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Methods to Validate Binding and Kinetics of "Proximity-Inducing" Covalent Immune-Recruiting Molecules

Eden Kapcan,[1,2,3,4] Benjamin Lake,[1,2,3,4] Zi Yang,[1,2,3] and Anthony F. Rullo[1,2,3,5]

[1]McMaster Immunology Research Center (MIRC), McMaster University, Hamilton, Ontario, Canada
[2]Department of Medicine, McMaster University, Hamilton, Ontario, Canada
[3]Department of Chemistry and Chemical Biology, McMaster University, Hamilton, Ontario, Canada
[4]These authors contributed equally to this work.
[5]Corresponding author: *rulloa@mcmaster.ca*

The emergence of covalent inhibitors and chemoproteomic probes in translational chemical biology research requires the development of robust biophysical and analytical methods to characterize their complex interactions with target biomolecules. Importantly, these methods must efficiently assess target selectivity and accurately discern noncovalent binding from the formation of resultant covalent adducts. One recently reported covalent chemical tool used in tumor immune oncology, covalent immune recruiters (CIRs), increases the proximity of immune cells and cancer cells, promoting immune recognition and response. Herein we describe biolayer interferometry (BLI) biosensor, flow cytometry, and solution fluorescence-based assay approaches to characterize CIR:antibody binding and CIR-antibody covalent-labeling kinetics. BLI technology, akin to surface plasmon resonance, provides the unique opportunity to investigate molecular binding and labeling kinetics both on a solid surface (Basic Protocol 1) and in solution (Alternate Protocol 1). Here, recruitment of mass-containing proteins to the BLI probe via CIR is measured with high sensitivity and is used as a readout of CIR labeling activity. Further, CIR technology is used to label antibodies with a fluorescent handle. In this system, labeling is monitored via SDS-PAGE with a fluorescence gel imager, where increased fluorescence intensity of a sample reflects increased labeling (Basic Protocol 2). Analysis of CIR:antibody target-specific immune activation is demonstrated with a flow cytometry–based antibody-dependent cellular phagocytosis (ADCP) assay (Basic Protocol 3). This ADCP protocol may be further used to discern CIR:antibody binding from covalent adduct formation (Alternate Protocol 3). For the protocols described, each method may be used to analyze characteristics of any covalent-tagging or antibody-recruiting small molecule or protein-based technology. © 2020 Wiley Periodicals LLC.

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Support Protocol 2: Labeling streptavidin beads with biotinylated prostate-specific membrane antigen receptor

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INTRODUCTION

An emerging strategy in immunotherapy involves the use of synthetic small molecules to engage, redirect, or leverage the immune system for a therapeutic effect such as anticancer activity. One approach uses antibody engagers (AEs), also known as ARMs (Bandara, Bates, Lu, Hoylman, & Low, 2017; Murelli, Zhang, Michel, Jorgensen, & Spiegel, 2009; Sheridan, Hudon, Hank, Sondel, & Kiessling, 2014). These are bifunctional small molecules containing an antibody-binding domain (ABD) and a target-binding domain (TBD; Figure 1). The ABD contains a hapten for an endogenous serum antibody, such as dinitrophenyl (DNP; Rullo et al., 2016). The TBD contains a cancer receptor ligand such as glutamate urea lysine (GUL), which can bind to the highly expressed prostate-specific membrane antigen (PSMA) receptor on prostate tumors (Zhang et al., 2010). An AE is able to bind simultaneously through its ABD to an endogenous antibody and through its TBD, a cancer cell. The antibody is then able to recruit tumor-killing immune machinery, leading to an anticancer response (Lu, Sega, Leamon, & Low, 2004).

In an effort to probe antibody affinity requirements for AE antitumor efficacy, we developed a covalent immune-recruiting (CIR) strategy, where AEs are redesigned to contain an antibody-labeling domain (ALD). The ALD is composed of an electrophilic reactive center that, upon ABD binding, is able to undergo a nucleophilic attack (Mortensen, Nielsen, Palmfeldt, & Gothelf, 2019). This subsequently covalently attaches the TBD to the antibody and provides novel antitarget specificity. Consequent target opsonization may allow immune cell activation, which is intricately dependent on the stability of antibody engagement with immune receptors like Fcγ receptors, triggering receptor clustering and downstream activatory signaling. The stability of the CIR-antibody covalent complex is therefore hypothesized to affect increased immune activation. Furthermore, the increased serum half-life of CIRs (through their covalent linkage to antibodies) may increase efficacy in contrast to conventional AEs, which are rapidly cleared in vivo. In our recent publication, the synthesis, kinetic characterization, and immune recruitment activity of several CIRs was demonstrated (Lake, Serniuck, Kapcan, Wang, & Rullo, 2020).

CIR validation requires assessment of the following key parameters: (1) antibody reaction kinetics, (2) antibody selectivity, and (3) antibody recruitment to cancer receptors and resultant immune cell activation. Here we describe biolayer interferometry (BLI) assays on the Octet platform to determine CIR/antibody labeling kinetics on surface (Basic Protocol 1) and in solution (Alternate Protocol 1). Next, we describe a protocol to evaluate CIR/antibody labeling via fluorescence SDS-PAGE employing dye-modified CIR analogs (Basic Protocol 2). Finally, we describe how to employ antibody-dependent cellular phagocytosis (ADCP) assays (Basic Protocol 3, Alternate Protocol 2) to define the CIR mechanism of action and demonstrate its anticancer function. All CIRs investigated

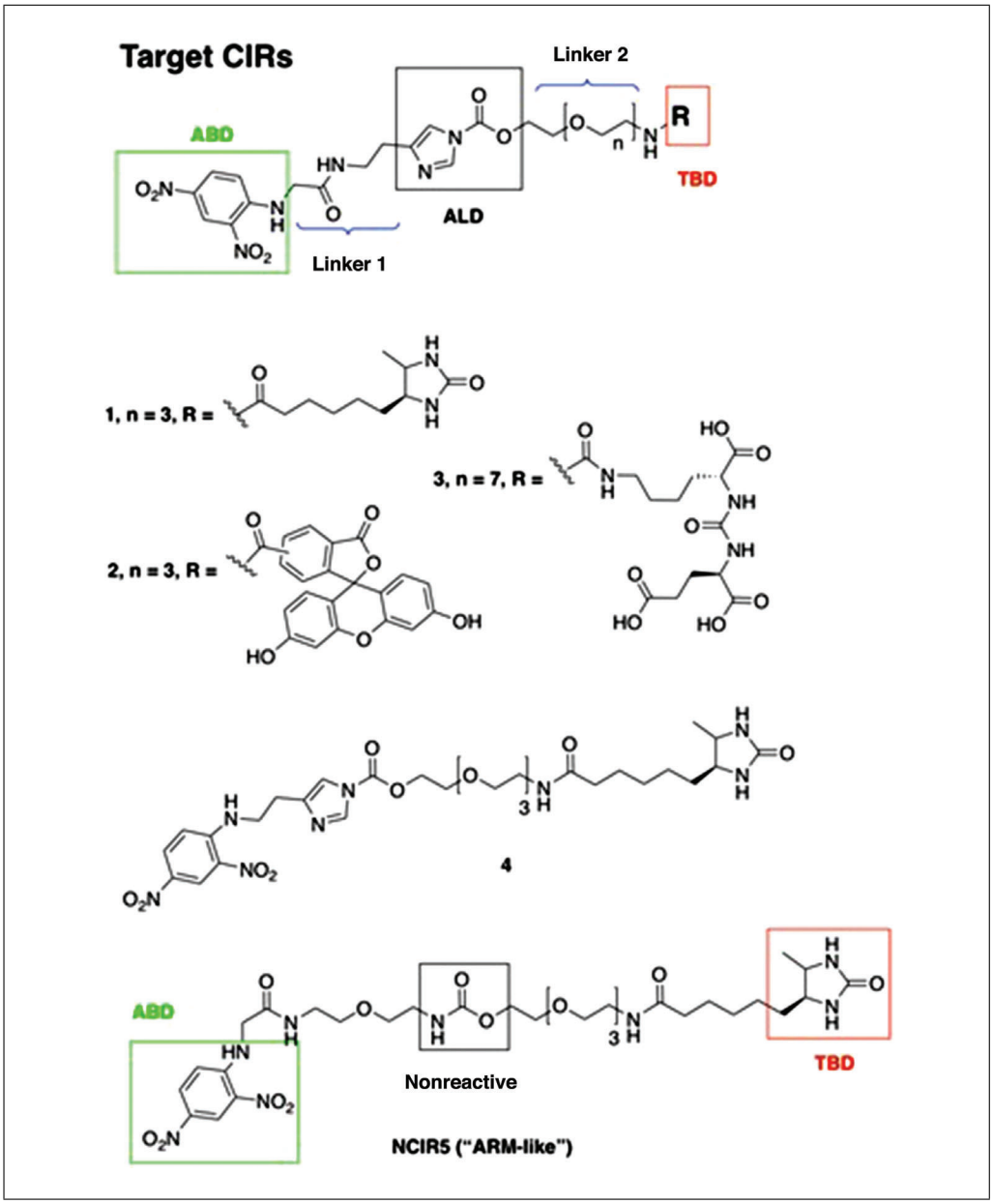

Figure 1 Demonstration of CIRs referenced in Basic Protocols 1, 2, and 3. Molecules as named in Lake et al. (2020) are shown. CIR1/2/3/4 and NCIR5 are referenced. ABD, antibody-binding domain; ALD, antibody-labeling domain; CIR, covalent immune recruiters; NCIR, nonreactive CIR; TBD, target-binding domain. Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

are depicted in Figure 1. Synthetic methods affording compounds CIR1/CIR4, NCIR5, and DNP-glycine can be found in Lake et al. (2020).

NOTE: All compounds were produced according to previously published work (Lake et al., 2020). Compound samples are available by request to the authors (*rulloa@mcmaster.ca*).

DETERMINING "ON-PROBE" REACTION KINETICS OF CIR1/CIR4 VIA BIOLAYER INTERFEROMETRY WITH OCTET RED96

BASIC PROTOCOL 1

To evaluate CIR/antibody reaction kinetics, we developed a BLI-based protocol employing the Octet RED96 system. BLI is an optical technique that analyzes the interference pattern of white light caused by addition of mass to an optical biosensor surface "probe"

(e.g., the addition of a ligand that binds a probe-immobilized receptor). Here, mass addition induces a wavelength shift proportional to the quantity of mass bound, and thus the rate of wavelength shift is proportional to the rate of mass binding to the biosensor.

