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Technical Note

A novel method for determining antibody-dependent cellular phagocytosis

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ABSTRACT

Antibody-based therapeutics are powerful tools to treat disease. While their mechanism of action (MOA) always involves binding to a specific target via the Fab region of the antibody, the induction of effector functions through the Fc region of the antibody is equally important for antibody therapeutics designed to deplete tumor cells. By binding of the Fc region to Fc gamma receptors (FcγRs) on the surface of immune cells or complement factors, antibody therapeutics exert effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), both of which induce target cell death and aid in the efficacy of treatment. Another major Fc effector function is antibody-dependent cellular phagocytosis (ADCP). ADCP is the mechanism by which antibody-opsonized target cells activate the FcγRs on the surface of macrophages to induce phagocytosis, resulting in internalization and degradation of the target cell through phagosome acidification. ADCP has been implicated as a major MOA of several biologics, but this activity is difficult to measure in in vitro. Most assays measure the association of target cells and macrophages; however, co-localization can represent cell attachment rather than internalization. Here, we describe the development of a novel method to accurately measure ADCP activity. By labeling target cells with a pH sensitive dye that only fluoresces in mature phagosomes, the ADCP activity of antibody therapeutics can be accurately quantitated via flow cytometry.

1. Introduction

Immune system activation by administration of targeted antibodies has been an effective therapeutic strategy to kill cancer cells. CD20 on B lymphoma cells was one of the initial targets for cancer immune therapy (Oflazoglu and Audoly, 2010). Preclinical studies showed that rituximab, the first anti-CD20 therapy to be approved (Oflazoglu and Audoly, 2010), induced B cell depletion by activating the immune system. The Fc region of rituximab can bind to Fcγ receptors (FcγRs) on immune cells. Binding and crosslinking of the FcγRs results in immune cell activation leading to B cell lysis through antibody-dependent cellular cytotoxicity (ADCC) (Reff et al., 1994). FcγR engagement can also lead to internalization and lysis of target cells via antibody-dependent cellular phagocytosis (ADCP).

As in ADCC, antibody-opsonized target cells are recognized by FcγR-expressing immune cells. In phagocytosis, however, the primary immune cell involved is the macrophage (Weiskopf and Weissman, 2015). Crosslinking of the Fcγ receptors on macrophages causes activation of intracellular signaling pathways, resulting in uptake of the target cell. The phagosome then proceeds along a highly regulated maturation pathway characterized by expression of different membrane markers, such as Rab5 and LAMP-1, as well as a gradual acidification of the phagosome until it fuses with the lysosome, ultimately degradation of the internalized cell (Levin et al., 2016).

While FcγR-mediated phagocytosis evolved as a mechanism to deal with infection, it can also provide a crucial therapeutic function in cancer treatment. Macrophages can employ antibody-dependent cellular phagocytosis (ADCP) to deplete tumor cells by internalizing and lysing them. Previous work has shown that a number of monoclonal antibodies, including antibodies targeting tumor antigens such as HER2, Ep-CAM, CD40, CD30, and MUC-1, induce phagocytosis in vitro (Bakema and van Egmond, 2014). Therefore, this mechanism is thought to be a major MOA of antibody-mediated clearance of tumor cells in vivo (Bakema and van Egmond, 2014). The most significant evidence demonstrating the importance of macrophages in Fc effector function was demonstrated in a mouse model with B cells expressing human

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Table 1
Staining reagents for macrophages.

Reagent	Target	Description	Source
CD16 V450	FcγRIII	V450 Mouse anti-human CD16	BD Biosciences (San Jose, CA)
CD32 PE	FcγRII	PE Mouse anti-human CD32	BD Biosciences
CD64 PE-Cy7	FcγRI	PE-Cy7 mouse anti-human CD64	BD Biosciences
CD14 APC	CD14	APC mouse anti-human CD14	Biolegend (San Diego, CA)

