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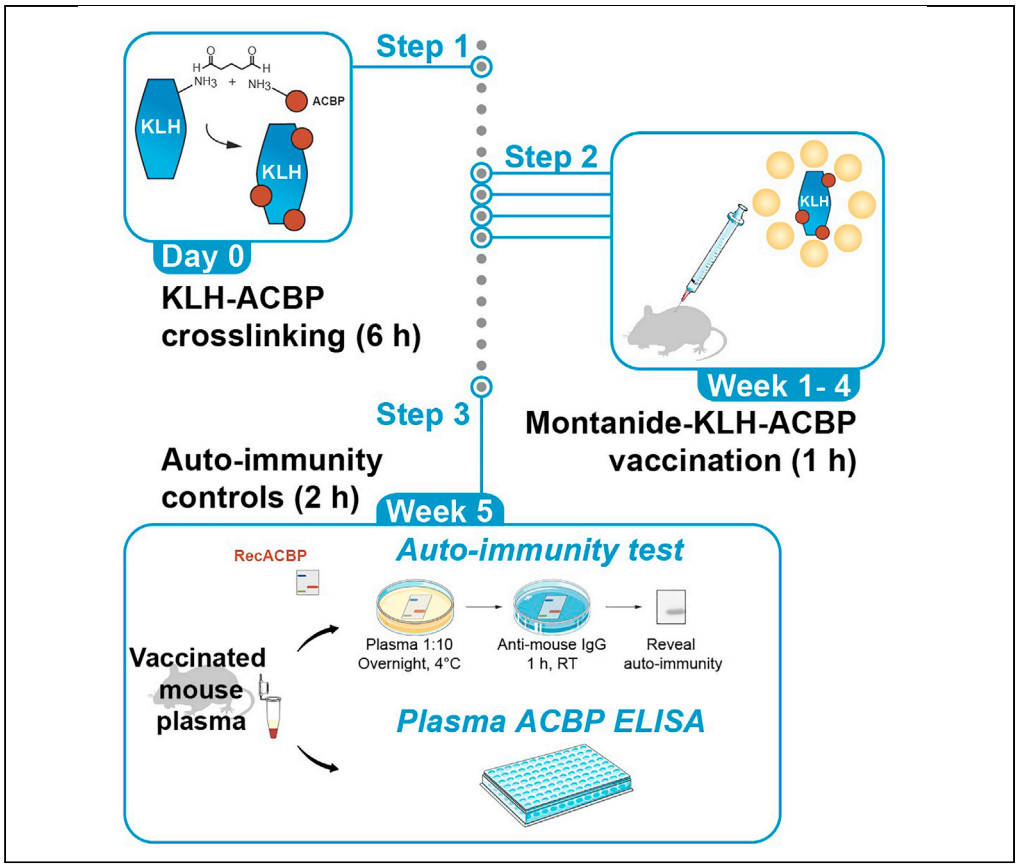

Keyhole limpet hemocyanin (KLH) is a glycosylated multi-subunit metalloprotein that elicits a strong nonspecific immune activation, thus inducing both cellular and humoral immune responses. The exceptional immunogenicity of this protein can be leveraged to vaccinate mice against self-antigens that otherwise would not induce an autoimmune response. This protocol describes the covalent conjugation of KLH with acyl-coenzyme A-binding protein (ACBP), the autovaccination of mice with ACBP-KLH conjugate together with a potent adjuvant, and the detection of the produced anti-ACBP autoantibodies.

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Highlights

ACBP can be glutaraldehyde-conjugated to the large immunogenic protein KLH

When coinjected with adjuvant, KLH-ACBP elicits autoantibodies against ACBP

Circulating ACBP protein can be quantified by a specific ELISA

Bioavailable ACBP decreases after successful autovaccination

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Immunization of mice with the self-peptide ACBP coupled to keyhole limpet hemocyanin

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SUMMARY

Keyhole limpet hemocyanin (KLH) is a glycosylated multi-subunit metalloprotein that elicits a strong nonspecific immune activation, thus inducing both cellular and humoral immune responses. The exceptional immunogenicity of this protein can be leveraged to vaccinate mice against self-antigens that otherwise would not induce an autoimmune response. This protocol describes the covalent conjugation of KLH with acyl-coenzyme A-binding protein (ACBP), the autovaccination of mice with ACBP-KLH conjugate together with a potent adjuvant, and the detection of the produced anti-ACBP autoantibodies.

