFSE labeling and thorough washing of 100,000 JIMT-1 cells in 24-well plates. 100 μ l samples were taken at specified time points from the wells. Data points are averages \pm SD of 5 wells.
- c. Leftover fluorescent dye remaining in the cells was measured. 4 hours after FDA or CFSE labeling of JIMT-1 cells, supernatant was removed and renewed with fresh indicator free medium. Data points are averages \pm SD of 5 wells.

Figure 2. Flow cytometric ADCC assay with CFSE and propidium iodide

- a. Representative flow cytometric dot plot of JIMT-1 cells treated with NK cells (at 1:5 ratio) + 10 μ g/ml trastuzumab (Tr) for 5 hours. Effector cells can be distinguished from CFSE-labeled target cells based on the fluorescence intensity of CFSE in FL1. Propidium iodide positive cells are considered dead or dying cells.
- b. Survival rates of target cells after treatment are calculated from PI negative / PI positive CFSE labeled cells (\pm SD). Survival rate of each sample is normalized to survival rate of NK-only treatment. Samples are measured after 5 hours of treatment. *: $p = 0.012$
***: $p = 0.001$, ns: $p = 0.51$).

Figure 3. ADCC assay with PanToxiLux

JIMT-1 cells were treated with NK cells + antibodies.

- a. Density plot from a PanToxiLux assay shows target JIMT-1 cells gated on the TFL4 label. Substrate positive and negative cells are well separated in the upper quadrants. Antibody: 10 μ g/ml trastuzumab.
- b. Survival ratio is calculated from PanToxiLux substrate negative and total target cells in each sample and normalized to that of the NK-only treated sample. Treatments are indicated in the figure. 7-11 independent experiments are averaged for each treatment (\pm SD). (***: $p = 0.0005$).

Figure 4. ADCC assay with an impedance-based cell analyzer

JIMT-1 cells were treated with NK cells (at 1:2.5 ratio) + 0.5-10 ng/ml trastuzumab and/or pertuzumab for 18 hours.

- a. The resistive component of the impedance was measured at 4000 Hz with an ECIS Z θ cell analyzer of attached JIMT-1 cells every 5 minutes. Treatment was administered after impedance reached plateau phase (in cc. 24 hours). Each trace is averaged for ≥ 2 replicates per ≥ 3 independent experiments. The midline of the traces represents the mean, and the faded halo represents \pm SEM. For a clearer view, some treatments are shown only in Figure 4b.
- b. The end-points of traces from Figure 4a are plotted (\pm SD). *: $p \leq 0.05$; ****: $p \leq 0.0001$

Figure 5. Background (aspecific) cytotoxic activity of NK cells detected by different methods.

Surviving fraction of target cells in NK-only treated samples was measured with PI/CFSE, PanToxiLux and impedance-based analysis, and normalized, for each assay modality, to survival measured for untreated (control) target cells. ns: $p \geq 0.87$; $n \geq 8$; 4-9 independent experiments, error bars indicate SD.

Table 1. Statistical analysis of differences among major treatments measured with PI/CFSE, PanToxiLux and impedance-based assays

The values in the table are the result of the statistical analysis of measured data presented in Figs. 2, 3, and 4. One-way ANOVA followed by Tukey's multiple comparison test was performed to analyze differences among three major treatments (untreated, NK-only, NK + Trastuzumab at 10 μ g/ml). Significant differences at $p < 0.05$ are marked bold.

