

Assay for Assessing Mucin Binding to Bacteria and Bacterial Proteins

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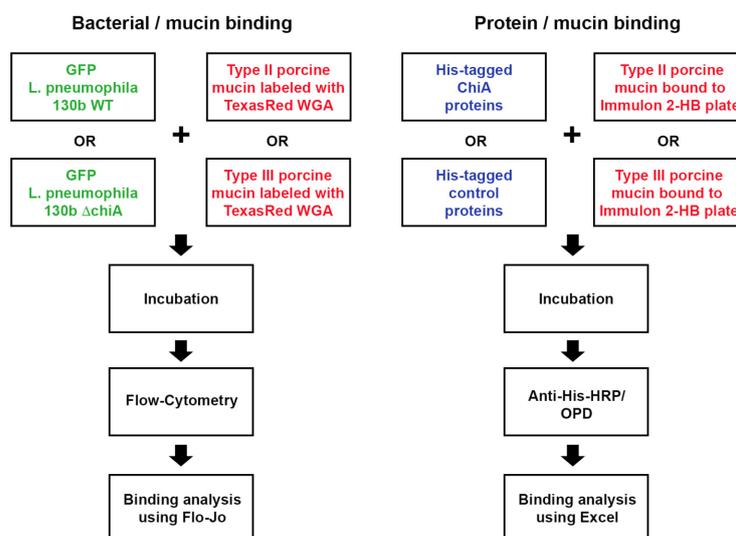
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[Abstract] *Legionella pneumophila*, a Gram-negative bacterium and the causative agent of Legionnaires' disease, exports over 300 effector proteins/virulence factors, through its type II (T2SS) and type IV secretion systems (T4SS). One such T2SS virulence factor, ChiA, not only functions as a chitinase, but also as a novel mucinase, which we believe aids ChiA-dependent virulence during lung infection. Previously published protocols manipulated wild-type *L. pneumophila* strain 130b and its *chiA* mutant to express plasmid-encoded GFP. Similarly, earlier studies demonstrated that wheat germ agglutinin (WGA) can be fluorescently labeled and can bind to mucins. In the current protocol, GFP-labeled bacteria were incubated with type II and type III porcine stomach mucins, which were then labeled with TexasRed-tagged WGA and analyzed by flow-cytometry to measure the binding of bacteria to mucins in the presence or absence of endogenous ChiA. In addition, we analysed binding of purified ChiA to type II and type III porcine stomach mucins. This protocol couples both bacterial and direct protein binding to mucins and is the first to measure Gram-negative bacterial binding to mucins using WGA and flow-cytometric analysis.

Graphic abstract:



Strategy for assessing bacterial and protein binding to mucins

Keywords: Mucin-bacteria binding, Mucin, ELISA, Mucin-binding proteins, Bacterial flow-cytometry, *Legionella pneumophila*

[Background] *Legionella pneumophila* (*Lpn*), a Gram-negative bacterium, is the causative agent of Legionnaires' disease, a severe form of pneumonia. *Lpn* is an intracellular pathogen that produces over 300 protein effectors that it secretes through either a type II secretion system (T2SS), or through a type IV secretion system (T4SS) (Hubber and Roy, 2010; White and Cianciotto, 2019). ChiA is one such T2SS protein effector. ChiA is an 81-kDa endochitinase that has a role in *Lpn* virulence during lung infection (Rehman *et al.*, 2020). *Lpn* carrying a deletion of the ChiA gene ($\Delta chiA$) shows decreased survival in the lungs of mice, compared to WT *Lpn* (DebRoy *et al.*, 2006). While humans do not produce chitin, they do produce analogous glycoproteins, mucins, that have known properties in interacting with and blocking infection of other pathogens. In our study, we showed, for the first time, that ChiA is able to both bind to and degrade mucins (Rehman *et al.*, 2020). To determine if live bacteria were able to bind to mucins, we utilized our previously published protocol to manipulate wild-type *Lpn* strain 130b and its *chiA* mutant to express a GFP-producing plasmid (DebRoy *et al.*, 2006; Rondelet and Condemine, 2013). Furthermore, earlier studies showed that wheat germ agglutinin (WGA) binds to mucins (Bhavanandan and Katlic, 1979; Valdizan *et al.*, 1992). Therefore, we utilized porcine stomach mucins which we labeled with TexasRed-conjugated WGA (Model *et al.*, 2009). To determine whether ChiA directly binds to these mucins, we also used an ELISA based-assay with purified recombinant (N-terminally His-tagged) ChiA and detected binding using anti-His₆ antibodies.

