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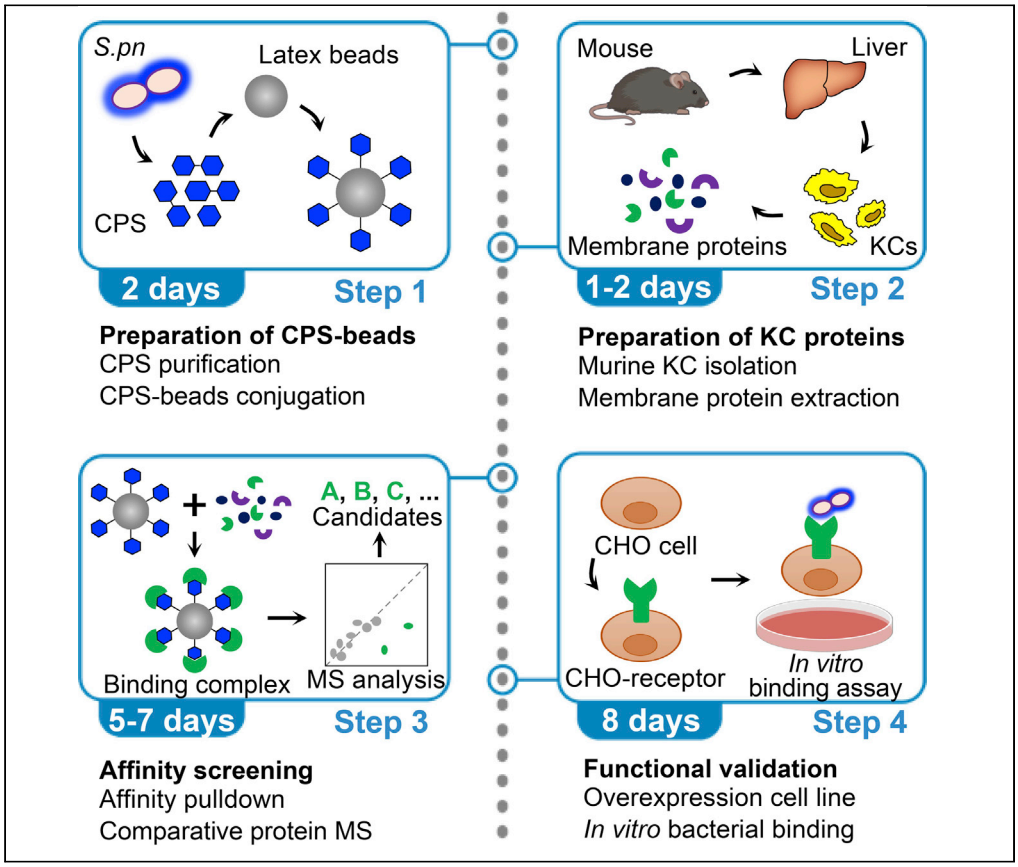
Identification of the mouse Kupffer cell receptors recognizing pneumococcal capsules by affinity screening



Kupffer cells (KCs) are the major sentinels to guard the bloodstream by recognizing diverse microbial ligands of blood-borne pathogens. Here, we establish a protocol for identifying the KC receptors recognizing the capsular polysaccharides (CPSs) of low-virulence *Streptococcus pneumoniae* in a mouse model. This protocol includes preparation of CPS-coated microspheres and KC membrane proteins, affinity pulldown of CPS-binding proteins, and functional validation of the CPS receptors. This protocol provides a platform to investigate the receptor-ligand interactions between KCs and encapsulated bacteria.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for preparation of pneumococcal capsule-coated microspheres

Protocol for extraction of membrane proteins from mouse liver Kupffer cells

High-throughput affinity screening of capsule-binding receptors

In vitro assay for functional validation of capsule-binding receptors

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Identification of the mouse Kupffer cell receptors recognizing pneumococcal capsules by affinity screening

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SUMMARY

Kupffer cells (KCs) are the major sentinels to guard the bloodstream by recognizing diverse microbial ligands of blood-borne pathogens. Here, we establish a protocol for identifying the KC receptors recognizing the capsular polysaccharides (CPSs) of low-virulence *Streptococcus pneumoniae* in a mouse model. This protocol includes preparation of CPS-coated microspheres and KC membrane proteins, affinity pulldown of CPS-binding proteins, and functional validation of the CPS receptors. This protocol provides a platform to investigate the receptor-ligand interactions between KCs and encapsulated bacteria.

For complete details on the use and execution of this protocol, please refer to An et al. (2022).[1]

BEFORE YOU BEGIN

Liver Kupffer cells (KCs) represent the largest population of tissue resident macrophages in the host. KCs have long been regarded as the primary phagocytes that rapidly eliminate blood-borne pathogens including viruses, bacteria, fungi, and parasites.[2] This immune function largely depends on the KC membrane receptors such as scavenger receptors, complement receptors, and Fc receptors. Conversely, many encapsulated bacteria, the most prevalent blood-borne pathogens, resist the KC surveillance by the capsule shield.[1,3] As exemplified, the interplay between KCs and *Streptococcus pneumoniae* determines the virulence of the pathogen in a capsular serotype-dependent manner. Although the high-virulence serotypes completely evade the KC recognition, some low-virulence capsules can be recognized by specific receptors on the KC surface leading to rapid clearance of the cognate pneumococcal strains.[1] This STAR protocol provides a feasible method for the *de novo* discovery of specific KC receptors that recognize and bind bacterial capsular polysaccharides (CPSs). This protocol can be also applied to identify the CPS receptors or CPS binding proteins of many other immune cells and plasma samples.

