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Flow cytometry-based assessment of direct-targeting anti-cancer antibody immune effector functions

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Abstract

Monoclonal antibody-based therapies are increasingly being used to treat cancer. Some mediate their therapeutic effects through modifying the function of immune cells globally, while others bind directly to tumor cells and can recruit immune effector cells through their Fc regions. As new direct-binding agents are developed, having the ability to test their Fc-mediated functions in a high-throughput manner is important for selecting antibodies with immune effector properties. Here, using monoclonal anti-CD20 antibody (rituximab) as an example and the CD20⁺ Raji cell line as tumor target, we describe flow cytometry-based assays for determining an antibody's capacity for mediating antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent-cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). These assays are sensitive, reliable, affordable and avoid the use of radioactivity.

Keywords

antibody; ADCC; ADCP; CDC; Fc receptor; complement; NK cells; macrophages

1. Introduction

1.1 Fc-dependent mechanisms of antibody effector functions

Antibodies can exert effector functions through their Fab domains by binding to their targets on cells and blocking key signaling pathways or, in certain settings, by activating certain pathways, either through mimicking the natural ligand or through crosslinking their target antigens. Based on this, antibodies can be classified as antagonistic or agonistic. The majority of effector functions of antibodies, however, are mediated through their Fc-regions that either bind to Fc receptors on immune effector cells, such as NK cells or macrophages (ADCC and ADCP, respectively), or to effector molecules that can initiate the complement cascade (CDC, Figure 1). Tumor-targeting antibodies such as rituximab (anti-CD20, Rituxan), alemtuzumab (anti-CD52, CAMPATH) and many others were initially thought to cause direct cytotoxicity through disruption of cell signaling pathways, but more recently studies have determined that they operate primarily through their Fc-mediated immune effector functions (reviewed in Josée Golay & Introna, 2012; Lindorfer, Wiestner, Zent, &

Taylor, 2012). One important determinant of Fc-mediated functions is the antibody isotype, as this dictates which Fc receptors (FcRs) the antibody interacts with.

Antibodies undergo class-switching from IgM to IgD, IgG, IgE and IgA Fc isotypes. In addition to these main isotypes, just within IgG there are four subclasses of Fc isotypes, IgG1, IgG2, IgG3, and IgG4 in humans and IgG1, IgG2b, IgG3 and IgG2a or IgG2c in mice. Binding of Ig Fc domains to their corresponding Fc receptors initiates and coordinates a targeted immune attack against many different classes of antigens expressed on a large variety of target cells. Most cancer-targeting therapeutic antibodies are of the IgG1 class, which is the most abundant IgG isotype in the serum that binds to Fc receptors with high affinity.

1.2 Fc optimization

Extensive work has been done to improve antibody effectiveness by optimizing their Fc regions, especially IgG1 Fc, through mutations and glycoengineering (reviewed in Bruhns & Jönsson, 2015; Rose et al., 2013). There are several known mutations that enhance ADCC, ADCCP, and CDC (Lee et al., 2017; Moore, Chen, Karki, & Lazar, 2010), [also see table 1 in (Wang, Mathieu, & Brezski, 2018)]. as well as selective binding to Fc γ Rs expressed on dendritic cells, which can enhance soluble antigen uptake and promote a vaccine-like effect (Bruhns & Jönsson, 2015).

Another modification that improves binding to Fc γ RIIIA and enhances ADCC is lack of fucosylation at the Fc N297 glycosylation site (Robert L. Shields et al., 2002). A recent study used directed evolution to identify mutations on aglycosylated Fc domains that still retain ADCC function (Jo, Kwon, Lee, Lee, & Jung, 2018). There are also engineered mutations (E345K and E430G and E345R/E430G/S440Y) that disrupt a salt bridge between CH2 and CH3 in the Fc domain (de Jong et al., 2016). Mutations at K326W, K326W/E333S, S267E, S239D/S267E/H268F/S324T and S239D/H268F/S324T/I332E increase C1q binding affinity (Idusogie et al., 2001; Moore et al., 2010; Tammen et al., 2017). Certain modifications that enable selective binding of C1q, such as aglycosylated (T299L) K320E/Q386R and aglycosylated (T299L) L245K/G246M/G247R/L351Q, can promote complement-dependent cell-mediated cytotoxicity (CDCC) and complement-dependent cell-mediated phagocytosis (CDCP) (Lee et al., 2017).