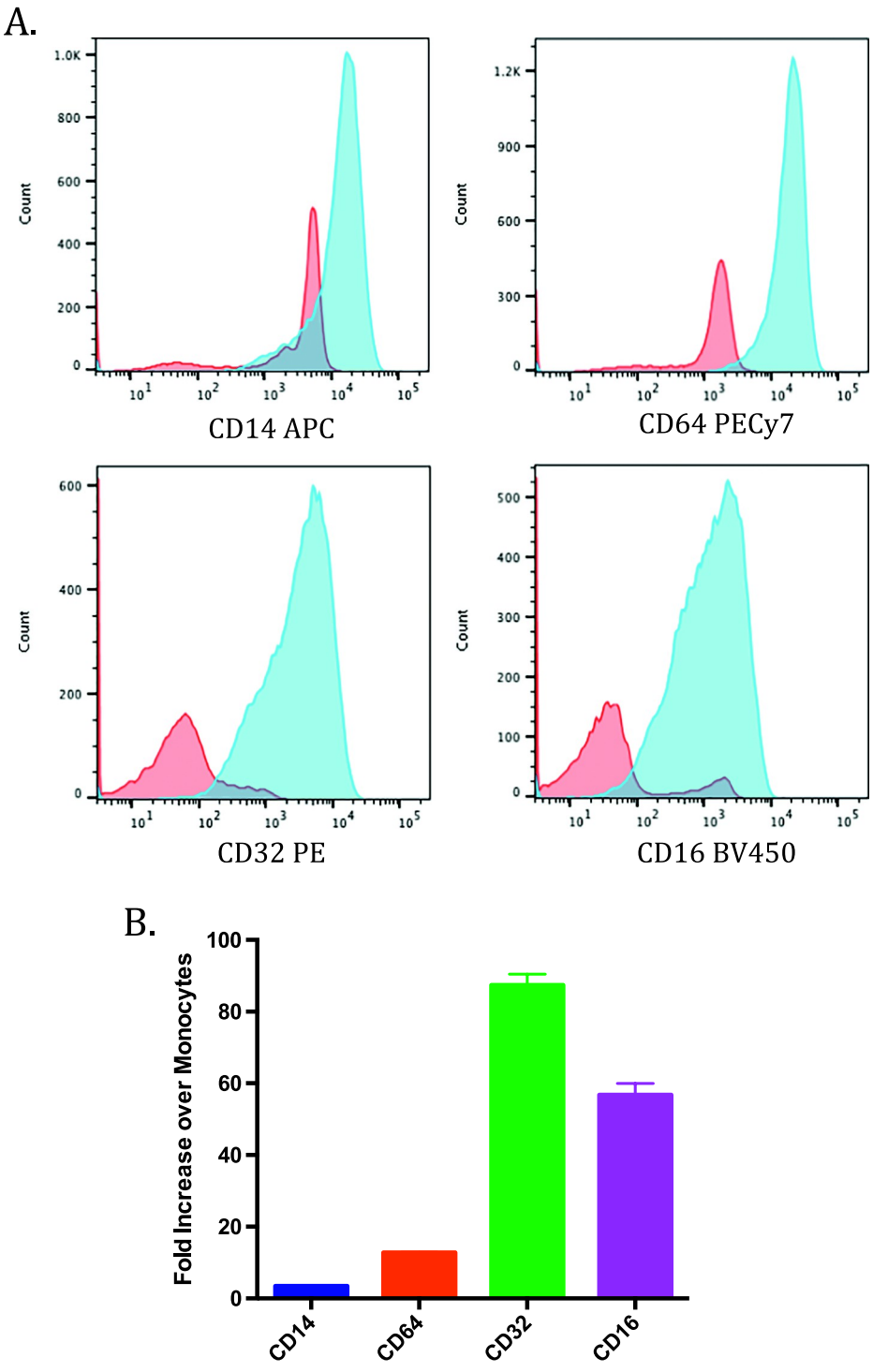


Fig. 1. FcγR expression on human monocytes and macrophages. Monocytes and in vitro differentiated macrophages were analyzed for expression of FcγRI, II, III and CD14. (A) The levels of expression on monocytes are shown in red, and the expression on macrophages are shown in blue. (B) The mean fluorescence intensity (MFI) for each macrophage marker was plotted as fold increase above the MFI on the control monocyte population. Data shown are a representative set taken from one of 2 donors analyzed for FcγR and CD14 expression on the monocytes and derived macrophages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CD20. When mice were depleted of macrophages, treatment with anti-CD20 no longer killed the circulating B cells, highlighting the importance of macrophage-mediated phagocytosis in IgG antibody-induced tumor cell depletion (Gong et al., 2005). Given the importance of phagocytosis upon the MOA of IgG therapeutics, it is important to have a robust in vitro assay that can accurately measure ADCP activity.