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Figure 1

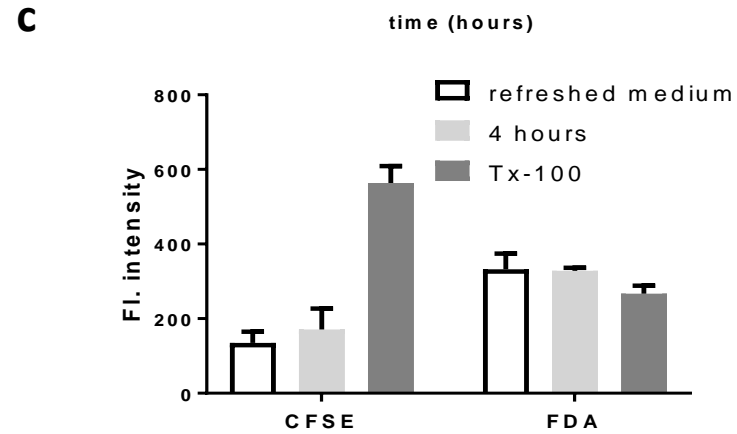
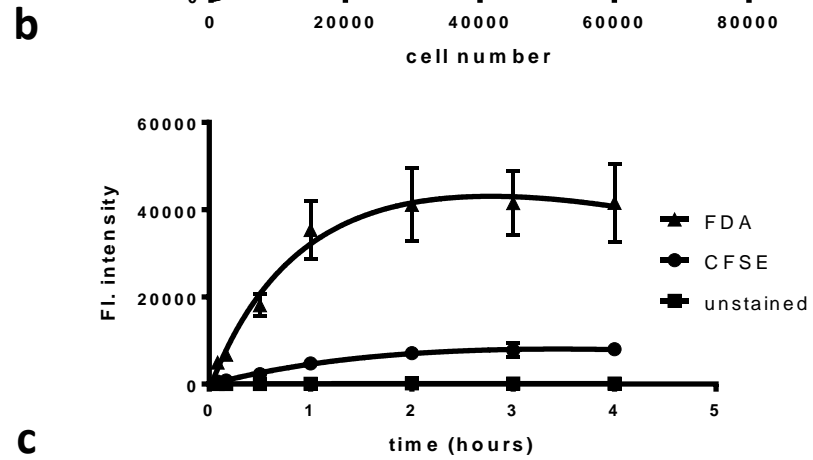
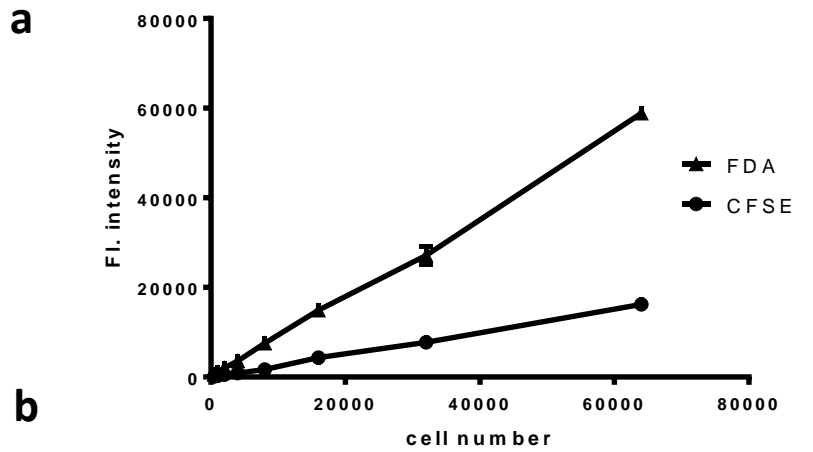
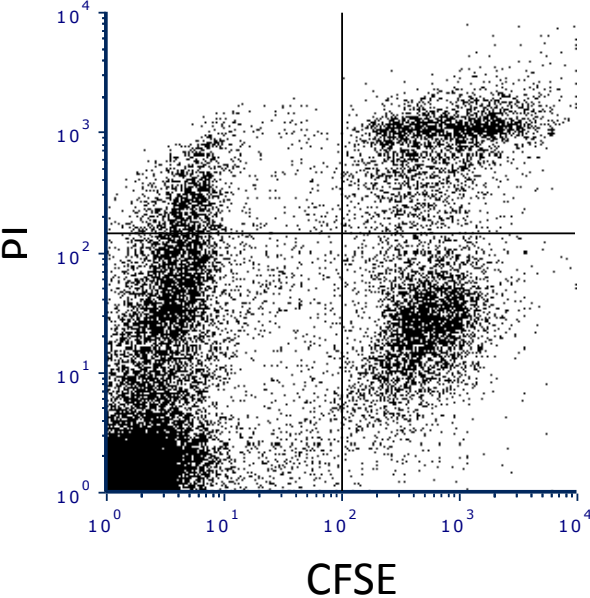