Modifications that abolish selective antibody effector functions are also known, such as the N297A mutation that eliminates an N-glycosylation site critical for FcR binding or production of antibodies in cells that do not glycosylate, such as *E. coli* (Simmons et al., 2002; Walker, Lund, Thompson, & Jefferis, 1989). A couple of mutations that have been identified that diminish binding to Fc γ RIIA and Fc γ RIIB, such as R292A and K414A (R. L. Shields et al., 2001) could improve the therapeutic antibody effect through selective reduction of binding to the inhibitory Fc γ RIIB while retaining binding to the activating Fc γ RIIIA. Insight into why these mutations may be effective came from data showing that a blocking antibody that binds Fc γ RIIB synergized with anti-CD20 antibodies *in vivo* (Roghani et al., 2015), and that a SHIP-1 inhibitor diminished Fc γ RIIB signaling to increase ADCC/ADCCP (Burgess et al., 2017). Another example is the addition of an A330L mutation to a S239D/I332E Fc variant that abolished CDC while retaining enhanced ADCC

(Lazar et al., 2006). Known polymorphisms in Fc receptors, including Fc γ RIIA 131H and Fc γ RIIA 158V with their higher affinity for IgG1 Fc correlate with better therapeutic outcomes (Cartron et al., 2002; Ferris, Jaffee, & Ferrone, 2010; Musolino et al., 2008; Yin, Albers, Smith, Riddell, & Richards, 2018), recently reviewed in (Mellor, Brown, Irving, Zalcborg, & Dobrovic, 2013).

1.3 Regulation of antibody effector functions

In addition to Fc domains and Fc receptors, other molecules can regulate ADCC, ADCP and CDC. Some cancer cells have intrinsic resistance to ADCC by their differential gene expression. This includes c-Abl (Murray et al., 2014), WEE1 kinase (Friedman et al., 2018), caveolin-1 (Sekhar et al., 2013) and oncogenic RAS (Kasper et al., 2013; Nakadate et al., 2014). Others have alterations in apoptotic factors including overexpression of survivin, Bcl-X_L and YY1 that correlate with resistance to antibody-mediated effects (Dalle et al., 2009; Haart et al., 2016). CD74 overexpression, increased histone- and interferon-related gene expression, and downregulation of HSPB1 and target antigen expression have also been found in ADCC-resistant cells (Aldeghaither et al., 2019). Surface expression of cell adhesion molecules is decreased in resistant cells and hyaluronan, an extracellular matrix protein, is overexpressed, suggesting the importance of a close synapse between effector and target cells (Aldeghaither et al., 2019; Singha et al., 2015). Similarly, disruption of E-cadherins increases susceptibility to ADCC through its disruption of tumor cell-tumor cell contacts (Green, Karlsson, Ravetch, & Kerbel, 2002). Other cancer cells express NK inhibitory ligands, such as HLA-E and HLA-G that could contribute to ADCC resistance (Diepstra et al., 2008; Levy et al., 2009; Lin et al., 2007). Finally, expression and cell-surface distribution of molecules that interact with the target (such as MUC1 and MUC4 with HER2 or EGFR) has been shown to cause resistance to ADCC (Aldeghaither et al., 2019; Mercogliano et al., 2017; Namba et al., 2019).

Some tumor cells upregulate CD47, the “don’t eat me signal,” which can lower ADCP by binding to SIRP α on macrophages (Chao et al., 2010). Adding blocking anti-CD47 antibodies increases phagocytosis *in vitro* and *in vivo* (Chao et al., 2010). Reduced ADCC/ADCP is also seen in macrophages with increased signaling through the inhibitory Fc γ RIIB (Burgess et al., 2017). Elevated amounts of immune complexes, as can occur during chronic viral infections and in autoimmunity, have been shown to inhibit Fc-mediated functions (Ahuja et al., 2011; Wieland et al., 2015; Yamada et al., 2015).

Increased production by tumor cells of complement regulatory factors including CD46, CD55 (decay-accelerating factor, DAF), CD59 and Factor H may limit tumor cells’ susceptibility to CDC (reviewed in Gancz & Fishelson, 2009). These factors may also be regulated by the tumor microenvironment (Kesselring et al., 2014). Downregulating these factors with siRNAs or blocking their function with antagonistic antibodies has been shown to restore CDC (Geis et al., 2010; Zell et al., 2007). Other mechanisms of CDC resistance involve increased anti-apoptotic factors (Dalle et al., 2009; Hussain et al., 2007), changes in sialylation in target cells (Bordron et al., 2018) and increased levels of heat shock proteins (Fishelson, Hochman, Greene, & Eisenberg, 2001). Low pH in the tumor microenvironment may also contribute to resistance to CDC (Dantas et al., 2016). The levels of surface antigen

expression and the epitope's proximity to the cell surface also affect CDC, with lower antigen levels and more distal epitopes being less favorable (Cleary, Chan, James, Glennie, & Cragg, 2017; J. Golay et al., 2001; Loeff et al., 2017; Moreno et al., 2019; Ragupathi et al., 2005).