For complete details on the use and execution of this profile, please refer to Bravo-San Pedro et al. (2019c).

BEFORE YOU BEGIN

This protocol describes the vaccination of mice against the self-protein acyl-coenzyme A-binding protein (ACBP, also known as diazepam binding protein, DBI). ACBP is a phylogenetically conserved protein (Charmpilas et al., 2020; Madeo et al., 2020) that is ubiquitously expressed intracellularly in mammals and released into the circulation upon starvation (Bravo-San Pedro et al., 2019b; Bravo-San Pedro et al., 2019c). ACBP exists in several isoforms, among which ACBP1 is the most abundant one in both human and murine tissues as well as in the circulation (Li et al., 2021). Extracellular ACBP stimulates appetite, reduces fatty acid oxidation, and stimulates lipid accumulation in adipose tissues. Since its plasma concentration correlates with the body mass index, it is a potential candidate target for the treatment of human obesity (Bravo-San Pedro et al., 2019a; Joseph et al., 2021; Joseph et al., 2020; Montégut et al., 2021). Indeed, when circulating ACBP is neutralized in mice, food intake as well as fat storage are suppressed (Bravo-San Pedro et al., 2019c; Pedro et al., 2019; Sica et al., 2020), spurring interest in protocols that induce long-term neutralization of the self-peptide.

Given its small size and ubiquitous presence in the body, ACBP is poorly immunogenic. Self-immunization requires a robust adjuvant strategy such as the one presented in this article.

The first step of this protocol consists in the conjugation of the ACBP1 protein to keyhole limpet hemocyanin (KLH), a marine mollusk hemolymph protein with strongly immunogenic properties that

has been used for decades for inducing immune responses, mostly against haptens conjugated to KLH (Harris and Markl, 1999; Swaminathan et al., 2014). KLH-hapten or KLH-peptide conjugates have previously been described in a wide variety of contexts and elicit B and T-cell-mediated immune responses (Bandivdekar, 2014; Ding et al., 2016; Haba and Nisonoff, 1995; Li et al., 2006; van Doorn et al., 2016; Zhang et al., 2020).

In a second step, the KLH-ACBP conjugate is emulsified with Montanide ISA 51 VG, a mineral oil-based adjuvant already used for human active immunotherapy trials (Ascarateil et al., 2015, van Doorn et al., 2016). Sequential subcutaneous injections of this KLH-ACBP/Montanide mixture into immunocompetent mice consistently induce humoral immune responses against the ACBP self-peptide. Such autoimmune responses can be easily monitored by measuring the generation of autoantibodies as well as by a decrease in circulating free ACBP1 protein.

We surmise that this protocol can be adapted to other self-peptides and is specifically relevant for small and weakly immunogenic proteins.

Borate buffer preparation

Timing: 30 min

We prepare a final volume of 100 mL 0.1 M borate buffer, pH = 10

1. Dissolve 620 mg in 90 mL deionized water.
2. Set the pH to 10 by slowly adding 10 M NaOH.

⚠ CRITICAL: Be careful to let the solution homogenize and the pH stabilize between each drop of NaOH.

3. Once pH = 10, complete the volume to 100 mL.

Protein mixes

Timing: 10 min

We use ACBP:KLH at a 1:1 mass ratio, i.e., a 30:1 molar ratio (ACBP is 13 kDa, KLH is 390 kDa).

4. Dilute 7.5 mg ACBP in 750 µL borate buffer.
5. Dilute 7.5 mg KLH in 750 µL borate buffer.
6. Mix these solutions 1:1 for a final volume of 1.5 mL.

⚠ CRITICAL: For control groups, a KLH-only mix should be prepared. Just replace the ACBP solution with borate buffer and proceed to the same steps as KLH-ACBP.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat Anti-Mouse IgG(H+L) Human ads-HRP	SouthernBiotech	Cat# 1031-05
Goat Anti-Rabbit IgG(H+L), Mouse/Rat/Human ads-HRP	SouthernBiotech	Cat# 4049-05
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Invitrogen	Ca# A-11037

