Development and validation of an antibody-dependent cell-mediated cytotoxicity-reporter gene assay

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Humanized monoclonal antibodies (mAbs) are the fastest growing class of biological therapeutics that are being developed for various medical indications, and more than 30 mAbs are already approved and in the market place. Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important biological function attributed to the mechanism of action of several therapeutic antibodies, particularly oncology targeting mAbs. The ADCC assay is a complicated and highly variable assay. Thus, the use of an ADCC assay as a lot release test or a stability test for clinical trial batches of mAbs has been a substantial challenge to install in quality control laboratories. We describe here the development and validation of an alternate approach, an ADCC-reporter gene assay that is based on the key attributes of the PBMC-based ADCC assay. We tested the biological relevance of this assay using an anti-CD20 based model and demonstrated that this ADCC-reporter assay correlated well with standard ADCC assays when induced with the drugable human isotypes [IgG1, IgG2, IgG4, IgG4S] and with IgG1 isotype variants with varying amounts of fucosylation. This data demonstrates that the ADCC-reporter gene assay has performance characteristics (accuracy, precision and robustness) to be used not only as a potency assay for lot release and stability testing for antibody therapeutics, but also as a key assay for the characterization and process development of therapeutic molecules.

Introduction

There are at least 30 monoclonal antibodies (mAbs) approved for the treatment of various diseases and more than 300 in clinical trials.1,2 Antibodies can function in a number of ways in the treatment of disease: blocking of ligand binding to a receptor, thereby blocking signaling (e.g., blocking of TNFα signaling by adalimumab), binding to a cellular receptor to block ligand binding and signaling (e.g., IL-6 receptor binding by tocilizumab), binding to a cell surface receptor and causing signaling (e.g., CD3 binding by muromonab), and binding to an antigen on the cell surface antigen and recruiting the immune system to cause effector function (induction of CDC and ADCC by rituxan).3,5

Antibodies induce effector function via interactions with the Fc domain of the antibody. The Fc portion of antibodies bound via their Fab domains to the surface of a cell can bind to C1q and cause the deposition of complement and the activation of a cascade resulting in complement-dependent cytotoxicity (CDC). The Fc region of the antibody can also signal through the FcγRIIa (CD16) receptor on natural killer (NK) and other myeloid cells, inducing these cells to release cytokines such as IFNγ and cytotoxic granules containing perforin and granzymes, which culminates in antibody-dependent cell-mediated cytotoxicity (ADCC). Upon crosslinking of FcγRIIa by the immune complex, the immunoreceptor tyrosine-based activation domain is phosphorylated by the SRC family tyrosine kinase LYN, thereby inducing the formation of a signaling complex.4 This signaling complex results in the activation of phospholipase C, which hydrolyzes phosphatidylinositol-3,4-biphosphate [PtdIns(4,5)P2] into diacylglycerol (DAG) and inosititol-1,4,5-trisphosphate [Ins(1,4,5)P3], thereby inducing a number of signaling events including calcium influx from the endoplasmic reticulum and the opening of calcium-release-activated calcium channels.5,6 The increased intracellular calcium induces the serine/threonine phosphatase calcineurin to dephosphorylate the nuclear factor of activated T cells (NFAT). Dephosphorylation of cytoplasmic NFAT exposes a nuclear localization sequence that causes translocation of this transcription factor to the nucleus where it induces the expression of a number of genes involved in the ADCC pathway.5,6,7 These effects are dependent on a number of factors including the density of the antigen on the cell surface and the isotype of the antibody.8 Thus, NFAT activation represents a robust and valid downstream target for FcγRIIIa activation via IgG1s.

Of the drugable human IgG isotypes, IgG1 is reported to be the best at inducing effector function and is selected as the
isotype for the antibody in indications such as the treatment of cancer where cell killing may be part of the mechanism of action. In cases where cell killing is not wanted, IgG2 or IgG4 are the isotypes of choice.1

While the IgG4 isotype is often reported to have limited ability to activate effector function, a number of reports suggest that in certain cases, IgG4 isotype antibodies can quite efficiently induce effector function. This induction can be decreased by changing two amino acids in the CH2 domain (F234A and L235A) where the interaction with FcγRIII is reported.9,12

The effector function potential of IgG1 isotypes can also be modified. In cases where decreased effector function is wanted, mutations in CH3, similar to those made in the above IgG4s can decrease the effector function of IgG1 antibodies.13 Moreover, other variations in the constant domains of IgG1 can increase effector function.14 Effector function of IgG1s is dependent upon glycosylation of Asn297.15 Changes in the fucose content of the glycan at Asn297 can also affect effector function. Specifically, the fucose content of the glycan is inversely proportional to ADCC activity.16-18