Institutional permissions

The animal experiments described in this protocol were conducted in accordance with the animal protocols (No. 14-ZJR1) approved by the Institutional Animal Care and Use Committee (IACUC) in Tsinghua University. Readers who want to conduct their animal experiments as described in this protocol will need to acquire the permission from the IACUC in their institutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-mouse CD31 (Clone: 390)	BioLegend	Cat# 102409; RRID: AB_312904
APC-Cy7 anti-mouse CD45 (Clone: 30-F11)	BioLegend	Cat# 103116; RRID: AB_312981
FITC anti-mouse F4/80 (Clone: BM8)	BioLegend	Cat# 123108; RRID: AB_893502
PE anti-mouse Tim-4 (Clone: RMT4-54)	BioLegend	Cat# 130005; RRID: AB_1227807
BV605 anti-mouse CD11b (Clone: M1/70)	BioLegend	Cat# 101257; RRID: AB_2565431
Anti-mouse CD16/32	BioLegend	Cat# 101301; RRID: AB_312800
Bacterial and Virus Strains		
Streptococcus pneumoniae TH2912, serotype 14	This study	N/A
Streptococcus pneumoniae TH2884, serotype 8	This study	N/A
Escherichia coli DH5α	Biomed	Cat# BC102-01
Chemicals, Peptides, and Recombinant Proteins		
DMEM	Gibco	Cat# 11965092
F-12K	Gibco	Cat# 21127022
Opti-MEM	Gibco	Cat# 31985062
Fetal bovine serum (FBS)	Biological Industries	Cat# 04-001-1ACS
Penicillin/Streptomycin	Gibco	Cat# 15140122
Sodium pyruvate solution	Sigma-Aldrich	Cat# S8636-100ML
Non-essential amino acid (NEAA) solution	Gibco	Cat# 11140050
0.25% Trypsin	Gibco	Cat# 15050065
Lipofectamine 2000	Invitrogen	Cat# 11668019
PLUS Reagent	Invitrogen	Cat# 11514015
Polybrene	Sigma-Aldrich	Cat# TR-1003-G
Puromycin	Invivogen	Cat# ant-pr-1
Zeocin	Invivogen	Cat# ant-zn-1p
Todd-Hewitt broth	Oxoid	Cat# CM0189
Tryptone soya agar	Oxoid	Cat# CM0131B
Tryptone	Oxoid	Cat# LP0042
Yeast extract	Oxoid	Cat# LP0021
Ampicillin	Millipore	Cat# 171254-5GM
Collagenase IV	Sigma-Aldrich	Cat# C5138-1G
DNase I	Roche	Cat# 10104159001
T4 DNA ligase	NEB	Cat# M0202S
HBSS	Corning	Cat# 21-022-CV
PBS	Corning	Cat# 21-040-CVC
RBC lysis buffer (10×)	BioLegend	Cat# 421301
Halt Protease and Phosphatase Inhibitor Cocktail	Thermo Scientific	Cat# 78442
DMTMM	Sigma-Aldrich	Cat# 74104-5G-F
Tween-20	Sigma-Aldrich	Cat# P9416-100ML
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# V900933-100G
Sodium azide	Sigma-Aldrich	Cat# S2002-100G
$CaCl_2$	Sigma-Aldrich	Cat# C4901-100G
$MgCl_2$	Sigma-Aldrich	Cat# M8266-100G
NaCl	Sigma-Aldrich	Cat# S9888-500G
Sodium deoxycholate	Sigma-Aldrich	Cat# D6750-10G
Acetic acid	Sigma-Aldrich	Cat# A6283-500ML
Sodium acetate	Sigma-Aldrich	Cat# S2889-250G
CTAB	Sigma-Aldrich	Cat# H6269-100G
Ethanol	Supelco	Cat# 1.00983
Methanol	Sigma-Aldrich	Cat# 34860-1L-R
5× SDS-PAGE loading	Beyotime	Cat# P0015
Tris base	Sigma-Aldrich	Cat# T1503-1KG
30% acrylamide mix	Sigma-Aldrich	Cat# A3574-100ML

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
SDS	Sigma-Aldrich	Cat# L3771-100G
Ammonium persulfate	Sigma-Aldrich	Cat# A3678-25G
TEMED	Sigma-Aldrich	Cat# T9281-25ML
Coomassie brilliant blue R 250	Sigma-Aldrich	Cat# 1125530025
Critical Commercial Assays		
TRIzol Reagent	Invitrogen	Cat# 15596018
Maxima H Minus First Strand cDNA Synthesis Kit	Thermo Scientific	Cat# K1651
Mem-PER Plus Membrane Protein Extraction Kit	Thermo Scientific	Cat# 89842
HiPure Plasmid EF Micro Kit	Magen	Cat# P1111
HiPure Gel Pure Micro Kit	Magen	Cat# D2110
HiPure Plasmid Micro Kit	Magen	Cat# P1001
Experimental Models: Cell Lines		
Human HEK293T cell	Dr. Wanli Liu laboratory	N/A
Chinese hamster ovary (CHO) cell	Dr. Wanli Liu laboratory	N/A
Experimental Models: Organisms/Strains		
C57BL/6 (8 week, female)	Charles River	Cat# C57BL/6NCrl
Recombinant DNA		
pCDH	System Biosciences	Cat# CD510B
pFUGW	Addgene	Cat# 14883
pMD2.G	Addgene	Cat# 12259
psPAX2	Addgene	Cat# 12260
Software and Algorithms		
GraphPad Prism v8.0.0	GraphPad Software	https://www.graphpad.com/
FlowJo v10.4	BD	https://www.flowjo.com/
Other		
70 μm strainer	Corning	Cat# CLS431751
0.22 μm syringe filter	Millipore	Cat# SLGSM33SS
0.2 μm Nalgene Rapid-Flow	Thermo Scientific	Cat# 566-0020
Carboxyl latex beads, 2 μm	Invitrogen	Cat# C37278
100 kDa ultra-centrifugal filter unit	Merck Millipore	Cat# UFC810008
10 kDa ultra-centrifugal filter unit	Merck Millipore	Cat# UFC801008
3 kDa ultra-centrifugal filter unit	Merck Millipore	Cat# UFC800308

MATERIALS AND EQUIPMENT

Prepare the reagents for CPS extraction and conjugation

Timing: 1 h

- Todd-Hewitt broth with 0.5% yeast extract (THY) for pneumococcal cultivation.

THY medium		
Reagent	Concentration	Amount
Todd-Hewitt broth powder	36.4 g/L	36.4 g
Yeast extract	0.5%	5 g
ddH_2O	N/A	1 L
Total	N/A	1 L
Autoclave at 115°C for 10 min		
Store at 20°C–25°C for several months.		

- 25 mM sodium acetate solution.

Dissolve 2.05 g sodium acetate in 1 L ddH_2O to a final concentration of 25 mM. Autoclave at 121°C for 15 min. Store at 20°C–25°C for several months.

- 1 M sodium chloride solution.

Dissolve 58.44 g sodium chloride in 1 L ddH_2O to a final concentration of 1 M. Autoclave at 121°C for 15 min. Store at 20°C–25°C for several months.

- 6% sodium acetate solution.

Dissolve 30 g sodium acetate in 500 mL ddH_2O to a final concentration of 6% (w/v). Autoclave at 121°C for 15 min. Store at 20°C–25°C for several months.

- DMTMM solution.

Dissolve 1 g 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) in 5 mL ddH_2O to a final concentration of 200 mg/mL. Filter through 0.2 μm filter and store at −20°C for several months.

- PBST solution.

Dissolve 50 μL Tween-20 in 100 mL PBS to a final concentration of 0.05%. Filter through 0.2 μm filter and store at 4°C for 2 weeks.

- Conjugation blocking buffer.

Conjugation blocking buffer		
Reagent	Concentration	Amount
BSA	1%	0.1 g
Sodium azide	0.05%	5 mg
PBS	1×	10 mL
Total	N/A	10 mL
Store at 4°C for several weeks.		

⚠ CRITICAL: Sodium azide is a highly toxic chemical with acute dermal and oral toxicity. Skin exposure, inhalation or ingestion of sodium azide may cause skin burns and blisters, cough, dizziness, headache, weakness and other symptoms within minutes. Avoid direct contact and handle the reagent with care in a fume hood.