Although mucin binding to bacteria has been studied previously (Naughton *et al.*, 2014), and fluorescently labeled WGA has been studied in the context of Gram-positive bacteria (Fife *et al.*, 2000) this is the first protocol to directly label mucins and bacteria and then utilize flow cytometry to measure mucin binding to a Gram-negative bacterium. Furthermore, by analyzing both bacterial binding to mucins and the binding of purified proteins to mucins, this protocol provides insight into synergistic binding of different surface exposed bacterial proteins to mucins. This protocol can be further applied to study mucin binding to other Gram-negative bacteria.

Part I: Bacterial Mucin Binding with Flow Cytometry

Materials and Reagents

1. *Legionella pneumophila* Brenner *et al.* (1979) (ATCC® BAA-74™)
 - a. WT 130b purchased from ATCC (see above)
 - b. ChiA mutant as previously reported (DebRoy *et al.*, 2006)
 - c. GFP plasmid (pMGFP), as previously reported, was derived from pMMB-GRN/pMMB-Gent (addgene 45475). GFP is expressed from a Ptac promoter and therefore IPTG is required (White and Cianciotto, 2016) (Sturgill-Koszycki and Swanson, 2000). While copy number in *Legionella* is unknown, the pMMB-Gent derives from pMMB67EH (ATCC 37622)

2. 1× Phosphate Buffered Saline (PBS) (Corning, catalog number: 21-040-CM)
3. IPTG (Sigma-Aldrich, catalog number: I6758)
4. Type II porcine stomach mucin (Sigma-Aldrich, catalog number: M1778)
5. Type III porcine stomach mucin (Sigma-Aldrich, catalog number: M2378)
6. TexasRed-tagged WGA (ThermoFisher Scientific, catalog number: W21405)
7. Sodium carbonate, Na₂CO₃ (Sigma-Aldrich, catalog number: S7795)
8. Sodium bicarbonate, NaHCO₃ (Sigma-Aldrich, catalog number: S5761)
9. Buffered Charcoal Yeast Extract (BCYE) agar plates (see Recipes)
 - a. ACES (Sigma-Aldrich, catalog number: A9758)
 - b. KOH (Sigma-Aldrich, catalog number: 221473)
 - c. Yeast Extract (Sigma-Aldrich, catalog number: Y1625)
 - d. α-Ketoglutaric acid sodium salt (Sigma-Aldrich, catalog number: K1875)
 - e. Activated Charcoal (Sigma-Aldrich, catalog number: C9157)
 - f. Bacteriological Agar (Sigma-Aldrich, catalog number: A5306)
 - g. L-cysteine HCl (Sigma-Aldrich, catalog number: C1276)
 - h. Ferric pyrophosphate (Sigma-Aldrich, catalog number: P6526)
10. 50 mM Carb/Bicarb Buffer (see Recipes)
11. Mucin solution (see Recipes)

Equipment

1. Flow Cytometer (<https://www.bdbiosciences.com/en-us/go-campaign/lsr-ii-comp-cont>) using Blue Laser (488 nm), Long pass Filter 600 and 505, Band Pass Filter 600-620 and 500-550 (BD Biosciences, model: LSR II)
2. General Purpose UV/Vis Spectrophotometer (Beckman Coulter, model: DU720)
3. Forced Air Microbiological Incubators (VWR, catalog number: 89511-430)

Software

1. GraphPad Prism version 8.0.0 for Mac (GraphPad Software, San Diego, California USA, www.graphpad.com)
2. FlowJo™ Software for Mac, Version 8.8.6. (Ashland, OR: Becton, Dickinson and Company, www.flowjo.com)

Procedure

A. *Lpn* Preparation

1. Streak out *Lpn* strain 130b and ChiA mutant NU319, both harboring a GFP-expressing plasmid, onto BCYE agar plates containing IPTG at 1 mM.

2. Incubate the plates for 3 days in a 37 °C incubator to grow out a lawn of bacteria.
3. Resuspend ~3 swabs of scraped bacteria into 5 ml of PBS and read the optical density (OD) of each suspension at 660 nm using a DU720 spectrophotometer. Use PBS to dilute the bacterial suspension to an OD of 0.3, which corresponds to approx. 1×10^9 CFU/ml.