Octet RED96 is able to measure binding interactions on probes that are commercially available with various immobilized proteins such as streptavidin. Streptavidin probes enable biotinylated or desthiobiotinylated receptors to be essentially irreversibly bound to the probe. Afterward, the association of ligand for the biotinylated receptor can be quantified.

Antibody-labeling reaction kinetics were studied using CIRs (CIR1/CIR4; Fig. 1) equipped with desthiobiotin (an analog of biotin) to serve as a model TBD with near-infinite binding affinity for a target receptor. This enables sufficiently high CIR binding affinity to streptavidin-coated probes to efficiently monitor "on-probe" antibody-labeling kinetics without concern for CIR dissociation from the probe. Note that CIR4 only differs from CIR1 through a shorter and more rigid linker between the ALD and ABD (see Fig. 1).

In these assays, CIRs and analogous nonreactive control molecules (NCIRs) are loaded onto a streptavidin probe. Next, the probe is submerged in a solution containing a saturating concentration (large dissociation constant [K_d]; 500 nM) of polyclonal anti-DNP immunoglobulin G (IgG) antibody for various amounts of time (0.25 to 8 hr). Noncovalent binding to the antibody can be distinguished from a covalent reaction by submerging probes corresponding to different reaction time points in a concentrated solution of DNP-glycine competitor. At equilibrium, a large excess of DNP-glycine competitor will remove all anti-DNP noncovalently bound to CIRs and NCIRs loaded onto the probe. As the reaction progresses, less antibody will be dissociated in the presence of competitor, corresponding to the formation of irreversible covalent complexes (CIR-antibody).

The resultant changes in signal amplitude following DNP competitor dissociation experiments versus time can be plotted to extract pseudo first-order rate constants describing CIR-antibody labeling kinetics. Repeating these reactions in the presence of a competitor (which competes for CIR binding to anti-DNP), or employing a nonspecific IgG antibody, serves as useful controls for reaction selectivity.

Materials

200 nM CIR1 (see recipe)
200 nM CIR4 (see recipe)
200 nM NCIR5 (see recipe)
1× kinetics buffer (see recipe)
500 nM anti-DNP antibody (see recipe)
5% (w/v) milk blocking buffer (see recipe)
1 mM DNP-glycine (see recipe)
Streptavidin probes (e.g., Forte Biosciences, cat. no. 18-5019)

96-well plate, black (e.g., Greiner Bio-One, cat. no. 655209)
Octet RED96
Computer running data acquisition and analysis software (e.g., GraphPad Prism)

Prepare plate

1. Prepare the following solutions as described in Reagents and Solutions: 200 nM CIR1, 200 nM CIR4, 200 nM NCIR5, 1× kinetics buffer, 500 nM anti-DNP antibody, 5% (w/v) milk blocking buffer, and 1 mM DNP-glycine.

2. Place solutions in a 96-well plate as follows:

   Column number refers to the column position on the plate from left to right.

   a. To column 1 add 200 µl of 1× kinetics buffer.
   b. To column 2 add 200 µl of 200 nM CIR1.
   c. To column 3 add 200 µl of 5% (w/v) milk blocking buffer.
   d. To column 4 add 200 µl of 1× kinetics buffer.
   e. To column 5 add 200 µl of 500 nM anti-DNP antibody.

      This is the association well.

   f. To column 6 add 200 µl of 1 mM DNP-glycine.

      This is the dissociation well.

3. Place 96-well plate in the designated location in the Octet. To a second 96-well plate, add 200 µl of 1× kinetics buffer.

   This is to be used to "wet" the probe prior to Octet reading.

4. Place streptavidin probes in the second 96-well plate.

5. Place second 96-well plate in the designated location in the Octet.

   For all Octet experiments, any well containing less than ∼180 µl will lead to erroneous data (stochastic spikes). Thus, it is necessary for longer time points to have a larger volume (250 to 300 µl to account for evaporation) in the experimental antibody association and dissociation wells. Evaporation-induced increase in concentration will not affect the rate of the reaction as the system is already at saturating conditions.

Measure streptavidin-targeting CIR (CIR1/CIR4) reaction kinetics with BLI using Octet RED96

The following steps are completed using a computer connected to the Octet instrument. The column number in parenthesis denotes the column of the plate in which the specific solution is loaded.

6. Acquire baseline signal for the streptavidin probes by placing them in 200 µl of 1× kinetics buffer for 3 min (column 1).

7. Place streptavidin probe in 200 µl of 200 nM CIR1 for 3 min to load the molecule onto the probe (column 2).

   To ensure data reproducibility, the integrity of aqueous CIR samples over time must be carefully monitored by liquid chromatography mass spectrometry. This is because the CIRs reported can hydrolyze with half-lives on the order of 5 to 20 hr.

8. Block probes using 200 µl of 5% milk blocking buffer to block nonspecific binding for 2 min (column 3).

9. Place probes in 200 µl of 1× kinetics buffer for 3 min to re-establish a baseline (column 4).

10. Place probes in 500 nM anti-DNP antibody for 0.25 hr to measure association (column 5).

    It is essential to use a saturating amount of antibody at >10× K_d to ensure the noncovalent CIR:antibody complex is saturated. This enables simple and reproducible calculation of the pseudointramolecular rate constant describing the antibody-labeling reaction.

    The remaining steps describe time points between 0.25 and 15 hr. The procedure can be adjusted to include any time point depending on the ALD chemistry and corresponding reaction kinetics.

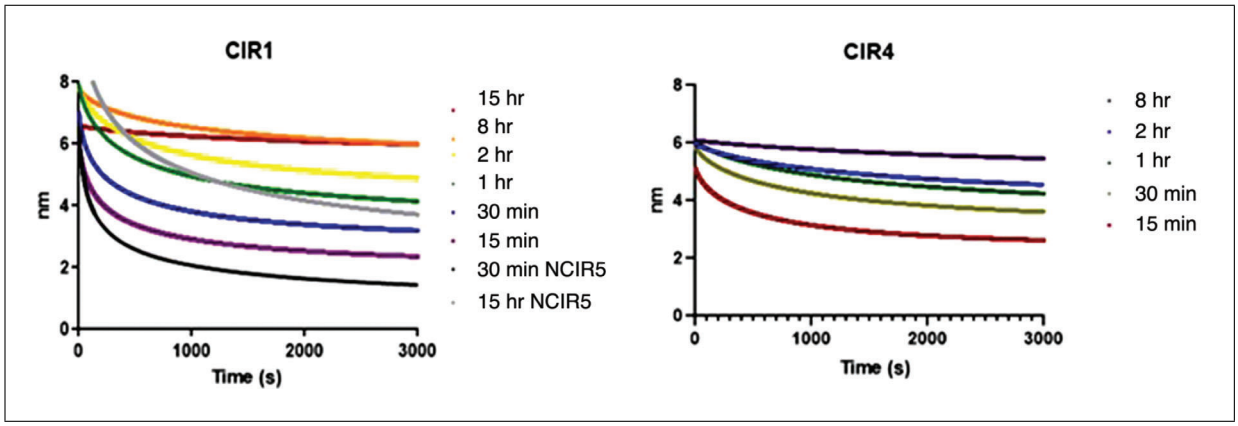

Figure 2 Dinitrophenyl (DNP)-glycine covalent immune recruiter (CIR) 1 and CIR4 dissociation curves after different preincubation (association) time points. These are the raw data of a spectrogram after incubation of CIR1 (left) and CIR4 (right) on probe with saturating amounts of antibody. Longer incubation times lead to less dissociation, indicating greater amounts of covalent reaction. Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

11. Place probes in 200 µl of 1 mM DNP-glycine for 50 min to measure dissociation (column 6).

    Using DNP competitor during dissociation is essential to disrupt avidity-driven binding of antibody to multivalent arrays of NCIR/CIR on bionsensor probes. Notably, the effective concentration of competitor that is functional in solution is likely less than anticipated due to the propensity of small organic molecules to aggregate at higher concentrations in aqueous solution.

    At high (mM) concentrations, DNP-glycine may become insoluble; ensure careful mixing.

12. Repeat steps 1 through 11 at 0.5, 1, 2, 8, and 15 hr.

13. Repeat steps 1 through 11 with 200 µl of 200 nM NCIR5 with an association time of 0.25 and 15 hr.

14. Repeat steps 1 through 11 with 200 µl of 200 nM CIR4.

    Through the above-described workflow sequence, you have generated a series of antibody dissociation curves from CIR- or NCIR-loaded strepativin probes. The amplitude of dissociation is proportional to the extent of CIR covalent reaction with antibody that will not dissociate from the probe. Therefore, on probes preloaded with only CIR (in contrast to NCIR, which cannot react), antibody dissociation will decrease as a function of on-probe CIR reaction time.

Process data

15. Set baseline of the signal to be the beginning of the association time.

16. Set time zero to be the beginning of the dissociation time.

17. To measure antibody labeling rate constants, calculate the fraction reaction conversion at each reaction (association time) point by measuring the dissociation signal at $t = 0$ (CIR_F, $NCIR_I$) and $t = 50$ min (CIR_F, $NCIR_F$) for each CIR and for NCIR5 to determine dissociation amplitude.

    Repeating these steps should produce data similar to that presented in Figure 2.

18. Plot fraction conversion ($CIR_F - NCIR_F$) / ($CIR_I - NCIR_I$) versus the on-probe association/reaction time, and fit using GraphPad Prism employing a "one-phase association" equation to determine the observed rate constant (k_{obs}).

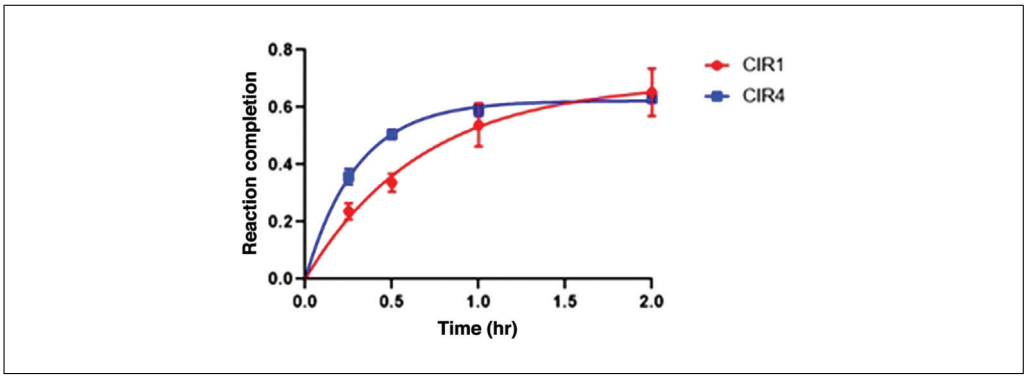

Figure 3 Fraction conversion of covalent immune recruiter (CIR) versus time. By using fraction conversion = (CIR_F – $NCIR_F$) / (CIR_I – $NCIR_F$), and a one-phase association equation, the observed rate constant can be estimated describing conversion of noncovalent CIR:antibody complex to covalent product (CIR-antibody). Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

A first-order kinetic treatment of the system is possible as the concentration of antibody is saturating and is in large excess over probe-loaded CIR/NCIR. Repeating these steps should produce data similar to that presented in Figure 3.