Prepare the reagents for extracting KC membrane proteins

Timing: 15 min

- Liver digestion buffer.

Liver digestion buffer		
Reagent	Concentration	Amount
Collagenase IV	0.5 mg/mL	7.5 mg
DNase I	0.02 mg/mL	0.3 mg
$CaCl_2$	0.5 mM	8.3 mg
HBSS	1×	15 mL
Total	N/A	15 mL

Note: Make 10 mg/mL collagenase IV and 2.5 mg/mL DNase I stock solution and store at −20°C for several months. Make 1 M $CaCl_2$ stock solution and store at 4°C for several weeks. Add indicated amount of the above constituents into 1× HBSS solution to make fresh digestion buffer just before use. Avoid repeated freezing and thawing. The volume of digestion buffer for one mouse liver is 15 mL.

- FACS buffer.

Dissolve 15 mL fatal bovine serum (FBS) in 485 mL PBS to a final concentration of 3%. Store at 4°C for 2 weeks.

- Antibody mixture for flow cytometry of mouse KC.

Antibody mixture		
Reagent	Concentration	Amount
APC CD31, 0.2 mg/mL	1:200	0.5 μL
APC-Cy7 CD45, 0.2 mg/mL	1:200	0.5 μL
FITC F4/80, 0.5 mg/mL	1:200	0.5 μL
PE Tim-4, 0.2 mg/mL	1:200	0.5 μL
BV605 CD11b, 0.2 mg/mL	1:500	0.2 μL
FACS buffer	1×	100 μL
Total	N/A	100 μL

Note: Prepare FACS buffer and make the antibody mixture in small volume just before use. The volume of antibody mixture for 10^7 non-parenchymal cells (NPCs), normally from one mouse liver, is 100 μL.

Prepare the reagents for CPS-receptor affinity screening

Timing: 10 min

- Binding buffer.

Binding buffer		
Reagent	Concentration	Amount
$CaCl_2$	2 mM	22.2 mg
$MgCl_2$	2 mM	19.0 mg
PBST	1×	100 mL
Total	N/A	100 mL

Note: Prepare 1 M $CaCl_2$ and 1 M $MaCl_2$ stock solution and add 200 μL of the stock into 100 mL PBST buffer. Filter through 0.2 μm filter and store at 4°C for 2 weeks.

- SDS-PAGE reagents.

1.5 M Tris (pH 8.8): dissolve 90.85 g Tris in 500 mL ddH_2O to a final concentration of 1.5 M. Adjust pH to 8.8 with hydrochloric acid and autoclave at 121°C for 15 min. Store at 4°C for several months.

1.0 M Tris (pH 6.8): dissolve 60.57 g Tris in 500 mL ddH_2O to a final concentration of 1.0 M. Adjust pH to 6.8 with hydrochloric acid and autoclave at 121°C for 15 min. Store at 4°C for several months.

10% SDS: dissolve 10 g SDS in 100 mL ddH_2O. Autoclave at 121°C for 15 min. Store at 20°C–25°C for several months.

10% ammonium persulfate (APS): dissolve 0.1 g APS in 1 mL ddH_2O, store at 4°C for 2 weeks.

- SDS-PAGE gels.

Make 12 resolving gel and 5% stacking gel just before use.

- Make Coomassie brilliant blue staining solution.

Coomassie brilliant blue staining solution		
Reagent	Concentration	Amount
Coomassie brilliant blue R 250	0.1% (w/v)	0.5 g
Methanol	50% (v/v)	250 mL
Acetic acid	10% (v/v)	50 mL
H_2O	40% (v/v)	200 mL
Total	N/A	500 mL
Store at 20°C–25°C for several months.		

⚠ CRITICAL: Methanol is toxic if swallowed or inhaled and in contact with skin. Avoid direct contact and prepare the staining solution in a fume hood.

Prepare the reagents for functional validation of CPS receptor candidates

Timing: 1 h

- Make Luria-Bertani (LB) medium for *Escherichia coli* growth.

LB medium		
Reagent	Concentration	Amount
Tryptone	1%	10 g
Yeast extract	0.5%	5 g
NaCl	1%	10 g
ddH_2O	N/A	1 L
Total	N/A	1 L
Autoclave at 121°C for 15 min		
Store at 20°C–25°C for several months.		

- Make mammalian cell medium.

293T medium		
Reagent	Concentration	Amount
FBS	10%	50 mL
Sodium pyruvate	1%	5 mL
Non-essential amino acids (NEAA)	1%	5 mL
Penicillin/Streptomycin	1%	5 mL
DMEM	N/A	435 mL
Total	N/A	500 mL
Store at 4°C for several months.		

CHO medium		
Reagent	Concentration	Amount
FBS	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
F12-K	N/A	445 mL
Total	N/A	500 mL
Store at 4°C for several months.		

Note: Add appreciate concentration of puromycin (pCDH vector) and zeocin (pFUGW vector) for maintaining CHO transfectants.

⚠ CRITICAL: Optimal concentrations in this protocol were 5 μg/mL puromycin and 300 μg/mL zeocin. The working concentration should be optimized by the researchers in their own laboratory.

Alternatives: In this protocol, CHO cells were used for overexpression of the CPS receptor candidates and subsequent *in vitro* bacterial binding assay. Other firmly adherent cell lines that are easily to transfect can be used, such as COS-7, MDCK, NIH/3T3, and HeLa cells.

STEP-BY-STEP METHOD DETAILS

Extraction of pneumococcal CPS

Timing: 1–2 day

The extraction procedure is adapted from the methods reported previously for *S. pneumoniae* that is sensitive to deoxycholate-triggered autolysis.[4] For capsule purification from Gram-negative and non-autolytic Gram-positive bacteria, the capsule component can be detached by mild detergent, e.g., zwittergent 3–14, according to the method reported elsewhere.[5] High-quality pneumococcal CPS with minimal nucleic acid and protein contaminants are generated at a yield about 10–40 mg per 1 L culture. The yield is highly dependent on the capsular serotypes of *S. pneumoniae*.

1. Culture the *S. pneumoniae* in a 2.5 L flask containing 1 L THY medium at 37°C with 5% CO_2.

   Note: Two pneumococcal strains TH2912 (serotype 14) and TH2884 (serotype 8) are used in this protocol. The serotype 14 strain is rapidly captured by liver KCs though the recognition of its capsule, and serotype 8 strain is a negative control with marginal binding on KCs.[1]

2. Monitor the optical density at 620 nm (OD_{620}) by a spectrophotometer and harvest the culture at $OD_{620\ nm}$ 0.5–0.8.

   ⚠ CRITICAL: *S. pneumoniae* is prone to autolysis at the late stationary phase. Do not culture the *S. pneumoniae* over 12 h or to an $OD_{620\ nm} > 0.8$. Overgrown bacteria may result in lower CPS production.