Table 2
Target cell viability. The viability of WIL2-S cells pre- and post-pHrodo labelling was measured on an automated cell counter. Shown is the average of n = 4 independent experiments.

	WIL2-S Percent Viability (pre-label)	WIL2-S Percent Viability (post-label)
Average	93.5	90.1
SEM	1.21	2.28

To quantify phagocytosis induced by antibody therapeutics, an assay must measure actual internalization of antibody-opsonized target cells. This assay could then be used to help screen candidate molecules in clinical candidate selection as well as evaluate critical quality attributes during later stage drug development. Therefore, it is necessary to develop a method that can not only differentiate bound versus internalized target cells, but also be run in a high-throughput assay design to generate dose-response curves for comparison across multiple antibodies. pH-sensitive rhodamine dyes have been used to measure the phagocytosis of bacterial particles (Miksa et al., 2009). The original formulation consisted of an activated succinimidyl ester that covalently

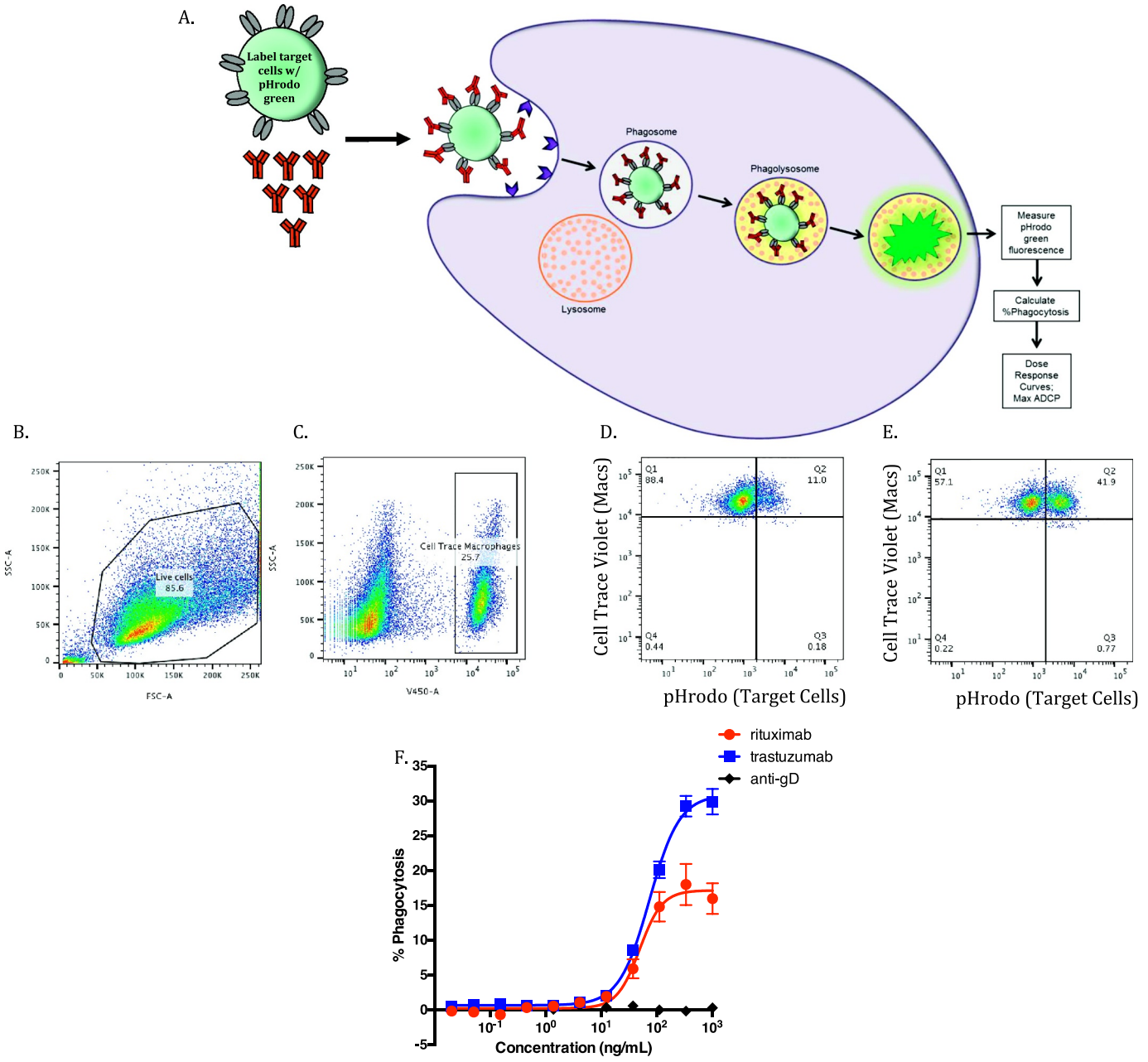
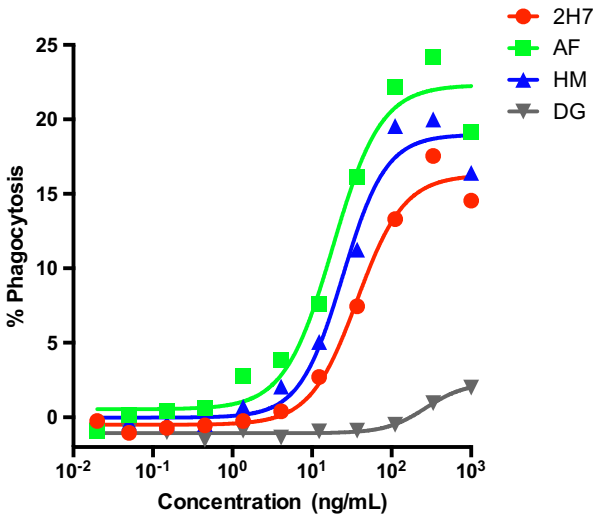