Figure 2

a



b

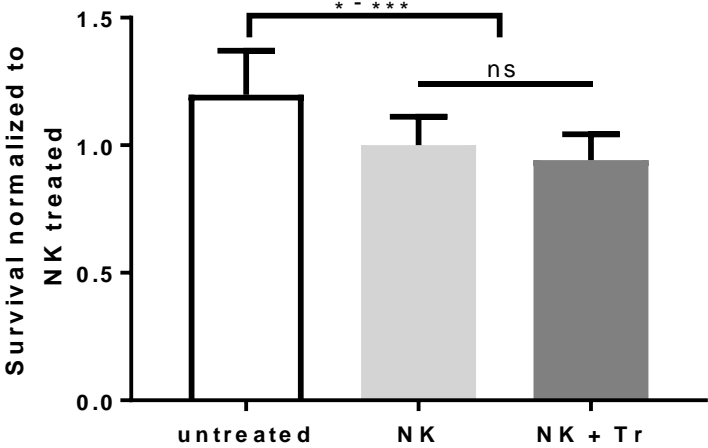
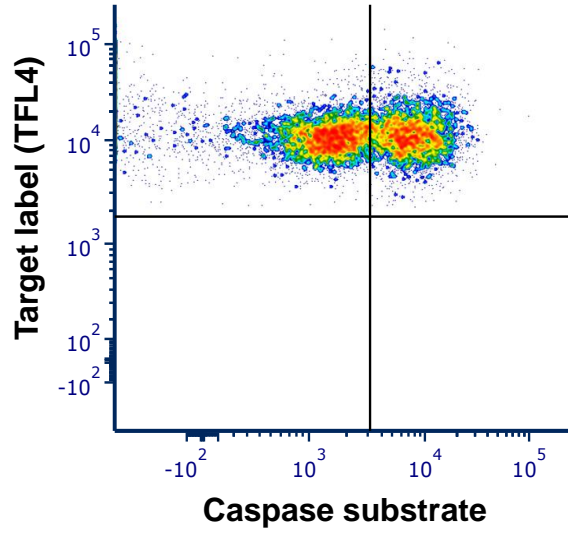


Figure 3

a



b

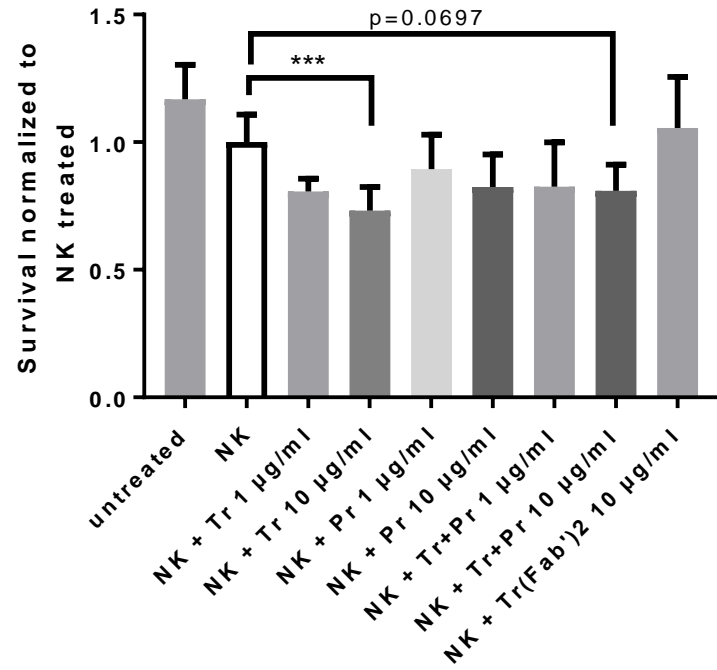
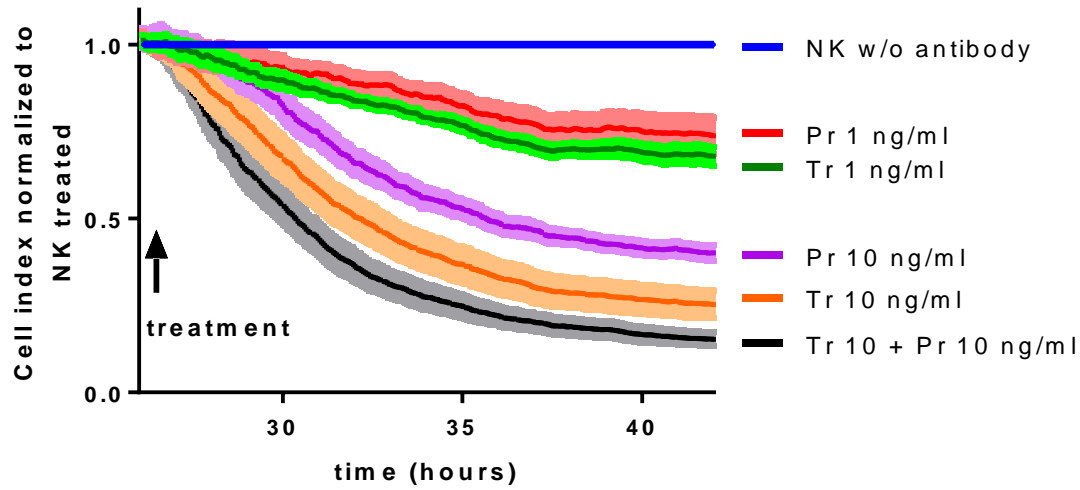