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-KLH antibody, clone 15F7E4G3	Invitrogen	Cat# MA5-28972
Capture antibody - Rabbit polyclonal antibody to Diazepam Binding Inhibitor (DBI)	Abcam	Cat# ab231910
Detection antibody - Biotin-Linked Rabbit Antibody to Diazepam Binding Inhibitor (DBI)	MyBioSource	Cat# MBS2005521
Chemicals, peptides, and recombinant proteins		
Mouse recombinant ACBP	Custom-made	n/a
Human recombinant ACBP (various isoforms)	Custom-made	n/a
Boric acid	Merck	Cat# 1.00165
Glutaraldehyde solution 25 %	Sigma-Aldrich	Cat# G6257
Formaldehyde solution 37 %	Sigma-Aldrich	Cat# F8775
Glycine	Sigma-Aldrich	Cat# 8898
Imject mcKLH Subunits, High Purity Research Grade	Thermo Scientific	Cat# 77649
Montanide ISA 51 VG sterile and endotoxin free	SEPPIC	Cat# 36362/FL2R3
Bovine Serum Albumin Standard (Fraction V)	Euromedex	Cat# 04-100-812-E
Skimmed milk	Dutscher	Cat# 711160
Tris Buffered Saline (TBS) 10×	Euromedex	Cat# ET220-B
Tween 20	Euromedex	Cat# 2001-C
NuPAGE LDS sample buffer (4×)	Invitrogen	Cat# NP0008
NuPAGE Sample Reducing Agent	Invitrogen	Cat# NP0009
MOPS SDS Running Buffer (20×)	Invitrogen	Cat# NP-0001-02
Tris-Glycine buffer 10×, pH 8.5	Euromedex	Cat# EU0550
Ethanol absolute	VWR	Cat# 20821.310
NuPAGE 4–12% Bis-Tris Gel 1.5 mm x 10 well	Invitrogen	Cat# NP0335BOX
NUPAGE 7% TA GEL 1.5MM10W	Invitrogen	Cat# EA0358BOX
Immun-Blot PVDF Membranes 0.2 µM	Bio-Rad Laboratories	Cat# 1620177
Amersham ECL Prime Western Blotting Detection Reagent	Cytiva	Cat# RPN2232
Ponceau S solution	Sigma-Aldrich	Cat# P7170
HRP Avidin	BioLegend	Cat# 405103
Tamoxifen	Sigma-Aldrich	Cat# T5648
Experimental models: Organisms/strains		
C57BL/6JOlaHsd mice	Envigo	Cat# 5704F
$Acbp^{fl/fl}$ UBC-Cre/ERT2 mice (loxP flanked Acbp exon 2)	OZgene	n/a
Other		
3 mL glass vials, such as serum vials with caps	DWK Life Sciences	Cat# 223684
Magnetic Stir Bar, for use with 3.0–5.0 mL vessels, PTFE	Sigma	Cat# 23227
RT Stirring Hot Plate with Aluminum Top 230V	Thermo Fisher	Cat# SP136320-33Q
Connector double female luer-lock STX100	SunMedical	Cat# DIDRACDLLFT
Injekt luer lock syringes 2 mL	B Braun	Cat# 4606701V
Magnetic stirring bar – 5 mm	Sigma	Cat# Z328839
Amicon® Ultra-15 Centrifugal Filter Unit	Sigma	Cat# UFC910024
Heparin Lithium Microvette CB 300 µL capillary tubes	Sarstedt	Cat# 16.443
XCell SureLock Mini-Cell Electrophoresis System	Thermo Fisher	Cat# EI0001
Mini Trans-Blot® Cell	Bio-Rad	Cat# 1703930
ImageQuant™ LAS 4000 camera	GE Healthcare	n/a
High Binding ELISA 96-well microplates	Corning Inc.	Cat# 9018
12 channels multichannel pipette 0.5–10 µL	Gilson	Cat# FA10014
12 channels multichannel pipette 20–200 µL	Gilson	Cat# FA10012
Victor X4 plate reader	Perkin Elmer	Cat# 2030-0050

MATERIALS AND EQUIPMENT

Solutions recipes

1 M glycine solution		
Reagent	Final concentration	Amount
Glycine	1 M	751 mg
ddH_2O	n/a	10 mL
Total	**n/a**	**10 mL**
Prepare on the day of KLH-ACBP conjugation and keep at 18°C–25°C.		

1 % glutaraldehyde solution		
Reagent	Final concentration	Amount
Glutaraldehyde (25 % solution)	1 %	400 µL
ddH_2O	n/a	9.6 mL
Total	**n/a**	**10 mL**
Prepare on the day of KLH-ACBP conjugation and keep at 18°C–25°C.		