Fig. 2. Flow cytometry analysis to determine ADCP activity. (A) A schematic showing the binding and internalization of pHrodo-labeled antibody-opsonized target cells by Cell Trace Violet-labeled macrophages. As the phagosome containing the target cells becomes increasingly acidic, the pH rhodo green fluorescent signal increases, which can be detected on a flow cytometer. (B) The cells were gated based on size and granularity. (C) Macrophages were gated on Cell Trace Violet fluorescence. Phagocytosis was determined by increased pHrodo Green fluorescence. (D) Macrophages treated with control unopsonized target cells (SKBR3) were used to determine the baseline level of pHrodo fluorescence or ADCP activity. (E) Opsonized target cells (300 ng/mL trastuzumab) show increased pHrodo Green fluorescence. (F) The percentage phagocytosis for trastuzumab, rituximab, and anti-gD are shown. The data shown are pooled from two independent experiments. All data points were collected in duplicate, and the mean of the % phagocytosis was plotted against antibody concentration. Error bars represent standard error of the mean (SEM).



Glycosylation Variant	EC50 (ng/mL)
2H7	37.0
AF	18.2
HM	24.4
DG	N/A

Fig. 3. Glycosylation variants tested in ADCP assay. Ocrelizumab (2H7) variants with high mannose (HM), afucosylation (AF), or deglycosylation (DG) were tested on CD20-expressing target cells. The data shown are a representative set taken from one of four independent experiments. All data points were collected in duplicate, and the mean of the % phagocytosis was plotted against antibody concentration.

labels cellular proteins. These dyes fluoresce brightly in the acidic pH of the late phagosome and can serve as a marker of bacterial uptake. Newer versions of pHrodo dyes (e.g. pHrodo AM) have been modified with acetoxymethyl (AM) ester groups to facilitate entry and retention in live mammalian cells. These pH-sensitive dyes are able to label mammalian cells and serve as a marker of phagocytosis. In this report, we detail the development of a novel method of ADCP quantification using the pH-sensitive pHrodo AM. This assay was used to evaluate the phagocytic activity of differing test antibodies on target cells, as well as evaluate the impact of glycosylation upon phagocytosis.