Use the equation $Y = Y_o + (plateau - Y_o) / (1 - e^{-K-X})$, where X = time, Y_o = y-intercept, and K = observed pseudo first-order rate constant.

As association time increases, more noncovalent complexes appear to form on the probe surface, which cannot be removed during the dissociation step. We hypothesize this represents slow higher-avidity binding of antibody to the surface or slow nonspecific antibody aggregation on the probe surface (see supporting information in Lake et al., 2020). To account for this we determined $NCIR_F$ at each time point and used this value to correct for the true extent of the covalent reaction.

DETERMINING "IN-SOLUTION" REACTION KINETICS OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN TARGETING CIR (CIR3) VIA BIOLAYER INTERFEROMETRY WITH OCTET RED96

ALTERNATE PROTOCOL 1

The study of lower-affinity tumor antigen–targeting CIRs via BLI presents unique challenges compared with the analysis using streptavidin-targeting CIR1 and CIR4. Due to the lower binding affinity for tumor antigens immobilized on the probe compared with streptavidin, the strategy employed in Basic Protocol 1 can be complicated by CIR dissociation from the probe. This is problematic if CIR affinity for the probe enables dissociation on the timescale of CIR reaction with antibody. As such, we developed an alternate protocol to enable analysis of tumor antigen–binding CIRs that can be used if tumor antigen affinity is limiting. In this protocol we synthesized CIR3 (Fig. 1), which substitutes the desthiobiotin ligand for GUL, a ligand that binds PSMA present in prostate cancers with $\sim 10^{-8}$ M affinity. In this protocol, the reaction kinetics of CIR3 were again determined using Octet RED96.

In this approach we evaluate probe–antibody reaction kinetics. Here, our sample molecule CIR3 is directly incubated with the antibody for varying amounts of time in solution (outside of the Octet system). These reactions are then quenched with an excess of DNP-glycine competitor enabling us to carefully stop the reaction at key time points. Next, streptavidin probes are incubated with biotinylated PSMA, followed by washing and baseline acquisition steps, before being placed in the aforementioned CIR3 + antibody solution. In this format, the slope of the association signal now corresponds to the fraction of covalent CIR3-antibody product formed with time. This can then be plotted versus time to extract k_{obs}.

Materials

200 nM CIR3 + 100 nM anti-DNP time course solution (see recipe)
1× kinetics buffer (see recipe)
5% (w/v) milk blocking buffer (see recipe)
1 mM DNP-glycine (see recipe)
10 mM glycine-HCl, pH 2.2 (see recipe)
75 nM biotin-PSMA (see recipe)
Phosphate-buffered saline (PBS)
Streptavidin probes (e.g., Forte Biosciences, cat. no. 18-5019)

96-well plate, black (e.g., Greiner Bio-One, cat. no. 655209)
Octet RED96
Computer running data acquisition and analysis software (e.g., GraphPad Prism)

Prepare plates

1. Prepare the following solutions as described in Reagents and Solutions: 200 nM CIR3 + 100 nM anti-DNP time course solutions, 1× kinetics buffer, 5% (w/v) milk blocking buffer, 1 mM DNP-glycine, 10 mM glycine-HCl, and 75 nM biotin-PSMA.

2. Place solutions in a 96-well plate as follows:

   Column number refers to the column position in the plate from left to right.

   a. To column 1 add 200 µl of 1× kinetics buffer.
   b. To column 2 add 200 µl of 75 nM biotin-PSMA.
   c. To column 3 add 200 µl of 5% (w/v) milk blocking buffer.
   d. To column 4 add 200 µl of 1× kinetics buffer.
   e. To column 5 add 200 µl of 200 nM CIR3 + 100 nM anti-DNP antibody. Allow reaction to proceed for 0.5 hr and then quench with 1 mM DNP-glycine.
   f. To column 6 add 200 µl of 1 mM DNP-glycine.
   g. To column 7 add 200 µl of 10 mM glycine-HCl.

      This is optional and is necessary if probe regeneration is intended to strip immobilized protein.

   h. To column 8 add 200 µl PBS.

3. To a second 96-well plate, add 200 µl of 1× kinetics buffer. Place streptavidin probes in the same wells containing 200 µl of 1× kinetics buffer in the second 96-well plate, and insert at the designated location in the Octet.

4. Place first 96-well plate in the designated location in the Octet.

Measure CIR3 reaction kinetics with BLI using Octet RED96

The following steps are completed using the software integrated with the Octet instrument.

5. Begin experiment by placing streptavidin probes in 1× kinetics buffer for 3 min to get a baseline (column 1).

6. Place probes in 200 µl of 75 nM biotin-PSMA for 5 min (column 2).

   A high biotin:receptor labeling ratio (employing >10 equiv. biotin) can disrupt proper binding if multiple biotins are linked nonspecifically to the receptor. For this reason we perform biotinylation with slightly excess biotin concentrations to maximize 1:1 labeling stoichiometry. Also ensure biotinylated proteins do not contain protease-labile linkages to biotin. This can lead to cleavage of biotin-peptide fragments that block the probe, disrupting analysis.

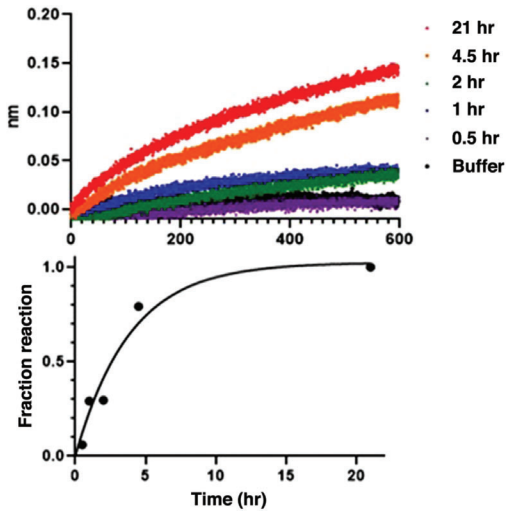

Figure 4 Covalent immune recruiter (CIR) 3-antibody association after different incubation time points. Top: Raw spectrogram observed following the addition of solutions containing a fixed concentration of CIR3 plus antibody allowed to react for different amounts of time to probes coated with prostate-specific membrane antigen. Longer incubation times lead to greater association, reflecting greater conversion to CIR3-antibody covalent product. Bottom: By determining the initial slopes of the association of all time points, and setting the 21.5-hr slope to correspond to 100% product formation, the fraction reaction of all time points can be determined. Nonlinear-curve fitting analysis enables extraction of the observed rate constant. Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

7. Place probes in 200 µl milk blocking buffer to block nonspecific binding for 2 min (column 3).
8. Place probes in 1× kinetics buffer for 3 min to re-establish a baseline for 3 min (column 4).
9. *Optional:* Place streptavidin probes in 10 mM glycine-HCl (regeneration buffer) for 5 s and then in PBS for 5 s. Repeat three times in total for a pre-regeneration (columns 7 and 8). Repeat step 8.

   It is important to treat the probe with regeneration buffer (i.e., 10 mM glycine-HCl, pH 2.2) prior to binding steps in any assay where regeneration steps are employed to recycle the probe. The first regeneration cycle lowers the association amplitude significantly; however, this is substantially lessened in subsequent regenerations. A pre-regeneration ensures the association amplitude is relatively conserved across multiple binding repetitions conducted on a single probe. If the probe-immobilized protein is highly stable to dissociation, the probe can be regenerated with simple kinetics buffer.

   It is essential to optimize probe regeneration conditions for each unique receptor and ligand pair studied by BLI (optimized for the compounds used here and detailed in step 9). Overly harsh conditions will destroy the receptor; overly weak conditions will not disrupt the receptor-ligand complex.

10. Place streptavidin probes in 200 nM CIR3 + 100 nM anti-DNP antibody incubated for 0.5 hr spiked with 1 mM DNP-glycine.
11. Place streptavidin probes in 1 mM DNP-glycine for 5 min.
12. *Optional:* Place streptavidin probes in 10 mM glycine-HCl for 5 s and then in PBS for 5 s. Repeat step three times in total to remove all noncovalent complex from the probe.

13. Repeat steps 9, 10, and 11 with a different solution of 200 nM CIR3 + 100 nM anti-DNP antibody incubated for 1, 2, 4, and 21.5 hr, followed by quenching with 1 mM DNP-glycine. In doing so, replace step 12 with a different well.

    One can either use new probes for every time point or reuse probes through the use of regenerations. Be sure to test whether the number of regenerations impacts the binding signal amplitude.

Process data

14. Set baseline of the signal to be the beginning of the association time.
15. Determine initial slopes.
16. Divide early slopes by the largest slope (21.5 hr) to determine percent product formation (CIR-antibody).
17. Plot a graph of reaction time versus percent product formation to determine k_{obs}.

    This procedure should enable replication of the data shown in Figure 4.

    This analysis relies on the assumption that, at the highest reaction time point, the reaction is complete and that conversion is 100%.

BASIC PROTOCOL 2

REACTION KINETICS OF COVALENTLY LABELED ANTIBODIES VIA FLUORESCENCE SDS-PAGE

It is essential for the antibody-labeling process to be significantly faster than the rate the molecule gets cleared from the body ($t_{1/2} = \sim$1 to 2 hr) and/or hydrolyzed/metabolized (Hirasawa et al., 2019). To further measure the solution-labeling rate (which is dependent on both CIR:antibody affinity and CIR:antibody intramolecular-labeling rate), we use CIR2 as our test compound, which contains a DNP antibody-binding terminus and fluorescein TBD. SDS-PAGE can effectively halt the reaction between CIR:antibody at chosen times and visually isolate free fluorescein (hydrolyzed and intact CIR2) from covalent CIR-antibody complex.

This protocol describes the analysis of fluorescence intensity of anti-DNP antibody protein bands in an polyacrylamide gel after treatment with CIR2 for 0 to 12 hr. Fluorescence intensity versus time is related to the CIR2-labeling rate via k_{obs}.