On the effector cell side, STING agonists have been shown to increase Fc gamma receptor expression on NK cells (Lekh N. Dahal et al., 2017). Similarly, TLR agonists, NKGD ligands and cytokines GM-CSF, G-CSF, IFN- α , IL-2, IL-12, IL-15 and IL-21 that activate effector cells can increase FcR expression (Cornet et al., 2016; L. N. Dahal, Gadd, Edwards, Cragg, & Beers, 2018; Shah et al., 2013). The addition of blocking antibodies for inhibitory receptors or agonistic antibodies to activating receptors on effector cells can augment effector functions (reviewed in Kohrt et al., 2012). Finally, effector cells may be exhausted by high circulating levels of therapeutic antibody, thus requiring dose and schedule optimization (Kennedy et al., 2004; Lindorfer et al., 2012).

2. Antibody-dependent cellular cytotoxicity (ADCC)

ADCC is mediated by the Fc-regions of antibodies bound to their cognate antigens on the target cells binding to activating Fc receptors on effector cells, such as NK cells and macrophages. Thus activated, effector cells release cytotoxic granules containing perforins and granzymes as well as cytokines such as IFN γ , that can kill the target cells. ADCC is primarily mediated by IgG1 and IgG3 antibodies that bind to Fc γ RIIA and IIIB. IgG3 has been shown to have higher affinity for activating Fc γ Rs than IgG1 but a shorter half-life *in vivo*. Most FDA-approved therapeutic antibodies are of the IgG1 class.

2.1 Experimental Protocol for ADCC

2.1.1 Reagents

- The medium used in all procedures is “complete RPMI:” RPMI-1640 with L-Glutamine (Corning, 10-040CV) supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin-streptomycin and 10% fetal bovine serum.
- Lymphocyte Separation Medium (LSM) gradient (Ficoll) (MP Biomedicals)
- Red Blood Cell Lysis buffer (Sigma)
- Dimethyl Sulfoxide (if using cryopreserved PBMCs)
- 24-or 48-well flat-bottom tissue culture plates
- 96-well round-bottom tissue-culture plates
- Items for magnetically enriching NK cells, including magnets, magnetic columns (LS columns, Miltenyi), antibodies, and magnetic particles
- 1X Phosphate Buffered Saline
- Buffer for enriching cells, “MACS buffer”, 0.5% Bovine Serum Albumin (BSA) in 1X PBS with 2 mM EDTA

- Buffer for staining cells, “FACS buffer” (1% BSA in 1X PBS)
- rhIL-2 (Miltenyi)
- CellTrace Yellow (ThermoFisher) or another amine-labeling cell dye, such as carboxyfluorescein succinimidyl ester (CFSE)
- Ghost Dye Red 780 (Ghost 780, Tonbo Biosciences) or another cell viability dye, such as 7-Aminoactinomycin D (7-AAD) or propidium iodide (PI)

2.2.2 Assay Procedure and Analysis

2.2.2.1 Isolation and culture of NK cells from PBMCs

1. To isolate PBMCs from buffy coats, add 15 ml of Lymphocyte Separation Medium (LSM) gradient (Ficoll) to the bottom of a 50 mL tube and carefully and slowly overlay 35 mL of buffy coat cells on top either dropwise or slowly letting liquid run down the side of the tube so as to not disturb the interface.
2. Centrifuge at $400 \times g$ for 30 min at room temperature with no acceleration and no brake in order to preserve the interface between the two liquid densities.
3. Discard some of the volume on top of the interface leaving about 10 mL and gently remove cells from the interface with a pipette into a new tube, trying to avoid taking the Ficoll. Red blood cells pellet to the bottom of the tube.
4. Wash the cells with 40 mL 1X PBS by centrifuging at $400 \times g$ for 15 minutes with full acceleration and brake.
5. Resuspend in 2 mL of hypotonic buffer, such as ACK lysis buffer or Red Blood Cell Lysis buffer (Sigma) for 5 minutes at room temperature to get rid of the few contaminating red blood cells that may have come along during isolation of cells at the interface.
6. Wash the cells two more times with 40 mL 1X PBS at $300 \times g$ for 10 minutes.
7. Aspirate supernatant and resuspend pellet in 5 mL 1X PBS.
8. Count the cells in a hemacytometer at 40X magnification.
9. Proceed to NK cell isolation. The data shown in Figure 2 used Miltenyi’s protocol for magnetic isolation of NK cells through negative enrichment on Miltenyi LS magnetic columns. Other enrichment protocols can also be used (Cosentino & Cathcart, 1987; Dittel, 2010; Shore, Melewicz, & Gordon, 1977), although the viability and effector function of NK cells post-enrichment was found to be better with magnetic enrichment (Hietanen, Pitkänen, Kapanen, & Kellokumpu-Lehtinen, 2016).
10. Resuspend the desired number of cells in MACS buffer (0.5% BSA in 1X PBS + 2 mM EDTA), 400 μ L per 100 million cells. Add 100 μ L of NK-cell biotin antibody cocktail and incubate at 4 °C for 5 minutes.
11. Add an additional 300 μ L of MACs buffer and 200 μ L of NK cell microbead cocktail per 100 million cells and incubate for 10 minutes at 4 °C.