2. Materials and methods

2.1. Reagents: test antibodies and cells

Rituximab and ocrelizumab are engineered mAbs that bind to human CD20 with different affinities and specificities. Rituximab is a chimeric IgG_1 antibody, while ocrelizumab is a humanized IgG_1 antibody. Trastuzumab is a humanized IgG_1 antibody that binds to human HER2. Anti-gD is a humanized anti-herpes simplex virus glycoprotein D IgG1 antibody that serves as a negative control. All four test antibodies are manufactured from engineered Chinese hamster ovary cell lines at Genentech (South San Francisco, CA).

Deglycosylated ocrelizumab was produced by incubating 1 mg of ocrelizumab with 275 units of PNGase F (New England Biolabs, Ipswich, MA) at 37 °C for 24 h and further purified through a protein A column. Afucosylated ocrelizumab was produced from a CHO cell line deficient in FUT8. The Man-5 glycoform of ocrelizumab was produced by addition of kifunensine (Cayman Chemical Company, Ann Arbor, MI) to the cell culture media at 5 mg/L as an α-mannosidase inhibitor to prevent removal of extra mannose molecules in the endogenous glycosylation pathway. The purified glycoforms at 10 mg/mL were then incubated with 20 mU/mL α-mannosidase I from *Aspergillus saitoi* (Prozyme, Hayward, CA) at 37 °C for 24 h to produce Man5 glycoforms in vitro.

WIL2-S, a human B lymphoblastoid cell line, was obtained from the American Type Culture Collection (Manassas, VA). WIL2-S cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Corning, Tewksbury, MA) supplemented with 10% fetal bovine serum (FBS: Hyclone, Logan, UT), 25 mM HEPES (Corning, Tewksbury, MA), 1% Glutamax (Gibco, Carlsbad, CA), and 1% penicillin/streptomycin (Gibco, Carlsbad, CA). SKBR-3, a human epithelial cell line derived from adenocarcinoma, was obtained from the American Type Culture Collection (Manassas, VA). SKBR-3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% Glutamax, and 1% penicillin/streptomycin.

Primary human macrophages were generated by isolating CD14-positive monocytes from heparinized human blood by negative selection (STEMCELL Technologies, Cambridge MA) and cultured in macrophage differentiation media (RPMI 1640, 10% FBS, 8% human serum (Sigma-Aldrich, St. Louis, MO), 20 mM HEPES, 1% Glutamax, 1% penicillin/streptomycin, and 50 ng/mL M-CSF (R&D Systems, Minneapolis, MN)). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 for 5–6 days.

2.2. Phenotyping FcγR expression on macrophages

To confirm macrophage differentiation, monocytes and the differentiated macrophages (from the same donor) were examined for FcγR and CD14 expression. Briefly, cells were resuspended in FACs buffer (PBS, 2% FBS, 0.02% sodium azide) at 1 million cells/mL and added at 100 µL/well in a 96-well U-bottom plate. The plate was centrifuged at 1400 rpm for 4 min at 4 °C. The supernatant was removed and cells were re-suspended and stained with FACs buffer containing antibodies to FcγRs and CD14 as detailed in Table 1. The cells were stained for 30 min and washed 3 times with FACS Buffer. After washing, the cells were fixed with 4% paraformaldehyde for 10 min. After fixing, the cells were washed, re-suspended in FACS Buffer and analyzed on a FACSCanto10 IVD flow cytometer (BD Biosciences; San Jose, CA). The fluorescence for each cell surface marker antibody was analyzed with Flow Jo software (Treestar; Ashland, OR).