3. Collect the bacterial cells by centrifugation at 10,000 g for 20 min at 20°C–25°C using Thermo Scientific centrifuge Sorvall RC6+.

   Note: Thermo Scientific centrifuge Sorvall RC6+ is used in following steps in this protocol for centrifugation at 10,000 g with large volume (i.e., more than 50 mL) of samples.

4. Resuspend cell pellet in 50 mL 2% sodium deoxycholate (DOC) and incubate at 37°C for 1 h to lyse the bacteria. Troubleshooting 1.

⚠ CRITICAL: Higher concentration (1%–2%) of the DOC is critical to induce complete lysis of *S. pneumoniae*. The suspension should be clear after the incubation. Lower concentrations (such as 0.1%–0.2%) may require longer incubation time.

5. Adjust the pH of the suspension to 3.5 with acetate acid and incubate at 20°C–25°C for 1 h.

Note: Acid precipitation removes most impurities, e.g., soluble proteins.

6. Remove any precipitate and collect the supernatant by centrifugation at 10,000 g for 30 min at 4°C.
7. Filter the supernatant through a 0.22 μm filter and ultracentrifuge the supernatant repeatedly using Eppendorf centrifuge 5810R at 3,220 g for 20 min at 4°C through the 100 kDa cutoff Ultra-centrifugal Filter Unit against 10-fold volume (at least 500 mL) 25 mM sodium acetate.

⚠ CRITICAL: In this step, low molecular weight cell wall polysaccharides (CWPS), ~ 10 kDa, are removed by repetitive ultracentrifugation. The more times of ultracentrifugation and higher cutoff of the filter, the less residual CWPS are contained.

8. Add the cetyltrimethylammonium bromide (CTAB) to a final concentration of 3%, then incubate at RT for 1 h.
9. Centrifuge at 10,000 g for 30 min at 4°C. Collect the pellet (anionic CPS) or supernatant (neutral CPS) according to the serotype of pneumococcal CPS.

⚠ CRITICAL: Negatively charged pneumococcal CPS (most serotypes except 7F, 14, and 33F) precipitate after CTAB treatment; the neutral serotype 7F, 14, and 33F CPS remain in the supernatant.

10. Resuspend the pellet in 50 mL 1 M NaCl (for serotype 8 CPS) or add the NaCl into the supernatant (for serotype 14 CPS) to a final concentration of 1 M.
11. Centrifuge at 10,000 g for 15 min at 4°C to remove any precipitated contaminants.
12. Add ethanol to the supernatant to a final concentration of 80% (v/v) and store at 4°C for 2 h.
13. Collect the precipitates by centrifugation at 10,000 g for 15 min at 4°C.
14. Resuspend the pellet in 100 mL 6% sodium acetate and adjust pH to 6.8 by acetate acid.
15. Add ethanol to the supernatant to a final concentration of 33% and store at 4°C for 1 h.
16. Collect the supernatant by centrifugation at 10,000 g for 15 min at 4°C.
17. Add ethanol to the supernatant to a final concentration of 80% and store at 4°C for at least 2 h.
18. Collect the CPS pellet by centrifugation at 10,000 g for 15 min at 4°C.
19. Air dry the pellet in a fume hood.
20. Dissolve the CPS in sterile H_2O.

⚠ CRITICAL: Dissolved CPS can be stored at −20°C for at least 1 year. Alternatively, air dried CPS can be stored at −80°C for more than 5 years.

Preparation of CPS-coated latex beads

Timing: 1 day

The purified bacterial CPS can be fixed on biologically inert platform for subsequent screening of potential CPS-binding proteins. In this protocol, the pneumococcal CPS are modified by DMTMM and then covalently conjugated to latex microspheres with carboxyl functional groups.[6] Latex beads

are conjugated with different types of CPS and used parallelly in affinity screening to identify serotype-specific CPS-binding proteins/receptors.

21. Dissolve 2.5 mg CPS in 2.5 mL distilled water in a 5 mL tube.
22. Add 200 μL of 200 mg/mL DMTMM and mix thoroughly.
23. Incubate for 1 h at 20°C–25°C on a Thermo Scientific Digital Waving Rotator (60 rpm).
24. Remove free DMTMM by ultracentrifugation at 4,000 g for 10 min at 20°C–25°C through a 10 kDa cutoff unit against 3.5 mL PBS.
25. Adjust the final volume with PBS to 2.5 mL.
26. Add 10^9 carboxyl microspheres (2 μm in diameter) and vortex briefly.
27. Incubate on a rotator (60 rpm) overnight at RT.
28. Wash the CPS-coated microspheres with 5 mL PBST and centrifuge at 10,000 g for 5 min at 20°C–25°C to remove noncovalently bound CPS, and repeat once.
29. Resuspend the CPS-coated microspheres in 5 mL conjugation blocking buffer and store at 4°C.

⚠ CRITICAL: The conjugation blocking buffer contains 0.05% sodium azide to inhibit microbial growth. Sodium azide is a highly toxic chemical with acute dermal and oral toxicity. Avoid direct contact and store the microspheres in tightly sealed tubes.

Extraction of KC membrane proteins

Timing: 1 day

The liver NPCs are isolated through a perfusion-digestion method modified from previously reported procedure.[7] Then, the KCs are enriched by flow cytometry-associated cell sorting with high purity (Methods video S1). Membrane proteins are extracted using commercial kit. Alternatively, membrane proteins can be extracted by traditional ultracentrifugation to separate membrane components and solubilization by detergents.

30. Euthanize the mouse (female, 8-week-old, C57BL/6, 18–20 g) by cervical dislocation.
31. Open the abdominal cavity to expose the liver and intestine, and turn over the intestine to the right and push the large lobe of the liver to the diaphragm (Figure 1A).
32. Load 5 mL digestion buffer into a syringe with a 24 G needle.
33. Perfuse the liver by injecting the needle into the portal vein and flowing out through the incision of the inferior vena cava (Figure 1B).

Note: Perform the perfusion immediately after dissection to avoid intravascular blood coagulation in the liver. Complete perfusion can be achieved by clamping and opening the inferior vena cava with forceps for several times during perfusion. The liver will become light brown after complete perfusion (Figures 1C and 1D).

34. Excise the liver lobes carefully into a 6 cm Petri dish and remove the gallbladder.
35. Mince the liver to small pieces (< 2 mm) with scissors (Figure 1E).

⚠ CRITICAL: Thorough mincing is critical for maximal dissociation of the tissue.

36. Add 10 mL digestion buffer into the dish and put on a horizontal rotator (360 rpm, MIULAB Thermo-Shaker ST70-2) at 37°C for 30 min.

Note: After complete digestion, the mixture can be easily pipetted by 1,000 μL tips.