Materials

13.33 μM anti-DNP-KLH, rabbit IgG polyclonal antibody (e.g., Fisher Scientific, cat. no. A-6430)
100 μM CIR2 in dimethyl sulfoxide (DMSO)
PBS, pH 7.4, no calcium or magnesium
100 mM DNP-glycine (see recipe)
4× Laemmeli sample buffer (e.g., Bio-Rad cat. no. 161-0747)
Protein ladder (e.g., Bio-Rad Precision Plus, cat. no. 161-0363)
Polyacrylamide gel
EZBlue Gel Staining Reagent (e.g., Sigma-Aldrich, cat. no. G1041-500 ml)

Heating block
Fluorescence gel imager (e.g., GE Typhoon)
Infrared gel imager (e.g., Odyssey CLX)
Computer running image analysis (e.g., ImageJ) and data analysis (e.g., Excel) software

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2012)

NOTE: This protocol describes the kinetic analysis of the labeling reaction performed in PBS. If serum is to be used, the reaction should be run in a maximum of 10% (v/v) serum diluted in PBS. In this case, inject less samples (down to 5 µl) into each gel lane. This is to ensure the gel is not overloaded with serum proteins.

NOTE: This protocol describes time points 0, 0.25, 2, 8, and 12 hr with a CIR:antibody ratio of 2:1. The procedure can be adjusted to include any time point or CIR:antibody ratio. Isotype controls can be added concurrently to ensure selectivity of the system.

NOTE: High µM/mM concentrations of CIR2 and DNP-glycine were chosen to minimize the amount of DMSO in the final solution (up to 2% DMSO [v/v] used herein).

Label antibody with fluorescent CIR2 and run gel

It is essential to keep track of time in steps 1, 2, and 3 as they directly relate to the reaction kinetics being measured. Read the protocol in its entirety BEFORE starting, and prepare a timer to ensure you can keep track of the exact time that solutions are mixed.

1. Prepare 25 µl of 1 µM anti-DNP antibody and 2 µM CIR2 in PBS. Note the time, as this will be the 12-hr time point.

   Use a reverse pipetting technique to improve accuracy when transferring small volumes.

2. After 4, 10, 11.75, and 12 hr, repeat step 1 for the 8-, 2-, 0.25-, and 0-hr time points, respectively.

3. Immediately quench all solutions with 1 µl of 100 mM DNP-glycine. Remove 21 µl of each solution from steps 1 and 2, and dilute with 7 µl Laemmeli sample buffer.

   DNP-glycine is added at a 2000× excess to CIR to reduce/inhibit further labeling prior to gel loading.

4. Heat each sample at 95°C for 2 min. Also heat 12 µl protein ladder.

   Heating helps denature protein tertiary structure prior to gel loading and aids SDS to bind highly hydrophobic protein regions to help achieve full denaturation of protein tertiary structures.

5. Load 20 µl sample into gel lanes. Load 10 µl protein standard ladder.

6. Run gel at 90 V while sample is in the stacking phase of the gel and at 120 V while the sample is in the resolving phase of the gel.

   The stacking phase should take about 15 min to run. The resolving phase should take about 40 min to complete. Turn off the gel tank when the blue dye band is close to the bottom of the gel.

7. Remove gel from casing, and rinse with deionized water three times. Transfer to analysis instruments in deionized water.

   Protect from light to minimize photobleaching of fluorescent moiety.

   Carefully cut off top stacking gel to reduce probability of ripping the gel during transfer to instruments or during rinsing. This can be done with a scalpel or by gently pressing the stacking gel with a lint-free wipe and applying force perpendicular and opposite to the direction of the resolving gel.

Analyze gel

8. Visualize gel first with a GE Typhoon fluorescence gel imaging system. Use Cy2 laser to observe fluorescein tag.

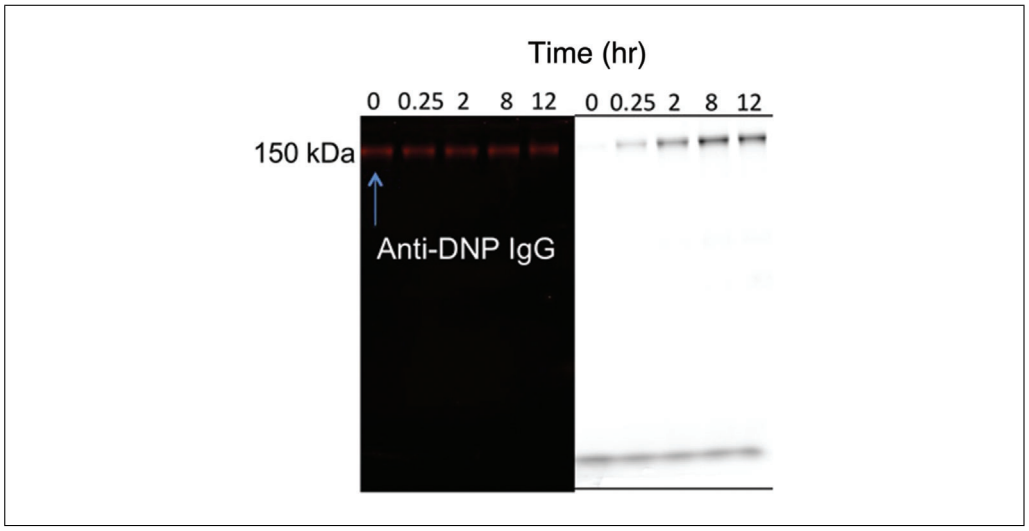

Figure 5 Covalent immune recruiter (CIR) 2 anti-dinitrophenyl (DNP) IgG reaction kinetics monitored by fluorescence SDS-PAGE. Coomassie staining (left) reports on the total protein loaded in each lane. The increase in fluorescence band intensity with time (right) is consistent with an increase in the amount of CIR2-labeled polyclonal anti-DNP IgG antibody (CIR2-antibody) formed. Excess CIR2 runs to the bottom of the gel. Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

If gel bands are too faint, increase the photomultiplier tube voltage. If the bands are too large and excessively bright, reduce the voltage. In most cases the automated setting will select a sufficient voltage.

This gel image will show only proteins that have been fluorescently tagged by CIR2. Fluorescence intensity can be used to quantify labeling.

9. Once fluorescent images are obtained, submerge gel in EZBlue Gel Staining Reagent. Gently shake solution for about an hour until the standard ladder is visible to the naked eye.

   EZBlue Gel Staining Reagent may be reused a few times if recollected after gel staining.

10. Rinse gel with deionized water three times. Transfer gel to an Odyssey CLX imager. Image gel using the 700-nm laser.

    For a representative gel see Figure 5.

    This image will show all protein in the gel, including the protein standard ladder. This can be used as a reference when analyzing the fluorescent gel images to determine the molecular weight of fluorescently tagged proteins.

11. Load fluorescent gel image into ImageJ software. Convert image to an 8-bit image. Complete densitometry analysis (see Internet Resources).

12. Use the rectangle tool to select the first band. Press Control and 1 (Command and 1 for Mac). Next, select the center of this box, and drag over the next band. Press Control and 2. Repeat for all bands; however, on the final band, press Control and 3, which will open a window with each band plotted.

13. Use the line tool to close the bottom of each plot. Use the magic wand tool to select and integrate each plot.

    The integration value will automatically appear.

14. Save data as an Excel file.

These values can be plotted and represent relative fluorescence values. These fluorescence values plotted against the reaction time can be used to reflect the reaction rate.

To determine the observed reaction rate, under the assumption all available free antibody is saturated with CIR over the time course of the reaction [CIR:antibody], employ the one-phase association equation, $Y = Y_o + (plateau - Y_o) / (1 - e^{-K-X})$, where X = time, Y_o is the y-intercept, and K is k_{obs}.

SMALL MOLECULE–DIRECTED ANTIBODY-DEPENDENT CELLULAR PHAGOCYTOSIS ON LIVE HUMAN CELLS MEASURED VIA FLOW CYTOMETRY

BASIC PROTOCOL 3

ADCP represents a main form of immune-mediated abhorrent cell (e.g., virally infected, cancerous cell) clearance mechanisms (Uvyn et al., 2019). Following target opsonization, antibody Fc regions can interact strongly with FcγI (CD64) receptors on phagocytes, inducing CD64 receptor clustering and downstream activitory signaling, leading to ADCP of the opsonized cell. This protocol describes the quantification of CIR-directed ADCP through the use of a flow cytometer. Herein, antibody is pretreated with CIR to allow labeling, and then the labeled antibody is diluted. Each concentration of antibody in this dilution series can be tested in effectiveness to enact ADCP through target–immune bridging. This may produce a generic autoinhibition curve, providing a measure of potency of CIR-antibody complex as a therapeutic. This procedure describes the activation and preparation of monocytes and target cells to allow for ADCP measurement, as well as the preparation of antibody and CIR stocks.

These steps produce a final experimental well plate with the following conditions: target cells only, effector cells only, target cells with effector and antibody alone, target cell with effector and isotype antibody alone, target cell with effector and CIR alone, target cell with effector and isotype antibody-CIR mix, target cell with effector and antibody-CIR mix–2-phosphonomethyl pentanedioic acid (PMPA) PSMA inhibitor, target cell with effector and antibody-CIR mix anti-DNP inhibitor, and target cell with effector and antibody-CIR mix (experimental conditions; antibody concentration: 80, 40, 13.33, 4.4, 1.5, and 0.5 nM). Here, all controls are completed at maximum 80 nM antibody concentration and CIR concentration = 2× antibody concentration only. Each well contains a final volume of 100 µl, and conditions are completed in duplicate (two wells each). Each duplicate condition is completed for two target cell lines (high PSMA expressing and non-PSMA expressing).

Materials

2.67 µM human monoclonal anti-DNP IgG1 antibody (e.g., ACROBiosystems, cat. no. DNP-M2)
RPMI 1640 medium (e.g., Thermo Fisher Scientific, cat. no. 31800)
500 mM DNP-glycine (see recipe) in DMSO
250 mM 2-PMPA (e.g., Sigma-Aldrich, cat. no. SML1612) in DMSO
76 µM polyclonal human IgG isotype antibody (e.g., Jackson ImmunoResearch, cat. no. 009-000-003)
100 µM CIR3 in DMSO
DMSO (e.g., Fisher Scientific, cat. no. D128-500)
Target cells
TrypLE Express (e.g., Thermo Fisher Scientific, cat. no. 12604013)
Activated monocytes (see Support Protocol 1)
DiD cell-labeling solution (e.g., Thermo Fisher Scientific, cat. no. V22887)
DiO cell-labeling solution (e.g., Thermo Fisher Scientific, cat. no. V22886)
Assay medium 1 (see recipe)

Centrifuge (e.g., Beckman Coulter Allegra 6R)
Hemocytometer or other cell counting method
96-well U-bottom plates, clear (e.g., Falcon)
Flow cytometer (e.g., BD LSR II)
Computer running analysis software (e.g., FlowJo)

NOTE: For effective quantification of ADCP, it is recommended to use 150,000 target cells and 150,000 effector cells at a 1:1 ratio. This ensures enough cells remain for adequate measurement on the flow cytometer and accounts for some cell loss due to wash steps.