2.3. Labeling macrophages with cell trace violet

Macrophages were harvested by washing the adherent differentiated cells in 10 mL of cold PBS, incubating for 10 min, and then gentle scraping to detach. Macrophages where counted using an automated cell counter that also calculated viability (Vi-CELL XR Cell Viability Analyzer, Beckman Coulter, Brea, CA). Macrophages were loaded with 10 µM Cell Trace Violet (Thermo Fisher Scientific, Eugene OR) as per the manufacturer's instructions. After labeling, macrophages were diluted in ADCP assay media (RPMI-1640, 10% FBS, 25 mM HEPES, 1% Glutamax, 1% penicillin/streptomycin) at 1×10^6 cells/mL and added at 50 µL/well 96 well low adherent U-bottom plate (Costar, Corning, NY).

2.4. Labeling target cells with pHrodo green AM

Target cells were diluted in ADCP assay media at 2×10^6 cells/mL and labeled with pHrodo (Thermo Fisher Scientific, Eugene, OR) as per

the manufacturer's instructions. After labeling, target cells were counted and assessed for viability as described above.

2.5. ADCP assay

pHrodo-labeled target cells were resuspended at 2.0×10^6 cells/mL and added (50 μL) at to the assay plate containing macrophages. 100 μL of test antibodies were added to the well and the cell/antibody suspension was thoroughly mixed. The assay plate was centrifuged at 700 rpm for 1 min to concentrate the cells at the bottom of the well. The plates were incubated for 4.5 h at 37 °C, 5% CO_2. After incubation, the cells were centrifuged at 1200 rpm for 5 min and washed in PBS before being fixed in 4% paraformaldehyde for 10 min at 4 °C.

The cells were analyzed with a flow cytometer (BD Biosciences FACSCanto IVD 10). Phagocytosis was analyzed via FlowJo. Cell Trace Violet fluorescence was used to gate the macrophages in the sample population. Phagoctyosis was determined by measuring the percentage of pHrodo Green positive macrophages. The degree of phagocytosis was normalized by subtracting the percent pHrodo Green positive macrophages from the control condition (no antibody present). Percent phagocytosis was plotted opposite antibody concentration and fitted to a four-parameter model using GraphPad Prism (LaJolla, CA).

3. Results

Tissue resident macrophages are the predominant cells that mediate ADCP (Weiskopf and Weissman, 2015). Macrophages express multiple FcγRs, including FcγRI, FcγRII, and FcγRIII (Weiskopf and Weissman, 2015). To generate surrogate effector cells that are able to undergo phagocytosis similar to tissue resident macrophages, human monocytes from peripheral blood were differentiated into macrophages through culture in macrophage colony stimulating factor (M-CSF) containing medium. The adherent macrophages were harvested on Day 5 of culture and analyzed for the expression of FcγRI, II, and III. As shown in Fig. 1, the in vitro-derived macrophages exhibited increased expression of all three FcγRs as compared to monocytes. Increased expression of the three FcγRs is in line with differentiation to macrophages and the potential to mediate ADCP.

Cells that undergo phagocytosis are contained in intracellular vesicles that become increasingly acidic. Taking advantage of both the unique pH of the phagosome and the pH sensitive property of pHrodo AM dyes, it was possible to develop a phagocytosis assay that measures only the internalized target cells and not those attached to the surface via non-specific binding or binding to FcγRs. pHrodo AM dyes are modified with AM ester groups, creating an uncharged molecule that can permeate cell membranes. These pHrodo AM dyes are able to label mammalian cells with minimal impact on cellular viability (Table 2). Labeling target cells with pHrodo AM allows for the specific detection of phagocytic events. Only target cells that are contained within phagosomes will fluoresce, making it a marker of true phagocytosis (Fig. 2A).