The clinical development of engineered mAbs with either enhanced or reduced ADCC activity is dependent on the development and validation of a robust ADCC assay as a quality control test. Over the years, numerous versions and variations of the ADCC assay have been developed. In principle, most of these versions require the isolation of peripheral blood mononuclear cells (PBMC) from donated blood. PBMC preparations serve as the source of the effector cells (NK cells). In some cases, NK cells have been further isolated from leukopheresis products of normal donors using MACS NK cell isolation kit.19 The earliest versions of the ADCC assay measured the release of the radioactive tracer 51Cr from preloaded target cells prior to the addition of the antibody and PBMC preparation.20 It is important to note that the successful ADCC assay is dependent on the optimization of the ratio of target to effector cells in the assay since the receptor densities (FcγRIIIa) will likely differ from donor-to-donor cell populations. In addition to 51Cr, target cells can also be preloaded with fluorescent dyes such as calcine-AM,21,22 CFSE,23 BCECF24 and the lanthanide fluorophore (Europium) used in time-resolved fluorescence assays.25 Although these approaches can be less sensitive than 51Cr release, they are preferred by many investigators because they alleviate the burden and cost of managing radioactive consumables. A particular lysis detection reagent may not be suitable for all target cell types. In some cases, the target cells do not readily uptake the given cell lysis reagent, and in other cases the reagents may leak out of the cell in the absence of membrane lysis. In addition to measuring the release of 51Cr or fluorescent dyes, the final read-out for ADCC activity have also been measured by standard approaches used to detect cell death and proliferation such as LDH release assay.26 Although, these types of read-outs are easy to implement, they do not discriminate between antibody-dependent killing vs. general cell death or necrosis induced independent of the antibody. More recently, the use of flow cytometry has gained favor for measuring ADCC activity since FACS allows the simultaneous utility of multiple labels to distinguish cell death between target and effector cells and to measure ADCC activity in vivo.27-29

Even with the modifications and variations made to the ADCC assay, the critical challenge to developing this assay as a robust analytical tool is the isolation of PBMC preparations. Not only does this step require human subjects for donor blood, the PBMC preparations need to be used within 48 h of isolation for optimal activity. In addition, the high degree of inherent assay variability has precluded use of the ADCC assay as a mAb product release assay in quality control labs. To mitigate these liabilities, we developed and validated an ADCC-reporter gene assay that is mechanistically relevant to PBMC-derived ADCC assays. To create a surrogate assay for ADCC, we transduced human Fcy, FcγRIIIa and a luciferase reporter under the control of the NFAT response elements of the IL-2 promoter into Jurkat cells that have previously been shown to signal through NFAT induction.6,30,31 Simultaneous binding of the mAbs to the target and the Jurkat reporter cell line leads to the activation of NFAT, which is monitored by performing luciferase assays. The ADCC-reporter gene assay has performance characteristics (e.g., accuracy, precision, robustness) that allow it to be used as a potency assay for lot release and stability testing and also as a assay for the characterization of therapeutic antibodies.

Results

Activation of NFAT reporter requires antibody-mediated cell bridging. In order to develop an alternate to the PBMC-based ADCC, we generated a recombinant Jurkat T cell line that stably expresses the FcγRIIIa complex and the luciferase reporter gene under the control of the NFAT response elements from the IL-2 promoter. We chose NFAT response elements to drive the reporter because this transcription factor is a well studied and validated transcription factor that is activated upon FcR activation, particularly FcγRIIIa activation in NK-cells.4,6,7 One critical aspect of the ADCC assay is the requirement for simultaneous binding of the target and effector cells by the mAb. That is, the direct binding of the antibody to the FcγRIIIa receptor should not be sufficient to activate the receptor complex; rather the FcγRIIIa receptor activation requires target cell engagement via binding of the antibody. We show that activation of the NFAT luciferase reporter requires the concomitant binding of IgG1v (anti-CD20 IgG1v) to the target Wil-2 cells (B cells expressing CD20 antigen) and the Jurkat-NFAT reporter cell line (Fig. 1). The reporter is not activated by an irrelevant IgG1 (anti-EGFR1). Additionally, NFAT activation can be blocked by the FK506, an inhibitor of NFAT signaling pathway (Fig. 1).