12. During this incubation prime the LS column with 3 mL of MACS buffer, which can be collected in a waste container or in a 15 mL tube labeled “bound fraction.”
13. Switch collection tubes. Place a new tube labeled “unbound fraction” underneath the column.
14. Add the 1 mL of sample to the column, collecting the flowthrough.
15. Add an additional 3 mL of MACS buffer to wash the column.
16. If the leftover cells are needed, switch back to the “bound cells” collection tube. Remove the column from the magnet, placing it at the top of the 15 mL tube. Add 5 mL of MACS buffer and quickly flush the liquid through the column with the accompanying plunger.
17. Count the cells in the “unbound” fraction and multiply the cells/mL by the total volume, in this case 4 mL for 100 million starting PBMCs.
18. Before centrifuging the cells, take a 50 μ L aliquot to check enrichment purity by flow cytometry, using fluorescently conjugated antibodies, anti-human CD56 and anti-human CD16 (Fc γ RIIIA). Compare these percentages to those in pre-enrichment or “unbound” fraction samples.
19. Centrifuge the cells at $300 \times g$ for 5 min, discard supernatant and resuspend pellet in complete RPMI supplemented with 100 IU/mL of rhIL-2 at 5 million cells/mL. Plate cells as close to 2 million/cm² as possible. Typically a 48-well or a 24-well plate offers the best surface-area to volume flexibility. Keep overnight in a 5% CO₂ incubator.

If fresh PBMCs are not available for NK cell isolation, frozen PBMCs may be used. We have successfully used PBMCs cryopreserved in 10% DMSO and 90% FBS at 50 million cells /mL for NK cell isolation. After overnight culture in 100 IU/mL rhIL-2, these thawed NK cells perform well in the assay.

2.2.2.2 Preparing effector cells and labeling target cells

1. Count the NK cells after overnight incubation, as this number will determine how many wells can be set up in the assay. The number of NK cells is usually the limiting factor, as they are used in ratios of 20:1 or 10:1 to target cells. If cell numbers allow, duplicate or triplicate wells are set up for each condition. After determining an optimal cell ratio and antibody concentration, multiple serial dilutions may not always be necessary, but for initial experiments, especially with new antibodies, they can be quite useful.
2. Count the target cells. In our experience, the assay can be reliably performed with as few as 5000 target cells/well. Take at least twice the number of cells needed for the assay because some will be lost during washes. For the experiment in Figure 2 we used the human lymphoma cell line Raji that grows in suspension. If using adherent target cells, trypsinize and wash the cells before counting.

3. If labeling 10 million target cells or fewer, wash cells once in 10 mL of 1X PBS and resuspend in 500 μ L of 1X PBS. The CellTrace dyes bind free amines. Therefore, the cells need to be in a solution without free proteins such as FBS or BSA. Make up a second tube with 500 μ L of 1X PBS and add 0.2 μ L of CellTrace Yellow, constituting a “2X”, 2 μ M solution. Add this to the cells with rapid mixing or vortexing to promote even labeling in a final 1 μ M concentration solution of CellTrace. A 10 μ M concentration is commonly used for labeling for cell division tracking and can also be used here if no increased toxicity from the higher concentration of dye is noted.
4. Incubate the cells for 4 minutes in the dark in a 37°C water bath. Gently mix the cells by flicking or pulse vortex them to continue promoting even labeling.
5. Incubate the cells for another 4 minutes. IMPORTANTLY, incubation past 8–9 minutes total will result in significant cell death and should be avoided.
6. At the end of the labeling incubation, immediately quench the reaction by adding 10 mL of pre-warmed complete RPMI containing at least 10% FBS and incubate the cells for 5 minutes in the 37°C water bath. Then centrifuge the cells at 300 $\times$ g for 5 minutes.
7. Discard the supernatant and resuspend the cells in 0.5 mL of complete RPMI and count. Resuspend the cells such that the desired number can be added to each well of a 96-well plate in 50 μ L.