NOTE: All culture incubations are conducted at 37°C with 5% CO_2.

NOTE: High μM/mM concentrations of CIR3, DNP-glycine, and 2-PMPA were chosen to minimize the percentage of DMSO in the final solution. DMSO should not exceed 1% (v/v) of the final volume with monocytes.

NOTE: ADCP can work using any antibody stock in which Fc can bind to human CD64 (such as polyclonal rabbit IgG). However, antibody affinity for hapten will vary between antibody sources, affecting antibody labeling and ADCP. Polyclonal anti-DNP antibodies employed in these studies possess high DNP binding affinity ($K_d = \sim 10^{-9}$ M), while human monoclonal anti-DNP binding affinity is weaker ($K_d = \sim 10^{-7}$ M). It is also important to ensure the DMSO concentration is consistent between conditions and is <1% (v/v) in the final well volume (to avoid significant DMSO-induced cytotoxicity).

Day 1: Prepare antibody solutions

1. Prepare stock solutions by diluting each solution:
   a. *Antibody stock (1):* Mix 70.6 μl human monoclonal anti-DNP IgG1 and 219.4 μl RPMI medium (650 nM final).
   b. *DNP-competition antibody stock (1D):* From antibody stock (1) remove 60 μl, and add 1 μl DNP-glycine.

      DNP-glycine is added here as a competitor for CIR. This is a control condition in which CIR cannot bind and therefore cannot selectively label antibody, preventing induction of ADCP. For effective competition to CIR, DNP-glycine is added in 6000× excess to CIR. Large excess is required as DNP-glycine effective concentration appears much lower than the actual concentration, likely due to a hydrophobic effect leading to self-complexation of the molecule in aqueous solution.

   c. *PMPA-competition antibody stock (1P):* From antibody stock (1), remove 60 μl, and add 1 μl PMPA. To adjust DMSO in antibody stock to be consistent with competition stocks, add 2.77 μl DMSO to the remaining 170 μl antibody stock.

      PMPA-competition is used here to compete for PSMA targeting. This is a CIR3-specific inhibitor and should be exchanged if different targeting ligands are used. For effective competition, 3000× excess to CIR is added.

   d. *Isotype antibody stock (2):* Mix 1 μl polyclonal human IgG isotype antibody with 1.9 μl DMSO and 117.1 μl RPMI medium (640 nM, 1.6% [v/v] DMSO final).
   e. *CIR stock (3):* Mix 4.48 μl CIR3 with 345.52 μl RPMI medium (1.28 μM, 1.3% [v/v] DMSO final).
   f. Prepare 1.3% (v/v) DMSO in RPMI medium (4).
   g. Prepare 1.6% (v/v) DMSO in RPMI medium (5).

      Note that the percentage of DMSO in CIR stock is less than that of antibody stock. All DMSO percentages will be made equivalent in step 2.

2. Using stocks from step 1, prepare experimental conditions as described in Table 1.

   All DMSO percentages are now consistent between conditions (at 1.45% [v/v]).

Table 1 Experimental Conditions for Small Molecule–Directed ADCP

Condition	Solution[a]	Mixture[b]
Antibody only	320 nM antibody	55 µl (1) + 55 µl (4)
DNP competition	320 nM antibody 4.1 mM DNP-glycine 640 nM CIR3	55 µl (1D) + 55 µl (3)
PMPA competition	320 nM antibody 2.05 mM PMPA 640 nM CIR3	55 µl (1P) + 55 µl (3)
Isotype antibody only	320 nM antibody	55 µl (2) + 55 µl (4)
Isotype antibody + CIR	320 nM antibody 640 nM CIR3	55 µl (2) + 55 µl (3)
CIR only	640 nM CIR3	55 µl (3) + 55 µl (5)
Antibody + CIR[c]	320 nM antibody 640 nM CIR3	110 µl (1) + 110 µl (3)

[a] Antibody as described in Basic Protocol 3 step 1
[b] See Basic Protocol 3 step 1 for a description of each solution.
[c] This condition is the experimental condition; remaining conditions are controls.
ADCP, antibody-directed cellular phagocytosis; CIR, covalent immune recruiter; DNP, dinitrophenyl; PMPA, 2-phosphonomethyl pentanedioic acid.

Isotype alone is included separately from isotype:CIR control, as isotype antibody Fc can interact differently with monocytes than anti-DNP antibody, creating a different baseline ADCP.

3. Allow solutions to incubate overnight for CIR:antibody labeling.

Day 2: Prepare effector and target cells

4. Suspend adherent target cells with TrypLE using a generic trypsin protocol.

   Interferon (IFN)-γ-activated monocytes (see Support Protocol 1) will be in solution already.

   TrypLE is a purified form of trypsin that conserves cellular surface proteins more effectively. Suspend an entire 150-cm^2 flask to ensure excess/adequate cells are available for use.

5. Wash cells (targets and monocytes) with RPMI medium three times. To do so, centrifuge 5 min at 500 × *g* (1100 rpm), room temperature, and decant. Then, resuspend pellet in RPMI medium, and repeat centrifugation and resuspension two additional times.

   The complete removal of serum is necessary for proper cell dye staining.

6. Resuspend cells in RPMI medium to a concentration of 1×10^6 cells/ml. Remove a small aliquot of cells for checking PSMA expression and for compensating on the flow cytometer.

   We have seen no evidence that cell dye alters PSMA expression levels. It would be prudent to check this for each new target cell used.

   To check expression of PSMA, add 1.5 µl anti-PSMA fluorescent antibody to proper wells only prior to step 11.

7. Add DiO cell dye to a concentration of 5.7 µM with target cells, and add DiD cell dye to a concentration of 1.9 µM with effector monocytes. Incubate at 37°C with 5% CO_2 for 30 min.

DiO/DiD lipid-based cell dyes provide individual recognition of each cell (target and phagocytic monocyte) on the flow cytometer, as well as recognition of cells containing both membrane dye colors (to be recognized as events of ADCP).

These concentrations should be adjusted and optimized for the flow cytometer used. Lower concentrations of dye may require higher voltages on the flow cytometer to visualize. We do not suggest lowering dye concentration, as we have found this causes a reduced final percentage of target phagocytosis signal.

8. Wash cells as described in step 5 using assay medium 1. Resuspend target cells to a concentration of 6.0×10^6 cells/ml. Resuspend monocytes to a concentration of 3.0×10^6 cells/ml.

Day 2: Incubate and perform flow cytometry

9. Prepare a dilution series for experimental conditions (antibody + CIR from Table 1). Complete a serial dilution of the current stock, which will be 80 nM in final well volume, from 80 to 0.5 nM.

10. Add 25 µl of each control condition (see Table 1) or experimental condition from step 9 to a 96-well U-bottom plate. For wells containing no conditions (e.g., cell only or PSMA expression check), add 25 µl assay medium 1.

11. Add 25 µl target cells to appropriate wells. For wells containing no targets, add 25 µl assay medium 1.

12. Add 50 µl monocytes to appropriate wells. Add assay medium 1 to wells with no monocytes.

    Ensure wells are properly and gently mixed by using a pipette.

    Use a multichannel pipette if possible to ensure incubations are as similar as possible. This is particularly important if a large number of samples is being tested concurrently.

13. Centrifuge plate 2 min at $270 \times g$ (800 rpm), room temperature, and incubate for 1 hr.

    Pelleting brings monocytes and targets in close proximity for incubation. It is crucial that a U-bottom plate is used to ensure cells pellet close together.

14. After 1 hr, place conditions on ice, and protect from light for analysis on flow cytometer.

    It is not necessary to fix cells; placing on ice sufficiently stops ADCP and cell death for same-day analysis.

Analyze output

15. Perform proper compensation on the flow cytometer prior to analysis. For target DiO-stained cells, use the Alexa 488 channel. For effector DiD-stained cells, use the APC-Cy7 channel. Determine proper voltage of the two channels, as well as forward scatter (FSC) and side scatter (SSC) to visualize cell populations. Export all data to be analyzed using FlowJo software.

    Here we use an FSC voltage of 390, an SSC voltage of 290, an Alexa 488 voltage of 260, and an APC-Cy7 voltage of 400.

16. Select for singlet populations in FlowJo software. For each sample, plot FSC area (FSC-A) versus FSC height (FSC-H), and select cells along the 45° line. Repeat for SSC (Fig. 6).