Figure 4

a



b

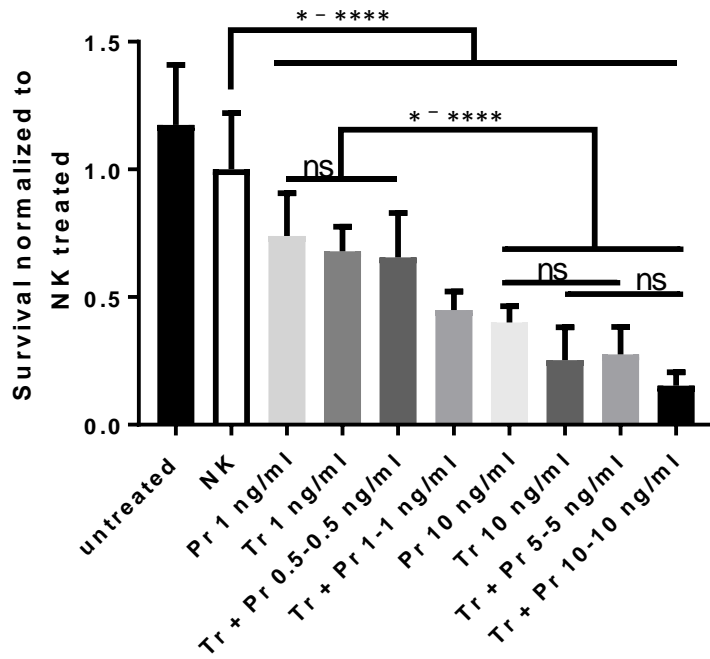


Figure 5

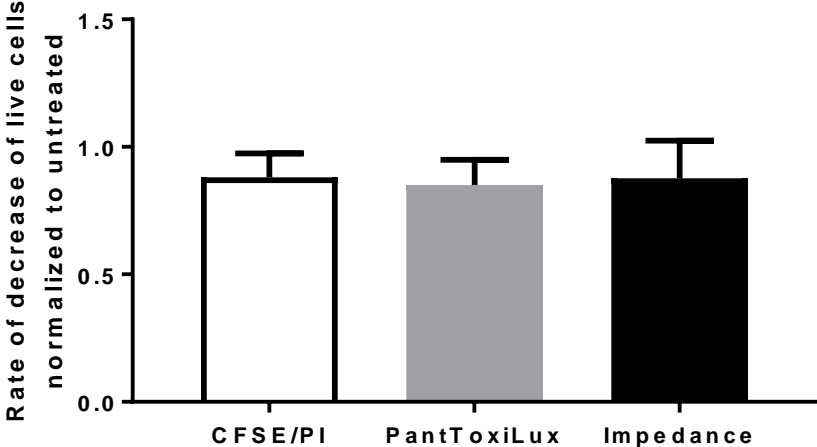


Table 1

Comparison	CFSE/PI	PanToxiLux	Impedance
Untreated vs. NK	0.012	0.013	0.196
Untreated vs. NK+Tr	0.001	<0.0001	<0.0001
NK vs. NK+Tr	0.516	<0.0001	<0.0001