B. Mucin Preparation

Prepare both type II and type III porcine stomach mucin solutions in carb/bicarb buffer according to the recipe below.

C. Mucin/*Lpn* Incubation

1. Statically incubate 1 ml of the *Lpn* suspension with 100 µl of either type II porcine stomach mucin, type III porcine stomach mucins, or PBS for 1 h in a 25 °C or 37 °C incubator. Repeat every condition in triplicate.

Note: Keep an aliquot of the type II and type III porcine stomach mucin solutions (no bacteria) as controls for the flow-cytometry analysis.

2. Centrifuge, at room temperature, the mixtures for 5 min at $4,000 \times g$ (for the 37 °C samples) or $8,000 \times g$ (for the 25 °C samples).
3. Wash each pellet in 1 ml of PBS
4. Repeat centrifugation and wash step (Steps C2 and C3) three times.
5. Resuspend pellet in 1 ml of PBS containing 7.5 µg of TexasRed-tagged WGA and incubate statically for 15 min at 25 °C.
6. Repeat the centrifugation and wash steps (Steps C2 and C3) three times.
7. Resuspend the pellet in 500 µl of PBS.

D. Flow Cytometry

Use BD LSRII flow cytometer to analyze suspensions via a TexasRed filter (Blue Laser – 488 nm: Long Pass 600, Band Pass 600-620) and GFP Filter (Blue Laser – 488 nm: Long Pass 505, Band Pass 500-550).

1. Set gating TexasRed and GFP parameters using only mucin and only bacteria respectively.
2. Collect a minimum of 500,000 events.

Data analysis

The experiment was replicated a minimum of three times, and each experiment had three technical replicates. FlowJo software was used to analyze percent of mucin binding to *Lpn*. Bacteria capable of expressing GFP and isolated mucin were used to set thresholds for both the TexasRed- and GFP-negative populations (unlabeled mucins and no IPTG-dependent GFP expression) and the TexasRed- and GFP-positive populations (TexasRed labeled mucins and IPTG-induced GFP expression). After setting these thresholds (see below for the four histograms on the left), the

percent population (see below for quadrant analysis in the right-most flow-plot) of single GFP-positive (Quadrant [Q]3), single TexasRed-positive (Q1), and double GFP and TexasRed positive (Q2) were calculated using FloJo quadrant tool. Thus, Q2 was used as a calculation of percent of mucin binding to bacteria. Due to variability in mean fluorescence between experimental days, data were normalized to GFP-Lpn incubated with TexasRed fluorophore alone (background), see sample data table below and Figure 4D (Rehman *et al.*, 2020). GraphPad Prism8 statistical software was used to graph and analyze the percent binding of bacteria to mucins. Prism8 analysis quantified standard deviation and used the two-tailed Student's *t*-test function to test for significant differences between groups tested. Only *P*-values less than 0.05 were determined to be significant.

Flow Cytometry Protocol (Figure 1)