37. Filter the mixture through a 70 μm cell strainer into a 15 mL tube that precooled on ice.

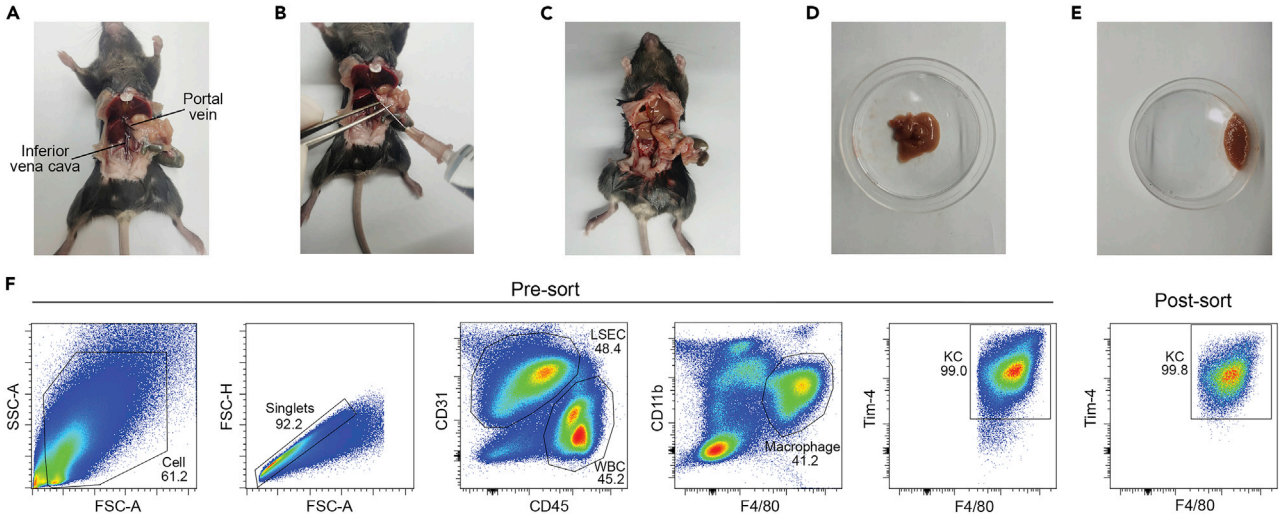

Figure 1. Isolation of mouse liver KCs

(A) Exposure of the portal vein and inferior vena cava after surgical dissection.

(B) Perfusion with digestion buffer by inserting into the portal vein and flowing out from the inferior vena cava.

(C and D) Liver turns light brown post thorough perfusion. (E) Liver homogenate post thorough mincing.

(F) FACS gating strategy and the purity of sorted KCs which are $CD31^-/CD45^+/CD11b^+/F4/80^+/Tim\text{-}4^+$.

See also Methods video S1.

⚠ CRITICAL: Macrophages such as KCs are strongly adherent and easily to attach on the plastic surface, thus the tubes should be kept on ice in following steps before proceeding to protein extraction.

38. Centrifuge the filtrate at 300 g for 5 min at 4°C and wash twice with ice-cold HBSS.
39. Lyse red blood cells with 1 mL RBC lysis buffer on ice for 1 min.
40. Add 10 mL HBSS to stop lysis, then centrifuge at 50 g for 1 min at 4°C.
41. Transfer the supernatant to a new 15 mL tube and centrifuge at 50 g for 1 min at 4°C.

Note: After the 50 g centrifugation, the pellet are mainly hepatocytes and most NPCs remain in the supernatant.

42. Transfer the supernatant to a new 15 mL tube and centrifuge at 300 g for 5 min at 4°C.
43. Resuspend the cell pellet in 100 μL FACS buffer (PBS + 3% FBS) and count the number of NPC using Countstar Automatic Cell Fluorescence Analyzer Mira FL as the manufacture's instruction.

Note: Generally, 1.0–1.5 × 10^7 NPCs are obtained from one mouse liver, in which KCs compose ~20%.

44. Adjust the cell density to 1 × 10^7 per 100 μL FACS buffer, and incubate with 1 μL of 0.5 mg/mL blocking antibody (anti-CD16/CD32) for 10 min at 4°C.
45. Wash with 1 mL FACS buffer and collect NPCs by centrifugation at 300 g for 5 min at 4°C.
46. Resuspend NPCs with FACS buffer containing staining antibodies and incubate for 20 min at 4°C.
47. Wash with 1 mL FACS buffer and resuspend the NPCs in 1 mL FACS buffer.
48. Sort KCs through a 70 μm nozzle into a 15 mL tube preloaded with 1 mL ice-cold FACS buffer. Troubleshooting 2.

Note: KCs are gated as $CD31^-CD45^+CD11b^+F4/80^+Tim\text{-}4^+$ cells (Figure 1F).

49. Collect sorted KCs by centrifugation at 300 g for 5 min at 4°C.
50. Carefully discard the supernatant and proceed to membrane protein extraction using Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific) as the manufacture's instruction.

⚠ CRITICAL: Add the protease and phosphatase inhibitors in the solutions when extracting the membrane proteins.

51. Exchange the buffer by ultracentrifugation using Eppendorf centrifuge 5810R at 3,220 g for 20 min at 4°C through 3 kDa cutoff unit against PBST buffer.
52. Measure protein concentration by BCA assay.
53. Aliquot at 100 μg total proteins per tube and store at −80°C.

⚠ CRITICAL: Avoid repeated freezing-thawing of the protein stocks.

Screening of CPS-binding candidates from KC proteins

Timing: 3–5 days

The CPS-binding proteins are captured on the CPS-coated beads after affinity reaction and subsequently dissociated by boiling in 1× SDS-PAGE loading buffer. Detached proteins are separated on SDS-PAGE gel and then subject to in-gel digestion and mass-spectrometry analysis. The MS identification can be conducted by professional researchers specialized on proteomics. Beads coated with different serotypes of the CPS are used to filtrate the type-specific CPS-binding proteins by comparative proteomics. Normally, the CPS isolated from high-virulence bacteria, e.g., serotype 8 *S. pneumoniae* in this STAR protocol, are used as negative control because these CPS are poorly recognized by any host receptor.

54. Use 10^8 CPS-coated beads for each binding reaction.
55. Wash the beads three times with 1 mL PBST and resuspend in 500 μL binding buffer (PBST with 2 mM $CaCl_2$/$MgCl_2$).
56. Add 100 μg total KC membrane proteins and incubate for 1 h at RT with constant vertical rotation (60 rpm).

⚠ CRITICAL: The binding reaction is more efficient at RT for 1 h than at 4°C for 12 h.

57. Discard non-binding proteins by centrifugation at 12,000 g for 5 min at 4°C.
58. Wash the beads for three times with 1 mL PBST.

⚠ CRITICAL: The CPS-protein complex may make the beads viscous. Do not touch the pellet when aspirating the supernatant, and leave small volume of the supernatant in each washing.

59. Resuspend the beads in 50 μL PBST containing 1× SDS-PAGE loading.
60. Boil the mixture at 100°C for 5 min to dissociate CPS-binding proteins, then quickly chill on ice.
61. Centrifuge at 12,000 g for 5 min at 4°C, and carefully transfer the supernatant to a new tube.