Phagocytosis was analyzed by flow cytometry. The gating strategy is shown in Fig. 2. HER2-expressing SKBR-3 cells were labeled with pHrodo Green AM and opsonized with trastuzumab. Target cells were added at a 2:1 target to effector cell ratio. The live cells were gated based on size and granularity (Fig. 2B) and macrophages were marked as Cell Trace Violet positive cells (Fig. 2C). Macrophages were then analyzed for pHrodo Green target cell fluorescence (Fig. 2D-E). As shown in Fig. 2D, macrophages treated with unopsonized SKBR3 cells showed little pHrodo Green fluorescence. However, macrophages treated with SKBR3 cells opsonized with trastuzumab had a detectable population of pHrodo green fluorescent macrophages (Fig. 2E). A macrophage that was positive for pHrodo Green fluorescence was counted as a macrophage that had undergone a phagocytic event. The percentage of phagocytic events in each sample was measured and normalized to the control (no antibody present) condition to calculate the % phagocytosis. The % phagocytosis was plotted against the antibody concentration to generate a dose-response curve (Fig. 2F). ADCP activity was detected with both rituximab and trastuzumab. As compared to the isotype control (anti-gD), trastuzumab induced dose-dependent phagocytosis with HER2-expressing SKBR3 cells (Fig. 2F). Similarly, rituximab induced dose-dependent phagocytosis with CD20-expressing WIL2-S cells (Fig. 2F). These results highlight the specificity of this phagocytosis assay.

Variation in Fc N-glycosylation profiles of antibodies has been demonstrated to impact antibody-dependent cellular cytotoxicity (ADCC). Absence of the core fucose in the Fc N-glycan of mAbs can lead to substantially increased binding to FcγRIIIA and enhanced ADCC activity (Chung et al., 2012). Similarly, higher levels of N-linked mannose-5 glycan (Man-5) has been shown to increase FcγRIIIA binding and ADCC activity while decreasing FcγRIIA binding activity (Yu et al., 2012). However, it is unknown how variation in glycosylation will impact ADCP activity of therapeutic antibodies. To investigate impact of glycosylation on ADCP, three glycosylation variants of ocrelizumab, afucosylated, high manose, and deglycosylated, was compared to the reference molecule in the ADCP assay. As shown in Fig. 3, the reference ocrelizumab (2H7) showed diminished ADCP activity compared with both afucosylated (AF) and high mannose (HM) ocrelizumab. The AF variant showed the largest increase in ADCP activity, highlighting the importance of FcγRIIIA in the process of ADCP. The HM variant showed moderately enhanced phagocytosis activity above the reference molecule, showing that FcgRIIA also plays a role in ADCP. The deglycosylated antibody, which cannot bind FcγRs, shows minimal ADCP activity, reflecting the important of glycosylation for antibody engagement with the FcγR receptors.

4. Discussion and conclusions

A quantitative ADCP method was developed using target cell labeled with pH-sensitive dyes and fluorocence of internalized cells as a readout of phagocytosis. pHrodo dyes exhibit low levels of fluorescence in physiological pH but increase in solutions of decreasing pH (mimicking the phagosome as it moves through maturation). As the macrophage internalizes the pHrodo-labeled target cells, the macrophage itself becomes fluorescent. Phagocytic events were calculated as the number of macrophages that are positive for the pH-dependent target cell fluorescence. This pH dependency makes the assay format unique since the readout is dependent upon target cell internalization.

This ADCP method was demonstrated to be reproducible and quantitative. By analyzing phagocytosis on the flow cytometer using the control (no antibody present) condition to determine the threshold for phagocytosis, the percentage of macrophages positive for pHrodo green fluorescence could be quickly calculated across multiple antibody concentrations. By normalizing the antibody dose-response curve to the control condition, the phagocytosis index could be pooled across multiple individuals, as shown in Fig. 2. This resulted in highly reproducible ADCP activity curves across macrophages derived from multiple individual donors.

Human macrophages express all three FcγRs, and since glycosylation is known to impact binding, it is expected that it will also affect phagocytosis. In fact, previous work has shown the impact of glycoengineering upon Fc effector functions including ADCP (Herter et al., 2014). Therefore for this assay to be a useful tool tfor both lead candidate selection and critical quality attribute monitoring during drug development, it is necessary that the ADCP assay be able to distinguish glycovariants. We have shown that this assay format is able to distinguish glycosylation variants, with enhanced binding to FcγRIIIA showing increased phagocytosis activity (Fig. 3).

The use of a pH-sensitive dye to measure phagocytosis in a flow cytometry assay has allowed for the development of a quantitative and reproducible ADCP assay. This assay is able to generate full dose-response curves for measuring the phagocytosis induced by IgG

antibodies to multiple target cell types. This ADCP method should prove to be a useful tool in engineering and development of therapeutic antibodies.

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