2.2.2.3 Setting up the co-incubation assay

1. Make up antibody dilutions as “4X” concentrations, for adding 50 μ L/well. For the example experiment, a 40 μ g/mL or 10 μ g/mL final concentration was used. This concentration has been shown to be effective for many antibodies, but a titration of lower antibody concentrations can be useful to find optimal effective dilutions, allowing for potential conservation of antibodies if lower concentrations retain high efficacy. Furthermore, a titration curve may reveal additional information about each antibody and its immune effector capabilities (Tóth, Szöllősi, & Vereb, 2017).
2. Aliquot labeled target cells into a 96-well round bottom plate. Round bottom plates allow for higher cell densities in the center, and more efficient interaction between effectors and targets than flat bottom plates.
3. Aliquot antibodies into the 96-well plates. Incubate the plate at room temperature for 15 minutes to allow antibodies to bind to the target cells before adding NK cells.
4. Add NK cells in 100 μ L/well. If interested in determining if the antibodies being tested have any direct cytotoxicity, leave one condition without NK cells.
5. Incubate the plate for 4 hours at 37°C in a 5% CO₂ incubator.

2.2.2.4 Measuring cell viability

1. Centrifuge the plate for a 30 sec pulse spin in a table-top centrifuge. If pulse spinning is unavailable as an option, spin at $300 \times g$ for 5 minutes.
2. Invert the plate rapidly to remove supernatant.
3. Wash the cells with 200 μ L/well of FACS buffer to remove residual unbound antibodies. Centrifuge the plate again as in Step 1 and afterwards invert the plate to remove supernatant.
4. Resuspend the pelleted cells in their residual volume by racking the bottom of the plate and add 60 μ L /well of 1:1000 Ghost Dye Red 780 in 1X PBS, or similar cell viability dye. If using this dye, it must be diluted in PBS as it is amine-reactive and needs to be in a buffer without free proteins in order to bind efficiently to cells. The Ghost dye will lightly label membrane proteins on live cells, but it will have access to all cytoplasmic proteins in dead cells, resulting in much greater labeling of dead cells.
5. For Ghost dye staining, incubate the plate at 4°C for 15 minutes. After this incubation, add 140 μ L of FACS buffer/well to bring up the volume to 200 μ L/well, and pulse centrifuge.
6. OPTIONAL: To determine the amount of remaining primary antibody bound to the surface of cells at the end of the assay, a fluorescently-conjugated secondary antibody recognizing the human IgG Fc domain can be used. This is another advantage that the flow cytometry-based assay affords over radiographic or other release-based protocols such as calcein dye or lactate dehydrogenase (LDH).
7. Resuspend the cells in the desired volume and proceed to analysis on a flow cytometer. Remember to include controls for setting up the voltages and determining all channels are working properly. At a minimum this includes unlabeled target cells, unlabeled effector cells, CellTrace labeled target cells and Ghost dye 780 labeled target cells. If also determining the NK cell purity post-enrichment and overnight culture, include single color controls for each of the markers used for that analysis. It is important to keep all samples on ice and to run individual replicates first before running duplicates or triplicates of the same condition to prevent any unwanted variation in the data during sample acquisition.
8. While we cannot provide a complete protocol for flow cytometry analysis, we can provide some helpful tips for users familiar with this analysis. Voltages for FSC-A and SSC-A need to be set such that both effector cells and target cells are visible in the same panel. Smaller, dead target cells should still be above the FSC-A threshold and visible on the graph. If the target cells are much bigger than NK cells, as can be the case for some epithelial tumor cell lines, it is better to have the target cells centered since the final analysis will be on the target cell viability. Although most first gates are made on a plot of FSC-A by SSC-A, much better resolution of target cells can be achieved if gating first on a FSC-A by CellTrace plot (see Figure 2A). Note, we typically do not include in this first

gate of target cells those that appear small by FSC-A and very CellTrace⁺ as these cells were dead before CellTrace labeling rather than in the co-incubation assay. The next gate is on single cells, to remove doublets from the analysis, and the final gate is on the Ghost 780⁺ cells. Therefore the “%Dead” values in Figure 2B represent the percentages of Ghost 780⁺ cells of single target cells that were killed during the co-incubation.