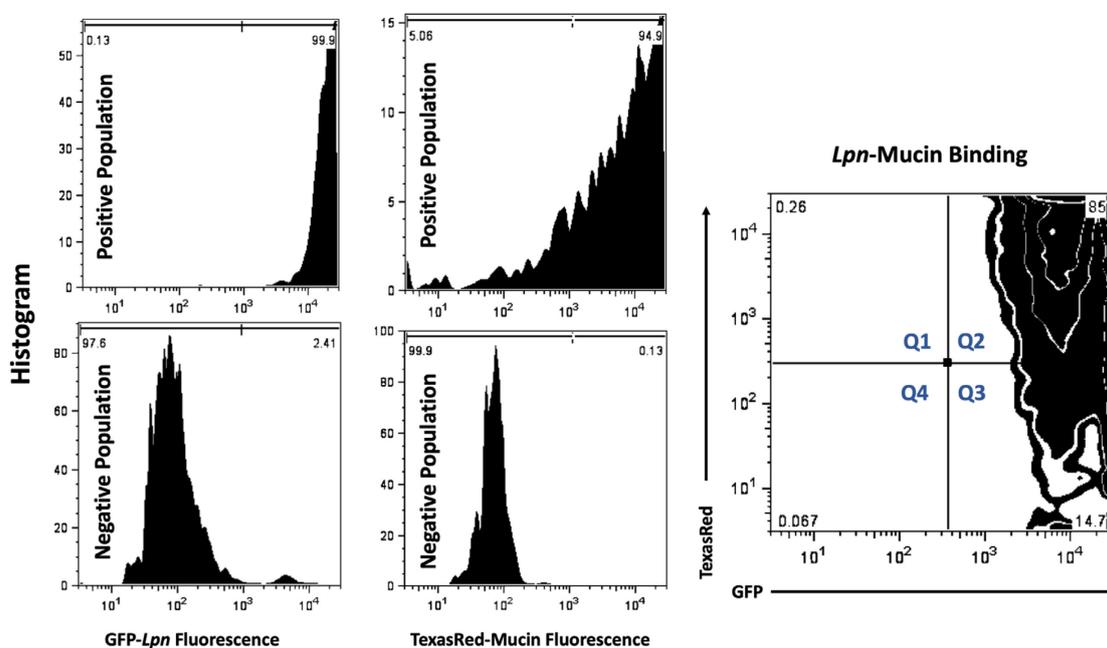


Figure 1. Flow Cytometry. Thresholds were set using single-positive samples 1. IPTG induced GFP production in *Lpn* (top left histogram) and 2. non-GFP bacteria with TexasRed labeled mucins (top middle histogram). Single negative populations were set using 1. Bacteria cultured without IPTG (bottom left histogram) 2. Non-GFP bacteria with un-labeled mucins (bottom middle histogram). For sample analysis, IPTG induced, GFP-expressing bacteria were co-incubated with TexasRed-labeled mucins. Gating parameters were used to setup quadrant percentiles for analysis. All events collected were partitioned into four quadrants using FloJo tool. Percentage of total population that were only TexasRed positive were labeled Q1, double GFP and TexasRed bacterial populations were labeled Q2, single GFP-positive populations were designated Q3 and non-fluorescent, all negative, populations were designated Q4. Percent of population that was GFP and TexasRed double positive (Q2) was used as a metric for mucin-bacteria binding and was analyzed in the sample table (Table 1) below.

Sample Data (Table 1)

Table 1. Percent of population that was double positive for TexasRed-mucin and GFP-Lpn (Q2) was used as a value for mucin binding to bacteria. In the single example of WT behavior in the flow plot shown above, this value was 85% (right-most plot in Figure 1). This value plus the values obtained from two additional independent replicates testing WT bacteria were added to the third row in the first three columns on the left, *i.e.*, under headers Rep1, Rep2, Rep3. The raw values for three technical replicates obtained from analysis of another bacterial sample; *i.e.*, a ChiA mutant, appear in the three left-most lanes in row-4. The value for each of the replicates was then normalized to GFP-bacteria (WT or mutant) incubated with TexasRed alone (as noted in rows 1 and 2), to quantify background TexasRed fluorophore binding to bacteria. For the two bacterial samples incubated with mucin, these values appear in columns 5, 6, and 7 for rows 3 and 4. Finally, the normalized values were averaged, and standard deviations calculated, as indicated in the last two columns. In this sample dataset, 22.5 + 5.4% of WT bacteria significantly bound the mucin.

	Rep1 GFP+ TexasRed+ Q2	Rep2 GFP+ TexasRed+ Q2	Rep3 GFP+ TexasRed+ Q2	Normalization	PBS Normalized Rep1	PBS Normalized Rep2	PBS Normalized Rep2	Mean	Stdev
WT_PBS_TexasRed	3	4	5	(GFP + TexasRed+ Quadrant 2)/(Quad2 WT PBS)	3/3=1	5/5=1	6/6=1	1	0
ChiA_PBS_TexasRed	2	3	4	(GFP + TexasRed+ Quadrant 2)/(Quad2 ChiA PBS)	5/5=1	6/6=1	7/7=1	1	0
WT_TexasRed_Mucin	85	87	88	(GFP + TexasRed+ Quadrant 2)/(Quad2 WT PBS)	85/3 =28.3	87/4=21.7	88/5=17.6	22.5	5.4
ChiA_TexasRed_Mucin	89	91	92	(GFP + TexasRed+ Quadrant 2)/(Quad2 ChiA PBS)	89/2=44.5	85/3=28.3	92/4=23	31.9	11.2