Optimization of ADCC-reporter gene assay. Optimization of cell-based bioassays often requires assessment of several key variables such as cell densities, assay incubation times, cell passage numbers, etc. In many cases, these parameters are optimized one at a time by changing one variable while holding the rest constant. Although this approach of ‘one factor at a time’ can be successful for optimizing assays, it can nevertheless be time-consuming and labor-intensive. Additionally, the ‘one factor at a time’ approach can be ineffective in optimizing parameters that can ‘interact’ with each other. Another approach for optimizing multiple variables at a time is to use statistical Design
of Experiments (DoE) tools. This approach is used routinely by other disciplines such as formulation development, as well as by process engineers for optimizing cell culture parameters for the production of biologics during the fermentation process. We used a fractional factorial DoE design to optimize cell numbers for Wil-2 cells, Jurkat NFAT cells and incubation times for the assay.\textsuperscript{32} Table 1 shows the fractional factorial design used to optimize the ADCC-reporter gene assay. The results of the DoE are shown in Figure 2. We used a statistical criterion called the L-term instead of parameters such as signal/noise ratio for the optimization of the assay since the L-term criteria correlates well with a 4-parameter logistic fit. The L-term is the log-width of the 95% confidence interval for the reported potency value. Thus, dose-response curves that fit the 4-parameter logistic model better have a lower L-term value than curves that have higher degrees of variability and systematic biases such as curve ‘hooking’ or aberrant shaped curve profiles. As seen in Figure 2, the most optimal condition requires a 15-fold ratio between Wil2/Jurkat reporter cells with an initial incubation time of 60 min for the Wil-2 cells on the plate and an 4 hr incubation time with all the components of the assay. The combinations with higher L-term values were due to increased curve ‘hooking’ at the higher concentrations of IgG1v. Although, the exact cause of the ‘hooking’ is not known, it is likely due to sub-optimal relative target to effector cell ratios. Plate layout experiments with alteration of the dilution layout on the plate did not ameliorate the ‘hooking’ indicating that it is not due to plate bias (data not shown). The DoE approach utilized here is not only a powerful tool for assay optimization, but also provides valuable information on the robustness of the system since it identifies parameters likely have a dramatic effect on the assay if not controlled. In order to demonstrate that the cell line continues to maintain the desired signal as a function of passage number, we performed additional studies to demonstrate that the overall signal, as well as signal/noise ratio is not adversely affected up to 30 passages in culture (data not shown).

### Validation of ADCC-reporter gene assay
For use as a product release assay, the ADCC-reporter gene assay was validated according to the ICH-QR2 guidelines for analytical method validation.\textsuperscript{33} The assay was validated to demonstrate specificity, linearity, accuracy and precision.

#### Specificity of the ADCC-reporter gene bioassay
Specificity is the ability to measure accurately and specifically the analyte of interest. Specificity of the ADCC-reporter gene assay was shown by demonstrating that an alternative, unrelated, IgG1 molecule (anti-EGFR) does not induce a measurable response in the assay. Testing of anti-EGFR Mab was conducted within the range of concentration evaluated for IgG1v and up to 2 log higher concentrations. As shown in Figure 1, the anti-EGFR Mab does not elicit a measurable response in this assay.

#### Accuracy of the ADCC-reporter gene bioassay
Accuracy is the measure of exactness of the analytical method, or the closeness of agreement between the measured value and value that is accepted either as a conventional true value or an accepted reference value. Accuracy was determined by either diluting or adjusting the starting concentration of IgG1v to 50% and 150% relative to set 100%. The 50% and 150% samples were run on three independent plates. As shown in Table 2, the accuracy of the assay is within 12% RSD of the two doses tested.

#### Precision of the ADCC-reporter gene bioassay
Precision is the measure of the degree of consistency of an analytical method
under normal operation. Precision is composed of repeatability and intermediate precision. Repeatability refers to the plate-to-plate variability of the method operating over a short interval under similar conditions (e.g., same day set-up, same analyst). As shown in Table 2, the repeatability estimate of the assay is 5.1% RSD. Intermediate precision refers to the variability of the final bioassay results; in this case the final result is an average value from three plates, which incorporates normal variation expected during routine operation within laboratory (e.g., different days, different analysts). As shown in Table 2, the intermediate precision estimate of the assay is approximately 3% RSD.