17. Using the single cell population, plot Alexa 488 versus APC-Cy7, and quantify ADCP of each condition (Fig. 7).

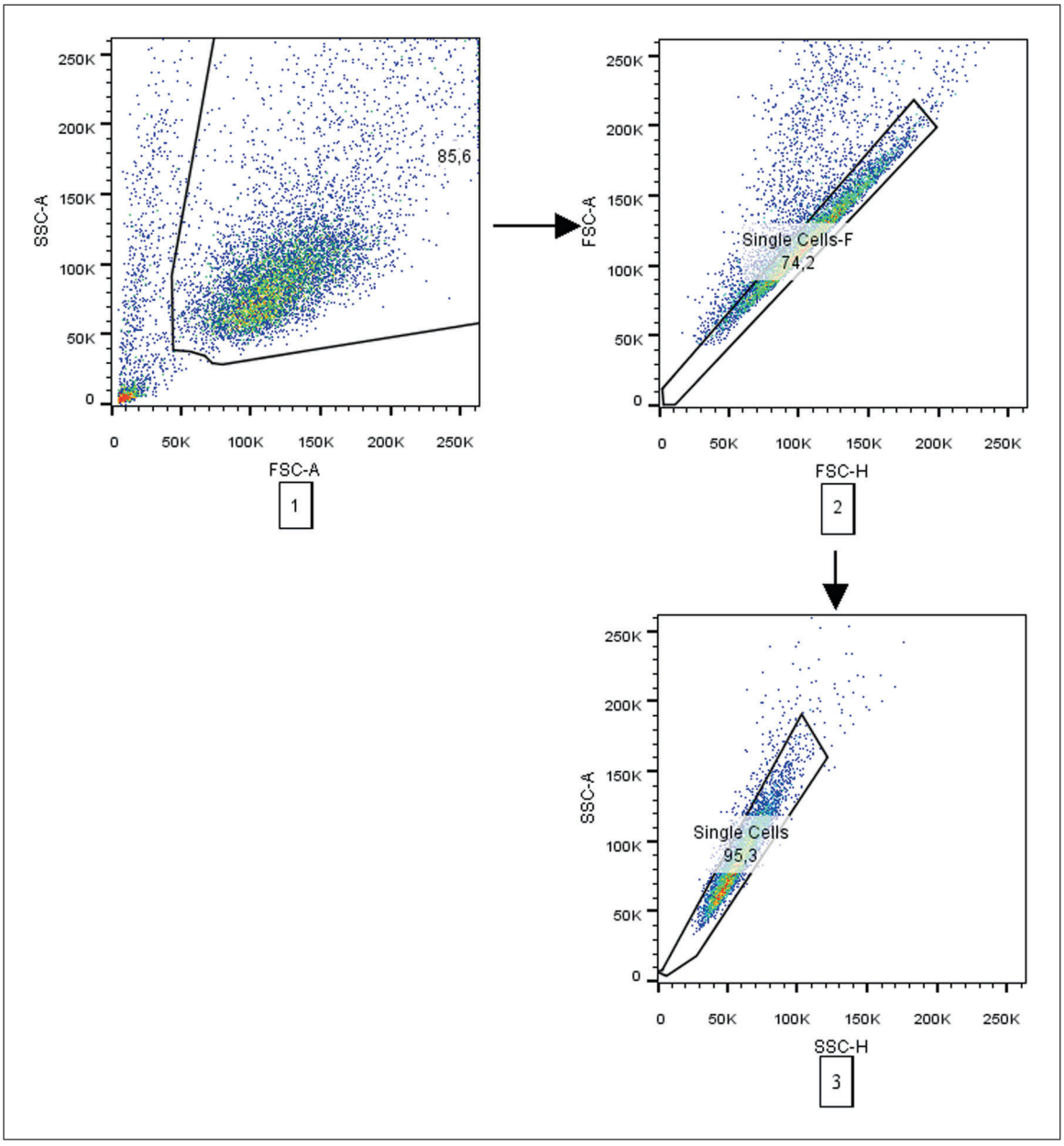

Figure 6 Gating for single cell populations. The target cell population is chosen from the FSC versus SSC plot. Within this population only single-cell events are analyzed. (1) Select original population of interest. (2) Isolate singlets from FSC parameters. (3) Isolate singlets from SSC parameters. All analyses are on this "single-cell" population. FSC, forward scatter; FSC-A, FSC area; FSC-H, FSC height; SSC, side scatter; SSC-A, SSC area; SSC-H, SSC height. Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

KINETIC ANALYSIS OF CIR3:ANTIBODY LABELING VIA ANTIBODY-DEPENDENT CELLULAR PHAGOCYTOSIS ON FLOW CYTOMETRY

ALTERNATE PROTOCOL 2

This protocol uses flow cytometry–based ADCP to probe kinetic labeling of antibodies and "covalent antibody recruitment" to targets. The procedure can be used for the measurement of any small molecule kinetic functions necessary for induction of opsonization for immune activation. Herein, a single concentration of antibodies and CIR is incubated for varying amounts of time prior to reaction quenching with DNP-glycine (CIR-antibody competitor). ADCP induction as a function of CIR:antibody incubation time provides an ability to observe the CIR:antibody labeling rate.

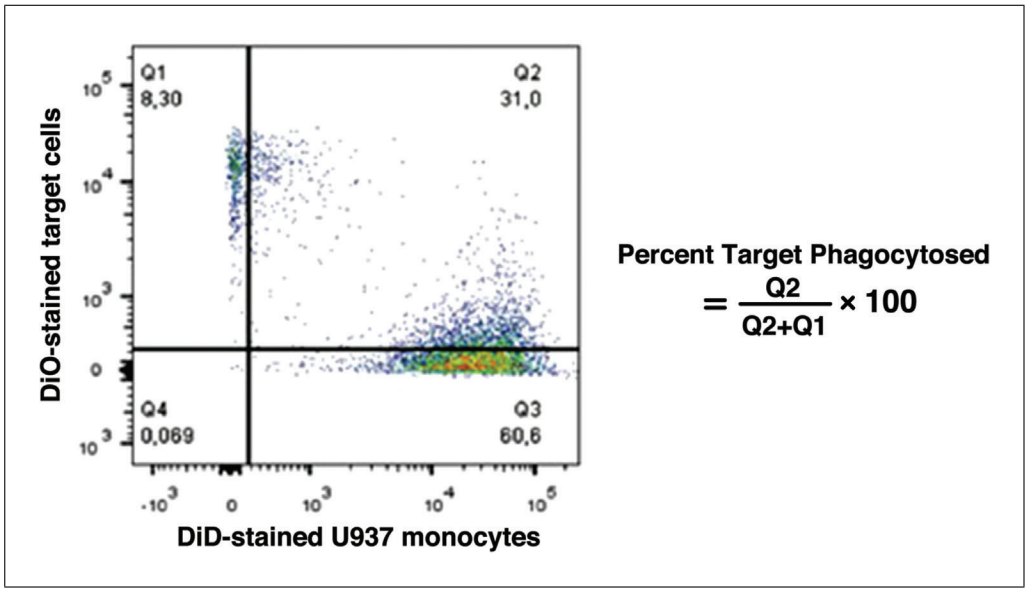

Figure 7 Quantitation of antibody-dependent cellular phagocytosis (ADCP). Target cells stained with DiO are shown in quadrant (Q) 1. Effector monocytes stained with DiD are shown in Q3. ADCP is quantified via the percent target phagocytosed equation shown. Adapted and reprinted with permission from Lake et al. (2020). *ACS Chemical Biology*, *15*, 1089–1095. Copyright 2020 American Chemical Society.

These steps produce a final experimental well plate with the following conditions: target beads only, effector cells only, target beads with effector and antibody alone, target cells with effector and CIR alone, target beads with effector and isotype antibody alone, target beads with effector and isotype antibody-CIR, target beads with effector and antibody-CIR mix-PMPA PSMA inhibitor, and experimental conditions (target beads with effector and antibody-CIR mix [100 nM antibody incubated with CIR for 0, 2, 4, 8, and 24 hr]). Here, all controls are measured at 24 hr, and CIR concentration = 2× antibody concentration. In this protocol, conditions should be completed in duplicate (two wells each).

Materials

13.33 μM anti-DNP-KLH, rabbit IgG polyclonal antibody (e.g., Fisher Scientific, cat. no. A-6430)
RPMI 1640 medium (e.g., Thermo Fisher Scientific, cat. no. 31800)
250 mM 2-PMPA (e.g., Sigma-Aldrich, cat. no. SML1612) in DMSO
DMSO (e.g., Fisher Scientific, cat. no. D128-500)
33.33 μM rabbit IgG isotype control antibody (e.g., Fisher Scientific, cat. no. 26102)
Assay medium 2 (see recipe)
100 μM CIR3 in DMSO
100 mM DNP-glycine (see recipe) in DMSO
Activated monocytes (see Support Protocol 1)
DiD cell-labeling solution (e.g., Thermo Fisher Scientific, cat. no. V22887)
Bead solution (see Support Protocol 2)

Centrifuge (e.g., Beckman Allegra 6R)
Hemocytometer or other cell counting method
96-well U-bottom plate, clear (e.g., Falcon)
Flow cytometer (e.g., BD LSR II)
Computer running analysis software (e.g., FlowJo)

Table 2 Conditions for Kinetic Analysis of CIR3:Antibody Labeling Via ADCP

Condition	Solution	Mixture[a]
PMPA competition (24 hr)	100 nM antibody 1.33 mM PMPA 200 nM CIR3	70 μl (1P) + 70 μl (3_24 hr)
Isotype antibody + CIR3 (24 hr)	100 nM antibody 200 nM CIR3	70 μl (2) + 70 μl (3_24 hr)
Antibody + CIR3 (24 hr)	100 nM antibody 200 nM CIR3	70 μl (1_24hr) + 70 μl (3_24 hr)

[a]See Alternate Protocol 2 step 1 for a description of each solution. All mixtures should be in assay medium 2 with 0.45% (v/v) DMSO.
ADCP, antibody-directed cellular phagocytosis; CIR, covalent immune recruiter; DMSO, dimethyl sulfoxide; PMPA, 2-phosphonomethyl pentanedioic acid.

NOTE: The use of streptavidin beads allows for tighter data and a cleaner kinetic analysis when compared with the use of target cells.

NOTE: All culture incubations are conducted at 37°C with 5% CO_2.

NOTE: High μM/mM concentrations of CIR3, DNP-glycine, and 2-PMPA were chosen to minimize the percentage of DMSO in the final solution. DMSO should not exceed 1% (v/v) of the final volume with monocytes.

Prepare antibody solutions

1. Prepare stock solutions by diluting each solution:
   a. *Antibody stock (1_24 hr):* Mix 2.26 μl anti-DNP-KLH antibody and 147.74 μl RPMI medium (201.1 nM final).
   b. *PMPA-competition antibody stock (1P):* From antibody stock (1_24 hr), remove 75 μl, and add 0.4 μl PMPA. To adjust DMSO in antibody stock (1_24 hr) to be consistent with competition stocks, add 0.4 μl DMSO to the remaining 75 μl.
   c. *Isotype antibody stock (2):* Mix 0.48 μl rabbit IgG isotype control antibody with 0.4 μl DMSO and 79.12 μl assay medium 2 (200 nM, 0.5% [v/v] DMSO final).
   d. *CIR3 stock (3_24 hr):* Mix 1.28 μl CIR3 with 318.72 μl assay medium 2 (400 nM, 0.4% [v/v] DMSO final).
2. At this time, mix 24-hr time point conditions as described in Table 2.
3. Immediately start a timer. Prepare and mix solutions from steps 1 and 2 as per Table 3.

   All conditions should incubate at room temperature until the 24-hr conditions are made, at which point the experimenter should proceed directly to step 4.
4. Immediately quench all conditions (from steps 2 and 3) with 0.4 μl of 100 mM DNP-glycine. Allow conditions to incubate for 30 min. After this time, be ready to add conditions to prepared targets (described below).

   This stops the reaction by blocking CIR-antibody interaction and dissociates noncovalent complexes, enabling efficient deconvolution between noncovalent binding and covalent reactions with the antibody.

   One should avoid using DNP-glycine at concentrations above 250 μM with beads, as we have found DNP-glycine shreds streptavidin beads significantly when used at concentrations above 250 μM.