Recipes

1. BCYE agar plates
 - a. Add 10 g of ACES to ~900 ml of double distilled water. Stir into solution
 - b. Add ~2.2 g KOH. Stir into solution
 - c. Add 10 g of Yeast Extract and 1 g α -ketoglutaric acid. Stir into solution
 - d. Adjust the pH of the broth to 6.85-6.95. Use concentrated HCl or 10 N KOH as required. Add double distilled water to 1 L
 - e. Dispense the broth into a 2-L flask
 - f. Add 1.5 g activated charcoal and 15 g of agar
 - g. Autoclave 20 min at 121 °C
 - h. Prepare cysteine and iron solutions respectively by adding 0.4 g of L-cysteine HCl to 10 ml double distilled water and 0.25 g ferric pyrophosphate to 10 ml double distilled water. Once each solution is made, sterilize the solution by filter (0.22 μ m).
 - i. Cool the medium to 50 °C in a water bath after autoclaving
 - j. Add separately 10 ml filter-sterilized (0.22 μ m) cysteine and 10 ml filter-sterilized (0.22 μ m) ferric pyrophosphate to the medium

- k. If making plates with IPTG, add 1 ml of 1 M Filter Sterilized (0.22 μ m) IPTG
- l. Cool to 40 °C
- m. Pour plates and store at 4 °C once solidified
2. 50 mM Carb/Bicarb Buffer
 - a. Dissolve 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ in 1 L of deionized water
 - b. Adjust the pH to 9.6 using HCl or NaOH
3. Mucin solution

Stir for 30 min to dissolve 10 mg of either type II or type III porcine stomach mucin in 100 ml of 50 mM Carb/Bicarb buffer above. Autoclave for 15 min at 135 °C to sterilize and to further dissolve the mucin*.

**Note: Mucin is difficult to dissolve and will not fully go into solution by mixing alone; thus, autoclaving is required to get a homogeneous solution. Fresh preparation of mucin is recommended for each experiment.*

Part II: Recombinant Protein Mucin Binding with ELISA

Materials and Reagents

1. Immulon 2-HB 96-well plates (VWR, catalog number: 735-0464)
2. Purified N-terminally His-tagged protein samples
3. Anti-His HRP-conjugated antibody (Sigma-Aldrich, catalog number: SAB4301134)
4. Mucins from porcine stomachs type II (Sigma-Aldrich, catalog number: M2378)
5. Mucins from porcine stomachs type III (Sigma-Aldrich, catalog number: M1778)
6. Sodium carbonate, Na₂CO₃ (Sigma-Aldrich, catalog number: S7795)
7. Sodium bicarbonate, NaHCO₃ (Sigma-Aldrich, catalog number: S5761)
8. Bovine serum albumin (BSA) (Melford, catalog number: A30075)
9. Phosphate buffered saline (PBS) (Fisher Scientific, catalog number: BP399-1)
10. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
11. Zinc chloride, ZnCl₂ (Sigma-Aldrich, catalog number: 229997)
12. Tween 20 (Sigma-Aldrich, catalog number: P1379)
13. o-Phenylenediamine dihydrochloride tablets (OPD) (Sigma-Aldrich, catalog number: P9187)
14. SnakeSkin® dialysis tubing (Thermo Scientific, catalog number: 68100)
15. Incubation buffer (see Recipes)
16. 50 mM Carb/Bicarb Buffer (see Recipes)
17. Mucin solution (see Recipes)
18. Blocking buffer (see Recipes)

Equipment

1. Plate reader (LabSystems iEMS Reader MF, catalog number: 5921200) using 450 nm filter
2. Incubator (New Brunswick Innova 4230)
3. Centrifuge (Eppendorf, model: 5810R)

Software

1. Ascent Microplate Reader Software version 2.7.0 for Windows
2. Microsoft Excel version 16.43 for Mac; although any database analysis software is appropriate

Procedure

A. Protein preparation

Dialyze sample proteins at 100 μ M into incubation buffer, prepared according to the recipe below. Adjust the final protein concentration after dialysis to 10 μ M.

B. Mucin preparation

Prepare both type II and type III porcine stomach mucin solutions in carb/bicarb buffer according to the recipe below.