9. The background killing by NK cells in the no antibody (no ab) control wells can be subtracted from the ones with antibody when reporting the extent of ADCC. In Figure 2 they are shown separately to emphasize the similarity to the control IgG1 group.

3. Antibody-dependent cellular phagocytosis (ADCP)

ADCP occurs though the Fc-domain of an antibody binding to and activating FcRs on effector cells such as macrophages and dendritic cells that then engulf target cells. The importance of this antibody-mediated function in cancer control has only recently been appreciated (reviewed in Gül & van Egmond, 2015). For example, an *in vivo* study of alemtuzumab (anti-CD52, CAMPATH) in human CD52-Tg mice showed that the major mechanisms of action were through ADCC and ADCP, not through CDC, as was previously thought from *in vitro* studies (Hu et al., 2009). Intravital imaging revealed ADCP by liver Kupffer cells could mediate a substantial amount of depletion of CD20-expressing B cells in the presence of rituximab (Grandjean et al., 2016), while increased slan⁺ monocytes were shown to correlate with better rituximab-mediated ADCP against diffuse large B-cell lymphoma (DLBCL) (Vermi et al., 2018). Phagocytosis has also been shown to be the dominant mechanism for anti-CD20 depletion *in vivo* (Burgess et al., 2017; Uchida et al., 2004) and an important therapeutic mechanism for trastuzumab, daratumumab (anti-CD38) and elotuzumab (anti-SLAMF7) (Kurdi et al., 2018; Overdijk et al., 2015; Shi et al., 2015; Yin et al., 2018). A key clue pointing to the role of phagocytic cells in these monoclonal antibody therapies is the requirement for and modulation by certain Fc receptors expressed primarily on these cell types (Clynes, Towers, Presta, & Ravetch, 2000; Minard-Colin et al., 2008).

3.1 Experimental Protocol for ADCP

3.1.1 Reagents—Complete RPMI

1X PBS

FACS buffer, 1% BSA in 1X PBS

CellTrace Yellow, or another amine-reactive cell labeling dye

CellTrace Violet, or another amine-reactive cell labeling dye in a second color

Ghost 780, or another cell viability dye, such as 7AAD or PI

Target Cells

Effector cells, here THP-1 cells, a human macrophage/monocyte cell line. As stocks of THP-1 cells can sometimes vary between laboratories, primary monocyte-derived macrophages can also be differentiated from human PBMCs with M-CSF and used as effectors (Herter et al., 2014).

3.1.2 Assay procedure and analysis

3.1.2.1 Labeling effector cells and target cells

1. Count THP-1 cells. If cell number allows, duplicate or triplicate wells are set up for each condition.
2. Count the target cells. Take at least twice the number of cells needed for the assay, because some cells will be lost throughout washes. In ADCP assays target cell number is usually the limiting factor as they are used at up to 5 times the number of effector THP-1 cells. For the example in Figure 3, we used Raji cells that grow in suspension. If using adherent cells, trypsinize and wash the cells before counting. In our experience, the assay can be reliably performed with as few as 10,000 THP-1 cells and 10,000 target cells at a 1:1 ratio.
3. Follow Steps 3–7 of the ADCC cell labeling protocol. Use two separate colors, one for targets and one for effectors. In Figure 3, CellTrace Yellow (on the PE channel) and CellTrace Violet (on the Pacific Blue channel) were used.
4. In addition to resuspending the target cells in a concentration where they can be added at 50 μ L/well, resuspend the effector THP-1 cells at a concentration such that they can be added to the plate in 100 μ L /well.

3.1.2.2 Setting up the co-incubation assay

1. Follow steps 1–3 of the ADCC co-incubation assay setup protocol for labeling and plating target cells with antibodies.
2. Add the THP-1 cells in 100 μ L/well.
3. Incubate the plate for 1 hour at 37°C in a 5% CO₂ incubator.

3.1.2.3 Measuring phagocytosis

1. Centrifuge the plate (a 30 sec pulse spin in a table-top centrifuge is sufficient).
2. Invert the plate rapidly only once to remove supernatant.
3. Wash the cells with 200 μ L/well of FACS buffer to remove residual unbound antibodies. Centrifuge the plate again as in Step 1. Afterwards, invert the plate to remove supernatant.
4. Resuspend the cells in the desired volume, place all of the samples on ice and proceed to analysis on a flow cytometer. Remember to bring along controls for setting up the voltages and determining all channels are working properly, at a minimum: unlabeled target cells, unlabeled effector cells, and CellTrace-labeled target cells and effector cells by themselves.