Table 3 Experimental Conditions for CIR3:Antibody Labeling Via ADCP

Time[a]	Condition[b]	Solution[c]
16 hr	Antibody + CIR3 (8 hr)	100 nM antibody 200 nM CIR3
20 hr	Antibody + CIR3 (4 hr)	100 nM antibody 200 nM CIR3
22 hr	Antibody + CIR3 (2 hr)	100 nM antibody 200 nM CIR3
23 hr	Antibody + CIR3 (1 hr)	100 nM antibody 200 nM CIR3
24 hr	Antibody only	100 nM antibody
24 hr	Isotype antibody only	100 nM antibody
24 hr	CIR3 only	200 nM CIR3
24 hr	Antibody + CIR3 (0 hr)	100 nM antibody 200 nM CIR3

[a]Here 16, 20, 22, 23, and 24 hr represent 8, 4, 2, 1, and 0 time points, respectively.
[b]For each condition, 140 µl total volume should be made with 0.45% (v/v) DMSO.
[c]All mixtures are made in assay medium 2.
ADCP, antibody-directed cellular phagocytosis; CIR, covalent immune recruiter; DMSO, dimethyl sulfoxide.

Prepare effector cells and target beads

5. Wash activated monocytes with 2 to 5 ml RPMI medium three times. To do so, centrifuge 5 min at 500 × *g* (1100 rpm), room temperature, and decant. Then, resuspend pellet in RPMI medium, and repeat centrifugation and resuspension two additional times.
6. Resuspend cells in RPMI medium to a concentration of 1×10^6 cells/ml.
7. Add DiD cell dye to a concentration of 1.9 µM with effector monocytes, and incubate for 30 min.
8. Wash cells as described in step 5 using assay medium 2. Resuspend monocytes to a concentration of 1.5×10^6 cells/ml in assay medium 2.
9. Pipette 10 µl prepared bead solution (see Support Protocol 2) into a 96-well U-bottom plate.

Incubate and perform flow cytometry

10. Add 60 µl of each antibody condition to the 96-well plate with beads. For beads alone, monocytes alone, or PSMA expression check, add 60 µl assay medium 2 (0.45% [v/v] DMSO). Mix gently by pipetting.
11. Allow antibodies to incubate with target beads for 20 min. Then centrifuge 5 min at 800 × *g* (1400 rpm), room temperature, and remove supernatant.
12. Resuspend beads with 100 µl monocyte solution. Mix gently by pipetting.
13. Centrifuge plate 2 min at 270 × *g* (800 rpm), room temperature, and incubate for 1 hr.

    Pelleting brings monocytes and targets in close proximity for incubation. It is crucial that a U-bottom plate is used to ensure cells pellet close together.

14. After 1 hr, place conditions on ice, and protect from light for analysis on flow cytometer.

It is not necessary to fix cells; placing on ice sufficiently stops ADCP and cell death for same-day analysis.

Analyze output

15. Perform proper compensation on the flow cytometer prior to analysis. For target beads use the PE-Cy7 channel. For effector DiD-stained cells use the APC-Cy7 channel. Determine proper voltage of the two channels as well as FSC and SSC to visualize cell populations. Export all data to be analyzed using FlowJo software.

    Here we use an FSC voltage of 430, an SSC voltage of 290, a PE-Cy7 voltage of 590, and an APC-Cy7 voltage of 400.

16. Quantify ADCP as per Figures 5 and 6.

ACTIVATION OF U937 MONOCYTES WITH INTERFERON γ

SUPPORT PROTOCOL 1

In this protocol U937 human monocytes are activated with IFN-γ for 24 hr prior to induction of ADCP. This treatment increases CD64 expression by about five-fold, priming the monocytes for recognition and engulfment of opsonized targets (Akinrinmade et al., 2017).

Materials

Monocyte culture
U937 complete growth medium (see recipe)
IFN-γ (e.g., Thermo Fisher, cat. no. PHC4031)

50-ml conical tube (e.g., Falcon)
Hemocytometer (e.g., Bright-Line)
Centrifuge (e.g., Beckman Allegra 6R)
25-cm^2 culture flask (e.g., Corning)

1. Remove monocytes to be activated from culture flask, and place in a 50-ml conical tube.

    Monocytes can be cultured in U937 complete growth medium and can be activated and used for ADCP prior to passage 20.

    Use proper aseptic technique and only sterile equipment to handle cells.

2. Ensure homogenous suspension by gently tilting capped tubes or by gently pipetting. Remove 100 µl cell suspension for cell counting, and count cells using a hemocytometer.

    For experiments described in this article, activation of six million monocytes is sufficient. If running alternative experiments, carefully plan experimental well plates, and calculate required monocyte number.

    Activated monocytes usually double in number after incubation (step 5); assume 1.5× the original cell count will be available on the day of the experiment.

3. Pellet cell suspension by centrifuging 5 min at 500 × *g* (1100 rpm), room temperature. Remove supernatant and seed 6×10^6 cells at a density of 500,000 cells/ml in a 25-cm^2 culture flask.

    For ease of use, resuspend cell pellet at 6×10^6 cells/ml in U937 complete growth medium. Add 1 ml cell suspension to 11 ml U937 complete growth medium in a 25-cm^2 culture flask.

4. Add IFN-γ to the seeded cells at a concentration of 0.1 mg/ml (15 µl per 10 ml cells).

Be sure to properly and gently mix the cells by gently tilting the flask to distribute IFN-γ.

5. Incubate for 24 hr.

SUPPORT PROTOCOL 2

LABELING STREPTAVIDIN BEADS WITH BIOTINYLATED PROSTATE-SPECIFIC MEMBRANE ANTIGEN RECEPTOR

Biotinylated PSMA can be attached to streptavidin-coated beads to provide a target for CIR3-labeled antibodies. Opsonized beads can be phagocytosed via ADCP as a measure of CIR3 labeling and recruitment efficacy of antibodies. The use of streptavidin beads provides a highly decorated "cell-like" surface for optimal opsonization and recognition by monocytes. This reduces the number of variables present when using live target cells, such as antibody internalization or cell lysis, and makes data analysis much clearer. This protocol describes the preparation of PSMA-coated beads for use in ADCP analysis.

Materials

Streptavidin Fluoresbrite® YG Microspheres, 6 μM, 1.05×10^8 beads/ml (e.g., Polysciences, cat. no. 24157-1)
PBS, pH 7.4
Biotinylated PSMA (e.g., ACRObiosystems, cat. no. PSA-H82Qb)
RPMI 1640 medium (e.g., Thermo Fisher Scientific, cat. no. 31800)
Microcentrifuge

1. Prepare beads: Aliquot desired number of beads from stock, and dilute in 200 μl PBS. Centrifuge 5 min at 18,000 × *g* (10,000 rpm), room temperature. Resuspend in 200 μl PBS. Repeat a total of three times.

   Each time beads are resuspended, loss occurs owing to sticking on the pipette tip. Add the desired volume of PBS to beads, and gently flick the microcentrifuge tube to resuspend beads.

2. Resuspend beads in concentrated biotinylated PSMA.
3. Incubate for 1 hr at 4°C.
4. Dilute with 200 μl RPMI medium, and wash three times with RPMI medium. Resuspend with RPMI medium to a concentration of 15×10^6 beads/ml.

   Here, 10 μl will have the necessary 150,000 beads.

REAGENTS AND SOLUTIONS

Anti-DNP antibody, 500 nM

11.3 μl anti-DNP antibody (e.g., Thermo Fisher Scientific, cat. no. A6430)
288.7 μl of 1× kinetics buffer (see recipe)
Store at 4°C for up to 8 months

Assay medium 1

RPMI 1640 medium supplemented with:
14% (v/v) low IgG fetal bovine serum (FBS; e.g., Thermo Fisher Scientific, cat. no. A3381901)
Store at 4°C for up to 1 month

Assay medium 2

RPMI 1640 medium supplemented with:
11.7% (v/v) low IgG FBS (e.g., Thermo Fisher Scientific, cat. no. A3381901)
Store at 4°C for up to 1 month

Biotin-PSMA, 75 nM

25 µg biotin-PSMA
3.3 ml of 1× kinetics buffer (see recipe)
Store at −80°C for up to 12 months

CIR1, 200 nM

10 µl of 10 µM CIR1 in DMSO
490 µl of 1× kinetics buffer (see recipe)
Prepare fresh before use

CIR3, 200 nM, + anti-DNP antibody, 100 nM, time course solution

1. Combine 11.3 µl of 13.3 µM anti-DNP antibody (e.g., Thermo Fisher Scientific, cat. no. A6430), 1443.5 µl of 1× kinetics buffer (see recipe), and 29 µl of 10 µM CIR3 in DMSO.
2. Prepare five 270-µl aliquots.
3. After 30 min, add 30 µl of 10 mM anti-DNP to aliquot 1.
4. After 1 hr, add 30 µl of 10 mM anti-DNP to aliquot 2.
5. After 2 hr, add 30 µl of 10 mM anti-DNP to aliquot 3.
6. After 4 hr, add 30 µl of 10 mM anti-DNP to aliquot 4.
7. After 21 hr, add 30 µl of 10 mM anti-DNP to aliquot 5.

The addition of DNP-glycine will compete with CIR3:antibody binding, quenching the labeling reaction. Over these different time points, one will be able to measure reaction conversion to form CIR-antibody.

CIR4, 200 nM

10 µl of 10 µM CIR4 in DMSO
490 µl of 1× kinetics buffer (see recipe)
Prepare fresh before use

DNP-glycine, 100 mM

24.1 mg DNP-glycine
1 ml DMSO
Store at −20°C

DNP-glycine, 500 mM

120.5 mg DNP-glycine
1 ml DMSO
Store at −20°C

Glycine-HCl, 10 mM, pH 2.2

100 ml Milli-Q water
111.5 mg glycine-HCl
Adjust pH to 2.2 using 1 M HCl (dropwise)
Store at 4°C for up to 3 months

Kinetics buffer, 1×

1 ml of 10× kinetics buffer (e.g., Forte Biosciences, cat. no. 18-1092)
8.9 ml PBS
100 µl DMSO
Prepare fresh before use

DMSO is not typically added to BLI buffers; the reason we do so is to ensure solubility of CIRs and DNP-glycine.