C. Incubation

1. Statically incubate an Immulon 2-HB 96-well plate with 50 μ l of either type II porcine stomach mucin, type III porcine stomach mucins, or PBS overnight in a 4 °C incubator. Repeat every condition in triplicate.
2. Remove the mucin solution and block the wells with 200 μ l of blocking buffer, according to the recipe below, statically for 1 h in a 24 °C incubator.
3. Remove the blocking buffer and wash once by adding 200 μ l of incubation buffer and incubating statically on the bench for 5 min. Next incubate the wells statically for 3 h in a 24 °C incubator with 50 μ l of purified protein samples or PBS.
4. Wash with 200 μ l of incubation buffer as described above and repeat for a total number of 4 washing steps.

D. Detection

1. Remove the buffer and incubate the wells with 50 μ l of anti-His-HRP antibody, diluted 1:2,000 in incubation buffer, statically for 1 h in a 24 °C incubator.
2. Wash four times as described above with 200 μ l of incubation buffer.
3. Add 150 μ l of *o*-Phenylenediamine dihydrochloride to each well and leave the plate in a 24 °C incubator without shaking for 30 min in the dark.

- Record at 450 nm using a plate reader.

Data analysis

The experiment was replicated a minimum of three times, and each experiment had three technical replicates. Data were imported into Microsoft Excel software and baseline corrected with the incubation with PBS alone samples. Sample data for ChiA, NttE (negative control) and SsIE (positive control) is given below for binding to type II porcine stomach mucin (also see Figure 4C, Rehman *et al.*, 2020). Excel analysis provided standard deviation values and a two-tailed Student's *t*-test function was used to test for significant differences between groups tested. Only *P*-values less than 0.05 were determined to be significant.

Sample Data (Table 2)

Table 2. Example data for protein binding to type II stomach mucin extracts

	Rep1	Rep2	Rep3	Mean (PBS)	PBS subtracted Rep 1	PBS subtracted Rep 2	PBS subtracted Rep 3	Mean (mucin)	Stdev (mucin)
ChiA_PBS	0.239	0.190	0.181	0.203	-	-	-	-	-
NttE_PBS (-)	0.084	0.074	0.062	0.073	-	-	-	-	-
SsIE_PBS (+)	0.130	0.164	0.156	0.150	-	-	-	-	-
ChiA_mucin	0.933	0.846	0.900	-	0.730	0.643	0.697	0.690	0.044
NttE_mucin (-)	0.138	0.119	0.151	-	0.065	0.046	0.078	0.063	0.016
SsIE_mucin (+)	0.718	0.912	0.910	-	0.568	0.762	0.760	0.697	0.111

Recipes

- Incubation buffer (prepare fresh and store at 4 °C)
 - Mix/dissolve 0.5 g BSA and 0.5 ml Tween-20 for every 1 L of PBS buffer
 - Cool to 4 °C
- 50 mM Carb/Bicarb Buffer (can be prepared in advance and stored at room temperature)
 - Dissolve 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ in 1 L of deionized water
 - Adjust the pH to 9.6 using HCl or NaOH
- Mucin solution (can be prepared the day before and stored at 4 °C)
 - Stir for 30 min to dissolve 10 mg of either type II or type III porcine stomach mucin in 100 ml of 50 mM Carb/Bicarb buffer above.
 - Autoclave for 15 min at 135 °C to sterilize and to further dissolve the mucin*.

**Note: Mucin is difficult to dissolve and will not fully go into solution by mixing alone; thus, autoclaving is required to get a homogeneous solution.*
- Blocking buffer (can be prepared the day before and stored at 4 °C)
 - Mix/dissolve 0.1 g BSA and 0.5 ml Tween-20 for every 1 L of PBS buffer
 - Cool to 4 °C

Acknowledgments

Work in the Cianciotto lab was supported by a National Institutes of Health grant R01AI 043987. LSG and RCW were also partly supported by National Institutes of Health training grants T32 GM08061 and T32 AI0007476, respectively. We thank the Northwestern Immunobiology Flow Cytometry Core for the maintenance and use of their equipment. Work in the Garnett lab was supported by MRC grants MR/M009920/1 and MR/R017662/1.

Competing interests

NPC and JAG have a pending patent application (63/005,592) that describes the use of ChiA for therapeutic applications.

Ethics

No human or animal subjects were used in this protocol.

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