⚠ CRITICAL: Do not aspirate up the beads and leave small residual supernatant in the tube.

62. Separate the dissociated proteins on SDS-PAGE gel (5% stacking gel and 12% resolving gel).

⚠ CRITICAL: Use MS-grade reagents for electrophoresis and wear gloves and mask to avoid any possible contamination.

63. Stain the gel with Coomassie brilliant blue for 1 h at RT with shaking.
64. Destain the gel with ultra-pure water until the protein bands appear.
65. Excise the protein bands at minimal size of the gel and put into 1.5 mL tubes.
66. Proceed to in-gel digestion and subsequent procedures for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.
67. Search the spectra from each LC-MS/MS run against the mouse Unreviewed TrEMBL with "Transmembrane" filtered FASTA database using Proteome Discovery algorithm.
68. Analyze the relative abundance of proteins with $\geq$ 2 unique peptide matches.
69. Compare the protein abundance enriched by indicated type of CPS to the negative control which is naturally resistant to recognition by host proteins.

Note: The resultant list of enriched CPS-binding proteins may vary up to over hundreds candidates which depends on the criteria, e.g., enrichment fold. The criteria can be adjusted according to the researchers' requirements.

Overexpression of CPS-binding receptor candidates in CHO cells

Timing: 2 weeks

The top hits of the CPS-binding proteins candidates are overexpressed in nonphagocytic cell lines, e.g., CHO cells. The cell line should be tightly adherent and have marginal or no binding activity to the encapsulated bacteria.

70. Clone the candidate CPS receptor genes in expression plasmids using *E. coli* system.
    a. Extract the total RNA from sorted mouse KCs using TRIzol Reagent (Invitrogen) according to the manufacture's instruction.
    b. Synthesize the full-length cDNA library using a Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific).
    c. Amplify the target genes by PCR with specific primers. Troubleshooting 3.

    Note: Include suitable restrict enzyme cut sites in the primer sequences and a His_6 tag at the intracellular terminus.

    d. Purify the PCR products using HiPure Gel Pure Micro Kit (Magen).
    e. Extract the backbone plasmids (e.g., pCDH, pFUGW, or other plasmids) from the harboring *E. coli* DH5α strains using HiPure Plasmid Micro Kit (Magen).
    f. Digest the PCR products and the backbone plasmids with restrict enzymes.
    g. Purify the digested DNA using HiPure Gel Pure Micro Kit.
    h. Ligate the digested products by T4 DNA ligase (NEB) at 16°C overnight.
    i. Transform the ligation into *E. coli* DH5α and select the transformants on LB plates with indicated antibiotics, e.g., 100 μg/mL ampicillin for pCDH and pFUGW.
    j. Verify the recombinant plasmids by DNA sequencing.
    k. Extract the recombinant plasmids and lentiviral packaging vectors pMD2.G and psPAX2 using HiPure Plasmid EF Micro Kit (Magen).
71. Prepare lentiviruses carrying target genes in HEK293T cells.
    a. Seed 293T cells in a 6 cm dish and grow to 80%–90% confluence.
    b. Prepare the following lentiviral target mixture.
       For a 6 cm dish:

Component A

Reagent	Amount
Opti-MEM	450 μL
pMD2.G	2.19 μg
psPAX2	3.35 μg
Target plasmid	4.37 μg
Plus reagent	10 μL

Component B

Reagent	Amount
Opti-MEM	450 μL
Lipofactamine 2000	30 μL
Incubate each component for 5 min at RT.	

c. Add the component A to the component B, invert several times, and incubate for 10 min at RT.
d. Add the mixture dropwise to the 293T cells and place in a cell incubator for 4 h.
e. Replace the medium with 5 mL of prewarmed complete DMEM medium and incubate for 2 days.
f. Pool the lentivirus supernatant and filter through a 0.45 μm filter to remove cellular debris.
g. Aliquot and store at −80°C before use.

72. Optimize the antibiotic selection concentration for CHO cells.
    a. Seed CHO cells in 96 well plate and grow to 50% confluence.
    b. Replace the medium with fresh F-12K containing a series of concentrations of corresponding antibiotics, e.g., 0–10 μg/mL puromycin for pCDH and 0–500 μg/mL zeocin for pFUGW.
    c. Replace the medium with the same fresh medium at day 3.
    d. Compare the cell viability at day 7, and choose the minimal concentration for complete killing of the cells.

Note: In this protocol, 5 μg/mL puromycin and 300 μg/mL zeocin are selected for CHO cells.

73. Construct stably transfected CHO cells overexpressing target genes.
    a. Seed CHO cells in 6 cm dish and grow to 50% confluence.
    b. Replace with 3 mL fresh medium.
    c. Add 0.5 mL lentivirus stock and 8 μg/mL polybrene, then mix the medium with gently shaking.

Note: Set up a non-transfection control and a blank plasmid control.

    d. Incubate the cells for 24 h, normally to 80%–100% confluence.
    e. Subculture the cells in fresh medium containing selective antibiotics.
    f. Incubate for 3–5 days until the non-transfection control CHO cells die completely.
    g. Harvest the CHO transfectants and make stock vials.
    h. Verify the successful overexpression by western blot using anti-His_6 tag antibody. Troubleshooting 4.

Functional validation *by in vitro* bacterial binding assay

Timing: 3–5 days

The CHO transfectants are subjected to an *in vitro* bacterial binding assay to evaluate the gain-of-function phenotype. The specific CPS receptors result in a significant bacterial adherence to the CHO transfectants compared to marginal bacterial binding to the native CHO cells. The specific bacterial binding is inhibited in the presence of the corresponding type of CPS.

74. Grow the CHO transfectant cell lines in complete F-12K medium containing selection antibiotics in 6 cm dish to 90% confluence.
75. Digest with 1 mL 0.25% trypsin for 1 min at 37°C.
76. Add 4 mL complete F-12K medium to stop digestion and collect cells by centrifugation at 300 g for 5 min.
77. Resuspend in 1 mL fresh complete F-12K medium and count the cell number.
78. Adjust the cell density at 2×10^5 cells per 1 mL medium.
79. Seed the cells in 48 well plate at 10^5 cells per well, i.e., 500 μL suspension.
80. Incubate for 12 h or overnight in a cell incubator.

Note: The cell density should be at 90%–100% confluence that equals 1.5×10^5 cells per well. Do not overgrow the cells.

81. Prepare the *S. pneumoniae* strains in basic F-12K medium at a bacterial density at 7.5×10^5 CFU/mL.
82. Wash the CHO cells twice with fresh basic F-12K medium.
83. Add 200 μL bacterial suspension into the well at a multiplicity of infection (MOI) of 1:1.

Note: Normally, each well contains 1.5×10^5 CHO cells in 48 well plate at 90%–100% confluence, and equal numbers (MOI 1:1) of bacteria are added into each well, e.g., 200 μL bacterial suspension at a density of 7.5×10^5 CFU/mL.