NCIR5, 200 nM

10 μl of 10 μM NCIR5 in DMSO
490 μl of 1× kinetics buffer (see recipe)
Prepare fresh before use

Milk blocking buffer, 5%

2 ml of 1× kinetics buffer (see recipe)
100 mg skim milk powder
Prepare fresh before use

U937 complete growth medium

RPMI 1640 medium supplemented with:
10% (v/v) FBS
2 mM L-glutamine
1% (v/v) penicillin/streptomycin (e.g., Thermo Fisher Scientific, cat. no. 15140-122)
Store at 4°C for up to 1 month

COMMENTARY

Background Information

BLI is a label-free biophysical technique for the measurement of a receptor–analyte interaction on the surface of a probe. It is an optical technique that analyzes the interference pattern of white light reflected from two surfaces (Concepcion et al., 2009). The first layer is an internal reference layer, and the second is a layer of immobilized protein. This immobilized protein (e.g., streptavidin) can be functionalized by a receptor (e.g., biotin-PSMA). As ligands bind to the immobilized protein, there is a slight increase in the thickness of the biosensor tip. Thus, the reflected white light has a slight wavelength shift compared with the internal reference. This wavelength shift is proportional to the amount of ligand binding to the probe. The change in wavelength shift over time is thus proportional to the rate of binding.

Key benefits of this assay are the real-time kinetics, label-free nature, and flexibility. Real-time kinetics give a greater understanding of the system. The label-free nature allows these assays to remain relatively simple. Flexibility of the system allows more assay orientation and formats to be attempted (e.g., incubating CIR and antibody separately or together, placing the CIR on the probe or in solution).

Flow cytometry allows for analysis of individual cells within a complex solution and conveys fluorescence information obtained from each individual cell (Adan, Alizada, Kiraz, Baran, & Nalbant, 2017). In this technique a cell suspension (most commonly treated with unique receptor-targeting fluorophores) is passed into the flow cytometer. Here, cells individually pass excitation laser(s) specific to known fluorophores used to stain these cells. The light passing straight through the cells is referred to as the FSC parameter and is generally a reflection of cell size. Light vectoring perpendicular to the cell is composed of both fluorescence emission of fluorophores, as well as refracted light known as the SSC parameter. The SSC parameter is generally a reflection of cell granularity. Alternatively, the fluorescence emission along this perpendicular vector is further selected for by various light filters and is individually detected. Each fluorescence signal on the cell reflects an individual receptor present on the plasma membrane surface. In this way, flow cytometry allows for rapid analysis of cell populations within a complex cell suspension. Alternatively, as discussed herein, a lipid-based dye can be used to nonselectively label all cells with a fluorescent dye. DiO and DiD dyes work in this manner, using long alkyl chains to hydrophobically anchor into plasma membranes. In this way, if an individual cell contains only the DiD dye, it is known to be a monocyte. Alternatively, if a cell contains only the DiO dye, it is known to be a target cell. Finally, if a cell contains both dye colors, it is concluded to be a monocytic cell that has consumed some or all of a target cell surface, a reflection of an ADCP event.

U937 human monocytes contain both Fcγ receptor IIa (CD32) and Fcγ receptor I (CD64), which bind with variable affinity to the Fc domain of human IgG antibodies (Akinrinmade et al., 2017). CD32 is capable of binding antibodies with moderate affinity around 10^{-7} to 10^{-8}, while CD64 binds tightly with

an affinity around 10^{-8} to 10^{-9} depending on the extent of avidity binding and clustering involved. Further, it is known that these monocytes can be further activated to induce five times more CD64, as well as cluster this CD64 through treatment with IFN-γ (Akinrinmade et al., 2017; Chen, Song, Li, & Zhang, 2019). A multivalent complex between clustered surface CD64-Fc domains on targets can induce monocyte membrane curvature and downstream signaling through the CD64 γ_2 domain. This induces ADCP, as well as cytokine (TNF-a, IL-6) and superoxide production for enhanced material digestion, further immune activation, and eventual presentation of antigens to adaptive immune components (Akinrinmade et al., 2017; Chen et al., 2019; Nimmerjahn & Ravetch, 2008). For this reason, ADCP represents a vital component of the innate immune system and a vital gatekeeper toward adaptive immune response.

Critical Parameters and Troubleshooting

It is essential to have confidence in the quality and consistency of reagents. CIRs hydrolyze over time, rendering them unable to recruit antibody. Furthermore, hydrolyzed CIR fragments may act as competitive inhibitors, leading to suboptimal results. For this reason CIR should be treated as a protein reagent, being aliquoted and frozen for individual-use stocks. Similarly, if a portion of the PSMA-biotin linkages cleaves, the biotin fragment will competitively block binding to streptavidin for both BLI and flow cytometry analysis. DNP-glycine is prone to solubility issues; thus, the effective concentration of the inhibitor may be lower than expected. Lastly, ensure cell surface receptor expression is consistent. For each experiment, passage the same amount of cells (over the same time frame) prior to the experiment to ensure consistent confluency.

Moreover, it is important to ensure buffer stability and compatibility. For example, RPMI medium contains biotin, which may interfere with flow cytometry experiments containing streptavidin beads or CIR1. Noting avidity effects on biosensor/bead and cell surfaces decorated with a target protein is critically important to correctly evaluate covalent immune recruitment, as multivalent arrays of CIR/NCIR can bind with high avidity to antibodies. The effects of this apparent affinity increase on ternary complex stability and immune activation are currently under investigation.

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Author Contributions

Eden Kapcan: Data curation; formal analysis; investigation; methodology; validation; writing-original draft. **Benjamin Lake:** Data curation; formal analysis; investigation; methodology; validation; writing-original draft. **Zi Yang:** Data curation; formal analysis; investigation; methodology; validation. **Anthony F. Rullo:** Conceptualization; formal analysis; funding acquisition; project administration; supervision.

Literature Cited

Adan, A., Alizada, G., Kiraz, Y., Baran, Y., & Nalbant, A. (2017). Flow cytometry: Basic principles and applications. *Critical Reviews in Biotechnology*, *37*, 163–176. doi: 10.3109/07388551.2015.1128876.

Akinrinmade, O. A., Chetty, S., Daramola, A. K., Islam, M. U., Thepen, T., & Barth, S. (2017). CD64: An attractive immunotherapeutic target for M1-type macrophage mediated chronic inflammatory diseases. *Biomedicines*, *5*, 56. doi: 10.3390/biomedicines5030056.

Bandara, N. A., Bates, C. D., Lu, Y., Hoylman, E. K., & Low, P. S. (2017). Folate-hapten–mediated immunotherapy synergizes with vascular endothelial growth factor receptor inhibitors in treating murine models of cancer. *Molecular Cancer Therapeutics*, *16*, 461–468. doi: 10.1158/1535-7163.MCT-16-0569.

Chen, X., Song, X., Li, K., & Zhang, T. (2019). FcγR-binding is an important functional attribute for immune checkpoint antibodies in cancer immunotherapy. *Frontiers Immunology*, *10*, 292. doi: 10.3389/fimmu.2019.00292.

Concepcion, J., Witte, K., Wartchow, C., Choo, S., Yao, D., Persson, H., … Tan, H. (2009). Label-free detection of biomolecular interactions using biolayer interferometry for kinetic characterization. *Combinatorial Chemistry & High Throughput Screening*, *12*, 791–800. doi: 10.2174/138620709789104915.

Gallagher, S. R. (2012). SDS-polyacrylamide gel electrophoresis (SDS-PAGE). *Current Protocols Essential Laboratory Techniques*, *6*, 7.3.1–7.3.28. doi: 10.1002/9780470089941.et0703s06.

Hirasawa, S., Kitahara, Y., Okamatsu, Y., Fujii, T., Nakayama, A., Ueno, S., … Taki, M. (2019). Facile and efficient chemoenzymatic semisynthesis of Fc-fusion compounds for half-life extension of pharmaceutical components. *Bioconjugate Chemistry*, *30*, 2323–2331. doi: 10.1021/acs.bioconjchem.9b00235.

Lake, B., Serniuck, N., Kapcan, E., Wang, A., & Rullo, A. F. (2020). Covalent immune recruiters: Tools to gain chemical control over immune recognition. *ACS Chemical Biology*, *15*, 1089–1095. doi: 10.1021/acschembio.0c00112.

Lu, Y., Sega, E., Leamon, C. P., & Low, P. S. (2004). Folate receptor-targeted immunotherapy of cancer: Mechanism and therapeutic potential. *Advanced Drug Delivery Reviews*, *56*, 1161–1176. doi: 10.1016/j.addr.2004.01.009.

Mortensen, M. R., Nielsen, N. L., Palmfeldt, J., & Gothelf, K. V. (2019). Imidazole carbamate probes for affinity guided azide-transfer to metal-binding proteins. *Organic & Biomolecular Chemistry*, *17*, 1379–1383. doi: 10.1039/C8OB03017K.

Murelli, R. P., Zhang, A. X., Michel, J., Jorgensen, W. L., & Spiegel, D. A. (2009). Chemical control over immune recognition: A class of antibody-recruiting small molecules that target prostate cancer. *Journal of the American Chemical Society*, *131*, 17090–17092. doi: 10.1021/ja906844e.

Nimmerjahn, F., & Ravetch, J. V. (2008). Fcγ receptors as regulators of immune responses. *Nature Reviews Immunology*, *8*, 34–47. doi: 10.1038/nri2206.

Rullo, A. F., Fitzgerald, K. J., Muthusamy, V., Liu, M., Yuan, C., Huang, M., … Spiegel, D. A. (2016). Re-engineering the immune response to metastatic cancer: Antibody-recruiting small molecules targeting the urokinase receptor. *Angewandte Chemie (International Edition in English)*, *55*, 3642–3646. doi: 10.1002/anie.201510866.

Sheridan, R. T. C., Hudon, J., Hank, J. A., Sondel, P. M., & Kiessling, L. L. (2014). Rhamnose glycoconjugates for the recruitment of endogenous anti-carbohydrate antibodies to tumor cells. *ChemBioChem*, *15*, 1393–1398. doi: 10.1002/cbic.201402019.

Uvyn, A., De Coen, R., Gruijs, M., Tuk, C. W., De Vrieze, J., Van Egmond, M., & De Geest, B. G. (2019). Efficient innate immune killing of cancer cells triggered by cell-surface anchoring of multivalent antibody-recruiting polymers. *Angewandte Chemie (International Edition in English)*, *58*, 12988. doi: 10.1002/anie.201905093.

Zhang, A. X., Murelli, R. P., Barinka, C., Michel, J., Cocleaza, A., Jorgensen, W. L., … Spiegel, D. A. (2010). A remote arene-binding site on prostate specific membrane antigen revealed by antibody-recruiting small molecules. *Journal of the American Chemical Society*, *132*, 12711–12716. doi: 10.1021/ja104591m.

Key Reference

Lake et al. (2020). See above.

Introduced covalent immune recruiter (CIR) technology and demonstrated CIR capacity to selectively label specific antibodies in a discrete location to provide novel and chosen specificity.

Internet Resources

https://www.sybil-fp7.eu/node/95

ImageJ densitometry analysis.