0.2 % formaldehyde solution		
Reagent	Final concentration	Amount
Formaldehyde (37 % solution)	0.2 %	53 µL
ddH_2O	n/a	10 mL
Total	**n/a**	**10 mL**
Prepare on the day of KLH-ACBP conjugation and keep at 18°C–25°C.		

TBS 0.1% Tween 20 (TTBS)		
Reagent	Final concentration	Amount
TBS 10×	1×	100 mL
ddH_2O	n/a	900 mL
Tween 20	0.1 %	1 mL
Total	**n/a**	**1 L**
Keep at 18°C–25°C for up to two weeks.		

TTBS + 10 % bovine serum albumin solution (TTBS-BSA)		
Reagent	Final concentration	Amount
TTBS	n/a	100 mL
Bovine serum albumin	10 %	10 g
Total	**n/a**	**0.1 L**
Keep at 4°C for up to one week.		

TTBS + 5 % bovine serum albumin solution		
Reagent	Final concentration	Amount
TTBS	n/a	100 mL
Bovine serum albumin	5 %	5 g
Total	**n/a**	**0.1 L**
Keep at 4°C for up to one week.		

TTBS + 5 % skimmed milk solution		
Reagent	Final concentration	Amount
TTBS	n/a	100 mL
Skimmed milk	5 %	5 g
Total	**n/a**	**0.1 L**
Keep at 4°C for up to one week.		

ELISA washing buffer		
Reagent	Final concentration	Amount
TBS 10×	1×	100 mL
ddH_2O	n/a	900 mL
Tween 20	0.05 %	0.5 mL
Total	**n/a**	**1 L**
Keep 18°C–25°C for up to two weeks.		

ELISA blocking buffer		
Reagent	Final concentration	Amount
PBS 1×	1×	27.9 mL
BSA	2 %	0.6 g
Fetal Bovine Serum	5 %	1.5 mL
Tween 20	0.05 %	15 µL
Total	**n/a**	**30 mL**
Sterile-filter and keep at 4°C for up to one week.		

ELISA reaction buffer		
Reagent	Final concentration	Amount
PBS 1×	1×	99 mL
BSA	1 %	1 g
Tween 20	0.05 %	50 µL
Total	**n/a**	**100 mL**
Sterile-filter and keep at 4°C for up to one week.		

⚠ CRITICAL: Glutaraldehyde and formaldehyde are both toxic when inhaled and by contact. Wear appropriate personal protective equipment and manipulate under a chemical hood.

STEP-BY-STEP METHOD DETAILS

KLH-ACBP conjugation

Timing: 6 h

Glutaraldehyde is used as crosslinker that reacts with the amine groups of both proteins. Excess aldehyde groups are then saturated with glycine, and the reaction is stopped by formaldehyde.

1. Glutaraldehyde conjugation
   a. Pipet the KLH-ACBP mix into the 3mL glass vial and start to stir (approx. 500 rpm) 18°C–25°C.
   b. Slowly add 500 µL of 1 % glutaraldehyde.

⚠ CRITICAL: Proceed drop by drop. Do not hesitate to pipet 20 µL at a time for more precision.

c. Stir 2 h at 18°C–25°C.

Note: The solution will turn light yellow for KLH and dark yellow-to-brown for KLH-ACBP.

2. Glycine quenching
   a. Add 250 µL of 1 M glycine.
   b. Stir 30 min at 18°C–25°C.
3. Washes
   a. Transfer the vial content to an Amicon® Ultra-15 Centrifugal Filter Unit.
   b. Centrifuge 10 min at 4000 *g*.
   c. Rinse the walls of the chamber with 2 mL borate buffer.
   d. Centrifuge 10 min at 4000 *g*.
   e. Rinse the walls of the chamber with 2 mL borate buffer.
   f. Centrifuge 10 min at 4000 *g*.
4. Formaldehyde quenching
   a. Add 2 mL of 0.2 % formaldehyde solution.
   b. Leave 30 min at 18°C–25°C.
   c. Add 250 µL 1 M glycine.
   d. Leave 30 min at 18°C–25°C.
5. Washes
   a. Centrifuge 10 min at 4000 *g*.
   b. Rinse the walls of the chamber with 2 mL borate buffer.
   c. Centrifuge 10 min at 4000 *g*.
   d. Rinse the walls of the chamber with 2 mL borate buffer.
   e. Centrifuge 10 min at 4000 *g*.
   f. Carefully resuspend the content of the chamber in 1 mL PBS.
6. Quantify the protein concentration by the method of your choice: bicinchoninic acid (BCA) assay, absorbance…

Pause point: The solution can be stored at 4°C for the whole duration of the protocol (up to two months).