5. Set the voltages for FSC-A and SSC-A ideally such that both effectors and targets are visible in the same panel. If one cell type is much larger than the other, as can be the case for some epithelial cell lines, it is better here to have the THP-1 cells centered and the target cells perhaps out of range, since the final analysis will be on the percentages of THP-1 cells that took up target cells.
6. Here the first gates are made on a plot of FSC-A by SSC-A. The next plots look at CellTrace Violet (THP-1 cells) by CellTrace Yellow (target cells), in order to gate only on all of the THP-1 cells. The final gate takes the percentage of CellTrace Yellow⁺ THP-1 cells, which represent either entire target cells that were phagocytosed or cell conjugates. Because flow cytometry alone cannot distinguish this (microscopy and flow-based microscopy can), in single THP-1 cells the MFI of CellTrace Yellow is also reported as a readout of uptake of target cell fragments. These values are reported in Figure 3B. A very recent paper used a pH-sensitive dye to label target cells, so that it only fluoresced when the target cell was internalized in the phagocyte (Kamen et al., 2019). Others have used antibodies against additional cell surface proteins on target cells to identify phagocytosed targets as those lacking this additional staining, versus cell conjugates which would stain positively with the added target cell marker (Herter et al., 2014).

4. Complement-dependent cytotoxicity

CDC occurs when antibodies array themselves around a target antigen on the cell surface in an orientation that allows for C1q binding, clustering and initiation of the complement cascade (Taylor & Lindorfer, 2016). There are many components of the complement cascade that get recruited to ultimately form a membrane attack complex, which forms a pore in the cell membrane, killing the cell. To prevent spontaneous complement-mediated cytotoxicity on normal host cells, there are several complement regulatory factors that can inhibit the pathway.

4.1 Experimental Protocol for CDC

4.1.1 Reagents—Complete RPMI with and without FBS (serum-free), see ADCC section

Non-heat inactivated human AB serum

1X PBS

FACS buffer: 1% BSA in 1X PBS

Ghost 780 dye, or another cell viability dye

Target cells of interest

Antibodies of interest

4.1.2 Assay procedure and analysis

4.1.2.1 Preparing the target cells

1. Count the numbers of target cells, as here this number will determine how many wells can be set up in the assay.
2. Centrifuge the cells at $300 \times g$ and resuspend the cells in *serum-free* media, at a concentration of 50 μL cells/ well. We have been able to use as few as 5000 cells/ well.

4.1.2.2 Setting up the co-incubation assay

- 3 Make up antibody dilutions as “4X” concentrations in *serum-free* media, for adding 50 μL /well. For the example experiment, a 40 $\mu\text{g}/\text{mL}$ or 10 $\mu\text{g}/\text{mL}$ final concentration was used. As with the other assays, a titration curve may be useful.
- 4 Aliquot labeled target cells into a 96-well round bottom plate.
- 5 Aliquot antibodies into the 96-well plates. Incubate the plate at room temperature for 15 minutes to allow the antibodies to pre-bind to the target cells before adding the serum.
- 6 Add 30% human AB serum in 100 μL /well, thus the final concentration will be 15% human serum. Be careful to keep the serum at 4°C or on ice up until its addition to cells. Complement factors are heat-labile and can lose activity. Even with constant 4°C storage, we have noticed loss in activity over several weeks. It is best to carefully thaw the serum and aliquot it into smaller vials that can be refrozen once and thawed just prior to the experiment. To further show it is complement-mediated killing, a separate aliquot of serum can be heat-inactivated at 56°C for 30 minutes, and after it has cooled, added to the cells as an extra control.
- 7 Incubate the plate for 30 minutes at 37°C in a 5% CO_2 incubator.

4.1.2.3 Measuring cell viability

1. Follow steps 1–5 of the ADCC cell viability measurement protocol.
2. Resuspend the cells in the desired volume and proceed to analysis on a flow cytometer. If desired, one could also measure surface bound C1q and C4b by flow cytometry (Broyer, Goetsch, & Broussas, 2013). Remember to bring along controls for setting up the voltages and determining all channels are working properly, at a minimum: unlabeled target cells and Ghost dye 780 labeled target cells. It is important to keep all samples on ice and to run individual replicates first before running duplicates or triplicates of the same condition to prevent any unwanted variation in the data from coming through during sample acquisition.
3. For the analysis shown in Figure 4, the first gates are made on a plot of FSC-A by SSC-A, including both the normal lymphocyte gate and smaller cells that are likely dead. The second gate uses FSC-H and FSC-W to select single cells, and