84. Centrifuge at 500 g for 5 min to maximize the contact between bacteria and CHO cells.

⚠ CRITICAL: A low speed centrifugation enables synchronization of CHO cells and the planktonic bacteria. This step is critical for the ligand-receptor mediated binding but doesn't impact the non-specific adhesion.

85. Incubate the mixture for 30 min in a cell incubator.
86. Vigorously shake the cell plate 10 times to reduce non-specific adhesion.
87. Transfer the supernatant in a 1.5 mL tube and keep on ice.
88. Gently wash the cells with 200 μL basic F-12K medium and collect the supernatant in the above 1.5 mL tube.
89. Add 500 μL ice-cold sterile H_2O into each well and pipette several times to lyse the cells.
90. Collect the lysate into a 1.5 mL tube and keep on ice.
91. Count the bacterial numbers in the supernatant and the lysate fractions by gradient dilution and titration on TSA plate with 3% sheep blood or suitable agar plates for other bacteria.
92. Calculate the bacterial binding ratio by dividing the cell-associated CFU by the total CFU in each well.
93. Compare the bacterial binding level to the native CHO and CHO transfectants with empty plasmids.

Note: A specific CPS-binding proteins, i.e., the CPS receptors, significantly promote the adhesion of bacteria harboring the corresponding type of capsule used in affinity screening.

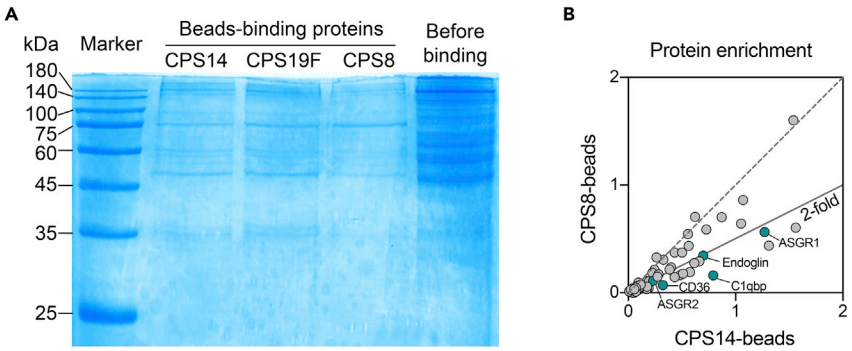

Figure 2. Identification of CPS-binding protein candidates by comparative proteomics

(A) Separation of conjugated proteins on CPS-coated beads and the extracted KC membrane proteins (Before binding).

(B) Plot of proteins enriched by CPS14-coated beads compared to the CPS8 counterparts. ©2022 An et al. Originally published in *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20212032.

The gain-of-function can be blocked in the presence of the same type of free CPS in a dose-dependent manner, which is not affected by heterologous types of CPS.

EXPECTED OUTCOMES

We applied this protocol to study the receptor-mediated interaction between bacterial capsules and KCs using a low-virulence serotype 14 *S. pneumoniae* as a model. The entire workflow can be adopted to the investigation of potential capsule receptors on cell membrane which recognize diverse polysaccharide capsules of other encapsulated bacteria.

We normally obtain 10 mg type 14 pneumococcal CPS (CPS14) and 40 mg CPS8 from 1 L of the bacterial culture. The contaminant protein and nucleic acid account for 1%–5% of the products as measured by Nanodrop 2000. The yield of KCs is 1–2 × 10^6 with a purify over 99% from one 8-w old female C57BL/6 mouse (Figure 1F). The estimated yield of KC membrane proteins is 100 μg per 5 × 10^6 KCs and used for one screening reaction.

After binding and dissociation, the CPS-beads captured proteins are separated on SDS-PAGE gel with clear protein bands (Figure 2A). Generally over hundreds of proteins are identified by LC-MS/MS and the raw list is filtered with further criteria, e.g., protein abundance, confidence score, annotated adhesion/binding activity, and enrichment fold change between two CPS types (Figure 2B). We constructed the CHO cells expressing the top 5 candidates individually and assessed the ability of the transfectants to capture serotype 14 *S. pneumoniae*, which revealed significant bacterial binding to CHO-Asgr1 (Figure 3A). Finally, we identified the first CPS receptor on the KC surface, i.e., the asialoglycoprotein receptor (composed by the subunit Asgr1 and Asgr2) (Figure 3B) that recognizes pneumococcal CPS7F and CPS14 (Figures 3C–3E).

QUANTIFICATION AND STATISTICAL ANALYSIS

The protein list (Table 1) illustrates the processing criteria for screening of candidate CPS-binding proteins/receptors. The proteins are ranked in descending order by the abundance in CPS14 group (Column D). Proteins with annotation of binding/adhesion activity (Column C) are labeled with asterisks. The enrichment (> 2 folds, Column F) is calculated through dividing the protein abundance in CPS14 group by the negative control CPS8 group.

LIMITATIONS

Before using this protocol, the existence and location of the CPS receptors should be confirmed by functional experiments, such as rapid capture of the encapsulated bacteria by liver KCs, and the

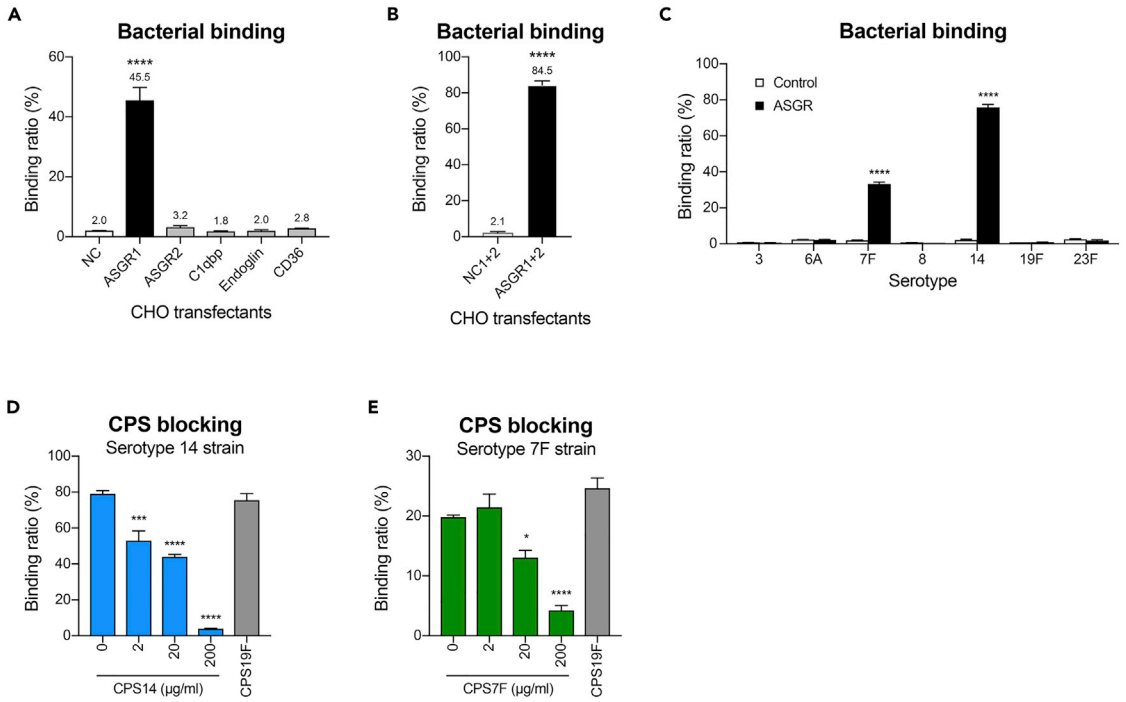

Figure 3. Functional validation of the CPS receptor ASGR

(A) Binding of serotype 14 *S. pneumoniae* to CHO transfectants expressing the candidate proteins.