### Biological relevance of the ADCC-reporter gene assay

To further demonstrate the utility of this assay as a suitable alternative to PBMC-based ADCC, we sought to establish the biological relevance of this assay relative to the PBMC based-ADCC. To address this question, we asked if the ADCC-reporter gene assay was similarly sensitive to changes in the Fc-glycosylation of mAbs as the PBMC based-ADCC assay. It is well known that Fc glycosylation is required for therapeutic mAbs to elicit effector functions. Particularly, it has long been recognized that the glycosylation in the CH2 domain of IgG1 is critical for complement activation and FcγRIIIa binding. Fucosylation of the Fc-glycan has been inversely linked to FcγRIIIa binding and ADCC activity.17,34,35 Interestingly, recombinant mAbs produced in CHO cells are heavily fucosylated,36 and thus even a small change in Fc fucosylation may have significant impact on ADCC activity. Thus, we generated IgG1v with varying levels of fucosylation (91–99%) by taking advantage of the observation that independent CHO clones of IgG1v had an inherent variation in fucosylation of IgG1v under typical cell culture conditions. We tested IgG1v derived from different CHO clones in both the ADCC-reporter gene assay and the PBMC based ADCC assay. The potency of all the samples in both the assays was determined relative to the same reference standard lot that was determined to be 96% fucosylated. The results of the comparison study (Fig. 3) show that the ADCC-reporter gene assay is similarly sensitive to changes in fucosylation of IgG1v as the PBMC-based ADCC assay.

### Table 1. Design of optimization studies using a fractional factorial DoE

| Run | Wil-2 cell (x1,000) | Wil-2 cell- Incubation Time (min) | Jurkat NFAT cells (x1,000) | Jurkat NFAT cell Inc. Time (h) |
|-----|---------------------|----------------------------------|---------------------------|-------------------------------|
| 1   | 25                  | 90                               | 100                       | 3                             |
| 2   | 40                  | 60                               | 150                       | 2                             |
| 3   | 40                  | 120                              | 50                        | 2                             |
| 4   | 10                  | 60                               | 150                       | 4                             |
| 5   | 10                  | 120                              | 50                        | 4                             |
| 6   | 25                  | 90                               | 100                       | 3                             |
| 7   | 25                  | 90                               | 100                       | 3                             |
| 8   | 40                  | 60                               | 50                        | 4                             |
| 9   | 25                  | 90                               | 100                       | 3                             |
| 10  | 10                  | 60                               | 50                        | 2                             |
| 11  | 25                  | 90                               | 100                       | 3                             |
| 12  | 40                  | 120                              | 150                       | 4                             |
| 13  | 25                  | 90                               | 100                       | 3                             |
| 14  | 10                  | 120                              | 150                       | 2                             |
| 15  | 10                  | 60                               | 150                       | 2                             |
| 16  | 25                  | 90                               | 100                       | 3                             |
| 17  | 40                  | 120                              | 150                       | 2                             |
| 18  | 40                  | 60                               | 50                        | 2                             |
| 19  | 10                  | 120                              | 50                        | 2                             |
| 20  | 40                  | 60                               | 150                       | 4                             |
| 21  | 10                  | 120                              | 150                       | 4                             |
| 22  | 25                  | 90                               | 100                       | 3                             |
| 23  | 10                  | 60                               | 50                        | 4                             |
| 24  | 40                  | 120                              | 50                        | 4                             |

### Table 2. Results of the validation of the ADCC-reporter gene bioassay

| Parameter               | Result     |
|-------------------------|------------|
| Accuracy                | %RP | % Recovery |
| 50.0                    | 108.3 |
| 100.0                   | 104.0 |
| 150.0                   | 111.8 |
| Repeatability           | Repeatability (%RSD): 5.1% |
| Intermediate Precision  | Intermediate Precision (%RSD): 2.9% |
| Linearity               | Regression Line (R²) = 0.997 |
developed based on employing different read-outs to assess the cell killing activity. However, these assays generally use freshly isolated PBMCs to kill the target cell after incubation with the antibody. While this format produces valuable data concerning the ability of the antibody to induce cell death, it is difficult to use in pharmaceutical development settings because fresh blood is required. These assays often are done using blood from individuals who are not genotyped. This is important since there are profound differences in IgG binding between the different FcγRIII allotypes, and this correlates with mAb efficacy in vivo for example, VV individuals have a 90% overall response rate to Rituxan at one year after treatment compared with 51% for VF and FF individuals. Thus, the implementation of these assays in quality control laboratories for use as a product release assay has been a substantial challenge due to the high inherent variability of these assays.