the final gate determines the percentages of cells that are dead (Ghost 780⁺). Therefore the “%Dead” values in Figure 4B represent the percentages of Ghost 780⁺ cells of single target cells. Due to the potential that dead cells could be fractionated into multiple smaller cell fragments that may or may not retain the ability to stain positively with the viability dye, an alternative analysis can focus on live cells to avoid any potential inflation or deflation of dead cell percentages. In this way, the starting numbers of live cells plated per well would be taken into account and normalization to no antibody or to heat-inactivated controls could be used. To quantify numbers of live cells accurately by flow cytometry, counting beads can be spiked into each well to account for any differences in flow rate during sample acquisition. As this method requires calculations across wells, particular care must be taken to plate equivalent numbers of cells in each well and acquire samples at a uniform speed. Some target cell lines may be more susceptible to fragmentation than others. For the example in Figure 4B, the percentages of CDC (%dead cells vs %reduction of live cells from no ab controls) is 13.4, 13.5 and 58.8% versus 0, 6.1, and 53.5% for no ab, control IgG and rituximab conditions respectively.

Concluding Remarks

We described here our specific flow cytometry-based methods for determining three separate antibody immune effector functions: ADCC, ADCP and CDC. Each of these methods utilizes fluorescent molecules to distinguish target cells from effector cells and to measure cell viability as indicator of efficiency of tumor killing. Several other groups have recently published variations on these experimental protocols (Alrubayyi et al., 2018; Carter et al., 2019; González-González et al., 2019; Kamen et al., 2019; Tanaka et al., 2019; van der Haar Àvila, Marmol, Cany, Kiessling, & Pico de Coaña, 2019; Yamashita et al., 2016). These techniques have several advantages over other methods, including the ability to simultaneously measure the amount of target antibody bound to the cells and cell viability. Importantly, these experiments avoid the use of radioactivity. The assays are reliable and sensitive even if the number of target cells are limiting. As more anti-cancer therapeutic antibodies are developed, fast, reproducible assays such as these will help identify the candidates with the greatest likelihood of therapeutic efficacy.

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Figure 1: Antibodies mediate immune effector functions through their Fc domains.

Antibodies bound to their antigens on target cells with their Fab domains and that also bind through their Fc domains, activating FcγRs on NK cells (such as FcγRIIIA) can mediate antibody-dependent cellular cytotoxicity (ADCC). Antibodies that opsonize target cells and also bind to activating FcγRs on macrophages can mediate antibody-dependent cellular phagocytosis (ADCP), in which the target cell is then engulfed by the macrophage. Antibodies that bind C1q in a set arrangement can facilitate activation of the complement cascade, leading to the formation of the membrane attack complex (C5-C9, the MAC), and complement-dependent cytotoxicity (CDC).

Figure 2: Assessment of ADCC by flow cytometry.

A. Representative gating strategy, showing CellTrace Yellow-labeled target cells alone and human NK cells on the left and two examples of gating co-incubated samples, 10:1 Effector:Target (E:T) cell ratio when incubated with control IgG1 mAb (Herceptin, top right) or rituximab (bottom right three panels). **B.** Bar graph depicting the percentages of dead cells (% Ghost 780⁺) in each condition. Two-way ANOVA with Bonferroni correction was used to determine statistical significance, ***p<0.001.

Figure 3: Measurement of ADCP by flow cytometry.

A. Representative gating strategy, showing CellTrace Yellow (CTY)-labeled target Raji cells alone and CellTrace Violet (CTV)-labeled THP-1 macrophages on the left and two examples of gating co-incubated samples, 1:1 Effector:Target (E:T) cell ratio when incubated with control IgG1 mAb (Herceptin, top right) or rituximab (bottom right three panels). **B.** Bar graphs depicting the percentages of CellTrace Violet⁺ THP-1 cells (top) in each condition, and also showing the relative mean fluorescent intensities of CellTrace Violet^{low} THP-1 cells for each condition. The MFIs from each group were normalized to the no ab control, which was set to 1. The data were analyzed by Two-way ANOVA with Bonferroni correction to determine statistical significance, **p<0.01; ***p<0.001.

Figure 4: Determination of CDC by flow cytometry.

A. Representative gating strategy, showing two examples of gating samples incubated with 15% human AB serum for 30 minutes and control IgG1 mAb (Herceptin, top) or rituximab (bottom three panels). **B.** Bar graph showing the percentages of dead cells (% Ghost 780⁺) in each condition. One-way ANOVA with Bonferroni correction was used to determine statistical significance, *** $p < 0.001$.