(B) Increased binding activity of the intact ASGR comprised of the two subunits ASGR1 and ASGR2.

(C) Specific binding of serotype-7F and -14 *S. pneumoniae* to CHO-ASGR cells.

(D and E) Inhibition of the homologous CPS to the bacterial binding to CHO-ASGR cells. All data are represented as mean ± SEM. ©2022 An et al. Originally published in *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20212032.

bacteria-KC interaction can be inhibited by the homologous CPS. This protocol enables the identification of CPS receptors that have relative high binding affinity to the CPS ligands and adequate expression on the host cells. Thus, this protocol may not be suitable for identifying capsule receptors with extremely low binding affinity or low expression level. In addition, some indirect receptors such as complement receptors and antibody Fc receptors play an important role in capturing complement/antibody-opsonized pathogens, but these receptors can't be found by this protocol in the absence of opsonin.

The contamination of cytosol proteins when extracting the membrane proteins is inevitable. This may result in many interferential hits in the LC-MS/MS raw data. Bioinformatics tool can be used to rule out undesired proteins in a high throughput way, but the researchers should take care of the balance between restrict criteria (fidelity) and the number of remaining candidates (potentiality).

The candidate proteins are overexpressed in CHO cells in this protocol. Some proteins may need to be expressed in other adherent cell lines beyond CHO. Alternatively, CRISPR-activation (CRISPRa) strategy can be used to activate the indigenous expression of the target gene if heterologous expression still fails.

This protocol includes functional validation of the CPS receptor by an *in vitro* binding model. However, the result doesn't fully reflect the situation in the host environment. Further verification is required to demonstrate the biological role of the putative CPS receptor, especially in *in vivo* conditions using genetic knock out mouse model.

Table 1. List of the CPS-binding protein/receptor candidates

A	B	C	D	E	F
Uniprot accession	Protein name	Annotation	Average abundance (CPS14)	Average abundance (CPS8)	Enrichment (CPS14/CPS8)
Q69ZN7	Myof	Myoferlin	2.30E+08	5.04E+07	4.56
Q8VDN2	Atp1a1	Sodium/potassium-transporting ATPase subunit alpha-1	1.56E+08	6.03E+07	2.58
Q9WTI7	Myo1c	Unconventional myosin-Ic	1.31E+08	4.38E+07	3.00
P34927	Asgr1[a]	Asialoglycoprotein receptor 1	1.27E+08	5.63E+07	2.25
Q8R5L1	C1qbp[a]	Complement component 1 Q subcomponent-binding protein	7.92E+07	1.60E+07	4.95
F7A1B4	Eng[a]	Endoglin	6.98E+07	3.43E+07	2.03
P17427	Ap2a2	AP-2 complex subunit alpha-2	6.64E+07	2.91E+07	2.28
Q3TWV4	Ap2m1	AP-2 complex subunit mu	6.15E+07	2.76E+07	2.23
Q08857	Cd36[a]	Platelet glycoprotein 4	3.22E+07	6.92E+06	4.65
J3QMY0	Asgr2[a]	Asialoglycoprotein receptor 2	2.33E+07	1.11E+07	2.10
Q8VI47	Abcc2	Canalicular multispecific organic anion transporter 1	1.89E+07	4.09E+06	4.64
P28660	Nckap1	Nck-associated protein 1	1.80E+07	5.57E+06	3.22
Q61009	Scarb1[a]	Scavenger receptor class B member 1	1.75E+07	5.53E+06	3.17
O35114	Scarb2[a]	Lysosome membrane protein 2	1.73E+07	3.56E+06	4.85
O55222	Ilk	Integrin-linked protein kinase	1.72E+07	6.23E+06	2.76
Q8BWG8	Arrb1	Beta-arrestin-1	1.70E+07	5.62E+06	3.02
J3QNY6	Abcb11	Bile salt export pump	1.69E+07	3.52E+06	4.82
Q08481	Pecam1[a]	Platelet endothelial cell adhesion molecule	1.13E+07	4.97E+06	2.27
…	…	…	…	…	…

[a]Proteins with annotation of binding/adhesion activity.

Although this protocol was originally developed for identification of bacterial capsule receptors, we anticipate that the molecular interaction-based screening strategy can be widely applied to study the ubiquitous ligand-receptor interactions in biology, such as immune receptors recognizing other microbial antigens (proteins, sugars, lipids, and nucleic acids) and molecular mechanisms of cell-to-cell communications in cell adhesion, migration, and signal transduction and immune cell activation.

TROUBLESHOOTING

Problem 1

Low yield of the extracted pneumococcal CPS.

Potential solution

- Increase the culture volume.
- Harvest the bacterial culture at mid-log phase to avoid autolysis of *S. pneumoniae* at step 2.
- Make sure complete lysis of the bacteria after DOC treatment, e.g., longer incubation at step 4.

Problem 2

Low yield of the KCs post FACS sorting.

Potential solution

- Fully perfuse the liver to enable thorough filling of digestion buffer in the liver sinusoids.
- Mince the liver lobe to small pieces as possible.
- Remove the RBC and hepatocytes as far as possible. This will increase the ratio of KCs in cell suspension and reduce the default loss of KCs in sorting process.

Problem 3
No products by specific PCR.

Potential solution

- Optimize the PCR reaction system.
- Synthesize the target genes in full-length.

Problem 4
Unsuccessful expression of candidate proteins in CHO cells.

Potential solution

- The lentivirus vector has a limit of < 6 kb in the length of inserted gene. Use other strategy, e.g., CRISPRa for large proteins.
- Try other adherent cell lines or vector plasmids.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jing-Ren Zhang (zhanglab@tsinghua.edu.cn).

Materials availability
Materials are available upon request.

Data and code availability
This study did not generate or analyze any dataset/code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102065.

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AUTHOR CONTRIBUTIONS
H.A. and J.-R.Z. contributed to setting up the whole protocol. All authors participated in optimizing the protocol and writing the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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