We describe here the development and validation of an alternate ADCC-reporter gene assay that is highly quantitative and robust. This cell line responds to incubation with antibody and target cells by inducing luciferase activity as a surrogate for ADCC activity. The data presented here demonstrate that this method correlates with the PBMC based ADCC assays. Similar data has been obtained with other antibodies where we have created IgG1 and IgG4PAA isotype pairs (data not shown). We further demonstrate that the ADCC-reporter gene assay compares favorably to the PBMC-based ADCC assay with regards to changes in Fc-glycoform structures of the antibody and different isotypes of the antibody. Not surprisingly, this assay can be adapted to cellular targets and non-standard mAb structures such as Fc fusion proteins.

To further establish the biological relevance of the ADCC-reporter assay, we compared the induction of luciferase activity in the ADCC-reporter assay to the amount of cell death (as measured by LDH release) in the PBMC based assay after incubation with anti-CD20 antibodies of different isotypes. Previous work suggests that IgG1 efficiently induces effector function whereas IgG2 and IgG4 isotypes do not. As expected the IgG1 isotype efficiently induced killing of the Wil-2 cells (Fig. 4A) and NFAT driven luciferase activity (Fig. 4B). Multiple reports demonstrate that in certain cases IgG4s can induce ADCC. The affinity engineered anti-CD20 antibody is capable of inducing ADCC as an IgG4 isotype antibody (either as a wild type IgG4 or as a stability engineered S > P variant) as measured by the PBMC based assay. This low level activity is also observed in the ADCC-reporter assay (Fig. 4B). In many of the reports concerning IgG4s inducing effector function, mutations in the FcyRIII binding site (F234A and L235A) are able to decrease the ADCC induced by these antibodies. As predicted by these reports, mutation of these two residues in IgG4 to create the IgG4PAA isotype decreased the ADCC to the levels of an IgG2 isotype. Similarly in the NFAT driven ADCC-reporter assay, neither IgG2 nor IgG4PAA isotypes of anti-CD20 induced luciferase activity (Fig. 4B).

Discussion

ADCC activity is a critical activity that is part of the mechanism of action of many therapeutic antibodies. Over the years, several variations of the PBMC-based ADCC assays have been

Figure 2. Results of the DoE study. Figure shows that effect of cell numbers for Wil-2 and JurkatNfat cell lines and incubation times on the L-term function.
Cell lines and reagents. Wil-2 (CRL-8885) and Jurkat cells (TIB-152) were obtained from ATCC. Both cell lines were cultures in RPMI 1640 supplemented with 10% fetal bovine serum and 100 μg/ml penicillin-streptomycin.

Cloning of jurkat-NFAT luciferase cell line. Jurkat FcγRIII(V) cells were created by co-transducing Jurkat T cells (ATCC) with the Murine Maloney Leukemia Virus (MMLV)-based vector pLHCX (Clontech) expressing the 158V or 158F allotype of human FcγRIIIa with a hygromycin resistance cassette and the MMLV vector pLNCX2 expressing human Fcγ with a neomycin resistance cassette. Dual resistant colonies were screened by FACS for high FcγRIIIa expression and confirmed by anti-FcR cross linking induced IL-2 release. The reporter line Jurkat FcγRIII(V)_NFAT_Luc was created by co-electroporating the luciferase reporter under the control of the NFAT promoter (Stratagene) and pPUR (Clontech) containing the puromycin resistance cassette. Puromycin resistant colonies were screened by anti-FcR induced luciferase expression with low non-induced background.

Isolation of effector cells. Peripheral blood mononuclear cells from donors of unknown FcγRIIIa phenotypes were isolated fromuffy coats diluted ½ in saline by Ficoll-Hypaque density gradient centrifugation. The mononuclear cells were collected from the interface with a Pasteur pipette after centrifugation for 20 min at 1,000x g at 4°C and treated with IL-2 (100 ng/ml) overnight at 37°C.

Antibodies. The anti-CD20 Fab #33 has been previously described by Bowles et al.14 The heavy chain variable domain

Materials and Methods

Pharmaceutical companies are creating many antibody treatments that target cellular membrane targets. In many cases, these cellular antigens are on cell types where effector function would be counter indicated. In these cases, the choice of antibody isotype is critical. IgG2 and IgG4 isotypes have been chosen to decrease the effector function. However, both IgG2 and IgG4 isotypes have issues that must be addressed before these antibodies can become pharmaceutical agents. As discussed above, IgG4 antibodies are engineered with S228P variation to prevent half-antibody formation and bispecific formation in vivo and the F234A and L235A variations to decrease residual effector functions. IgG2 isotypes have four disulfide pairs in the hinge (compared with two in IgG1 and IgG4). These disulfides have been shown to generate multiple structural variants during protein expression,65,67 rearrange in vivo68 and can affect the activity of the antibody. IgG1 and IgG2 but not IgG4 isotypes can bind to the 131 His allotype of FcγRII and, in the context of an anti-CD3 antibody, induce T cell proliferation.69 Certain IgG2s have also been reported to induce ADCC via cells of the myeloid lineage.50 Therefore, the IgG4PAA isotype may be the best choice for indications where ADCC would be counter-indicated.

Recent guidance demonstrates regulatory agencies’ increased awareness of the importance of effector function in antibody-based pharmaceuticals.39 This awareness dictates a renewed attention to how lot to lot variation, process changes, demonstration of comparability and even initial antibody isotype choice can influence effector function and highlights the need for a robust ADCC assay. Our data demonstrates that this novel ADCC-reporter assay can serve as this critical assay for therapeutic mAbs.
concentrations of the anti-CD20 isotypes. Peripheral blood mononuclear cells (PBMCs) from IL-2 treated donor blood was added to the Wil-2 cells and antibody containing wells at 1e6 cells/well and incubated for 4 h (a target: effector ratio of 1:25) at 37°C. Cell death was determined by lactate dehydrogenase (LDH) release by the addition of Roche Cytotoxicity Detection Reagent and measurement on a Molecular Devices SpectraMax II plate reader. 

**ADCC-reporter gene assay.** Wil-2, a CD-20 positive human B lymphoblastoma cell line, was seeded at 10,000 cells per well in a 96 well opaque tissue culture plate. Anti-CD20 antibody (IgGv) was serially diluted and incubated with the Wil-2 cells for approximately 1 h at 37°C, 5% CO₂. Following incubation, Jurkat NFAT luciferase reporter cells, were added to the Wil-2/antibody mixture at 150,000 cells per well. The mixture was amplified by PCR using Platinum Pfx (Invitrogen) and subcloned into vectors containing the constant domains of wild type human IgG1 (a,z), IgG1v (containing effector function enhancing variations in the constant domain), IgG2, IgG4wt, IgG4S > P and IgG4PAA. The expression cassettes of these heavy chain isotypes vectors were co-expressed with a vector expressing the Fab#33 kappa light chain in CHO cells and purified using well described process for therapeutic antibodies. Anti-EGFR IgG1 mAb was obtained via commercial sources.

**Antibody-dependent cell-cytotoxicity assay (ADCC) assay.** Immediately prior to the assay, CD20 positive Wil-2 cells were centrifuged and resuspended in RPMI + 0.1% BSA (Sigma), non-essential amino acids, sodium pyruvate, glutamine and plated at 4e4 cells well in Becton-Dickenson U bottom Microtest 96-well plates and incubated for 1 h with varying concentrations of the anti-CD20 isotypes. Peripheral blood mononuclear cells (PBMCs) from IL-2 treated donor blood was added to the Wil-2 cells and antibody containing wells at 1e6 cells/well and incubated for 4 h (a target: effector ratio of 1:25) at 37°C. Cell death was determined by lactate dehydrogenase (LDH) release by the addition of Roche Cytotoxicity Detection Reagent and measurement on a Molecular Devices SpectraMax II plate reader.

**Figure 4.** (A) Anti-CD20 isotype antibodies mediated ADCC. Cell death was measured by lactate dehydrogenase (LDH) release. The figure is a composite of two independent assays performed in duplicate, error bars are ± standard error of the mean. (B) Anti-CD20 isotype antibody induced NFAT driven luciferase activity in Jurkat cells expressing human FcγRIIIa. The figure is a composite of two independent assays, error bars ± standard deviation.
 incubated for approximately 4 h at 37°C, 5% CO2, and then measured for luciferase production using a luminescent substrate (Promega Steady Glo or Bright Glo).

**Statistical assessment of relative potency.** Potency of IgG1v test lot is determined by comparison of its activity relative to that of an independent reference standard lot of IgG1 that was produced using the same manufacturing process. Full 8-point dose-response curves comparing the test sample to the reference standard are generated and fitted to a 4-parameter nonlinear logistic regression model. The EC50 of both test sample and reference standard are calculated after the two curves have passed a statistical test for curve similarity or parallelism (e.g., F-test). The potency of test sample is defined as the ratio of EC50 of the test sample to the EC50 of the reference standard and is expressed as percent relative potency.

**Disclosure of Potential Conflicts of Interest Statement**
No potential conflicts of interest were disclosed.

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