Conjugation control

To verify the conjugation of the peptide antigen with KLH, a SDS-Page followed by Coomassie blue staining or Western Blot can be performed. Carefully adapt the procedure to account for the large size of these proteins (> 390 kDa).

7. Sample preparation
   a. Dilute the KLH and KLH-ACBP to a concentration of 0.2 µg/µL in LDS-reducing buffer.
   b. Denature the proteins by warming 5 min at 100°C.
   c. Place the samples in ice until same-day use or keep them at −20°C for later processing.
8. Migration
   a. Rinse a 7 % Tris-Acetate gel to ensure the wells are clean.
   b. Immerse the gel in the MOPS buffer and load 10 µL of each sample per lane.

Note: Duplicate all lanes to be able to do both Coomassie-blue staining and immunoblotting.

   c. Run for 2h30 under a constant voltage of 140 V.
9. Coomassie-blue staining
   a. Cut half the gel for Coomassie blue staining and keep the other half for step 4.
   b. Incubate for 1 h in Coomassie blue.
   c. Rinse in water.
   d. Take a picture of the Coomassie-blue stained gel to compare protein weights.

Note: The differences in height are subtle due to the very high protein weights.

10. Immunoblotting
    a. Transfer to an ethanol-activated 0.22 μm PVDF membrane for 2h30 in 1× Tris-Glycine buffer containing 10 % ethanol, under a constant voltage of 100 V.
    b. Block the membrane with TTBS + 5 % skimmed milk for 1 h at 18°C–25°C.
    c. Rinse the membrane 3 times 5 min with TTBS.
    d. Incubate 12–15 h at 4°C with primary antibodies diluted in TTBS + 5 % BSA (anti-DBI 1:1000, anti-KLH: 1:500).
    e. Rinse the membrane 3 times 5 min with TTBS.
    f. Incubate 1 h at 18°C–25°C with the secondary antibodies (anti-DBI: goat anti-rabbit AF594-conjugated 1:5000; anti-KLH: goat anti-mouse HRP-conjugated 1:5000).
    g. Reveal the membrane by immunofluorescence reading (anti-DBI), then chemiluminescence after a 1-min ECL incubation (anti-KLH).

Note: If immunofluorescence is not available for western blot revelation, the membrane can be revealed twice with chemiluminescence, with stripping and blocking steps in between.

Vaccination

Timing: 4 weeks

To vaccinate the mice, KLH-ACBP aqueous solution is brought to emulsion in the mineral oil adjuvant Montanide ISA 51 VG. The detailed protocol of emulsification can be provided by the manufacturer Seppic (protocol #5559).

11. KLH-ACBP + Montanide emulsion
    a. Dilute KLH-ACBP solution to the desired concentration according to Table 1. Load the aqueous phase (max. 1 mL) in a 2 mL luer lock syringe.

⚠ CRITICAL: Prepare the double amount of emulsion you will use. The final product is viscous and major loss can occur during syringe loading and injections.

Note: If the final mix volume is greater than 3 mL, 5 mL syringes can be used at this step instead of the reference given in the key resources table. The emulsion step will have to be carried out rigorously, meaning that larger volume requires more physical strength to achieve correct emulsification.

    b. Load 1 mL of Montanide into another 2 mL luer lock syringe.
    c. Connect the two syringes with a double female luer-lock connector as shown in Figure 1B.

⚠ CRITICAL: Make sure not to trap any air bubble in the system, since this can affect emulsion quality.

    d. Slowly transfer the liquid content from one syringe to the other, then back to the first side. Repeat this cycle 20 times.
    e. Perform the same movement as fast as possible 40 extra times.

Note: The emulsion will become white and thicker, which can be noticed by an increased resistance of the plunger.

12. Load the emulsion in a 1 mL syringe by plugging it on one side of the double female luer-lock connector.

Table 1. Injection planning and doses per animal

Injected dose/mouse	Week 1	Week 2	Week 3	Week 4
KLH-ACBP	30 μg in 50 μL	30 μg in 50 μL	30 μg in 50 μL	10 μg in 50 μL
Montanide	50 μL	50 μL	50 μL	50 μL
Total volume	100 μL	100 μL	100 μL	100 μL

13. Inject intraperitoneally 100 μL to each mouse with a 25 G needle.
14. Repeat these steps once weekly for 4 weeks using the doses presented in Table 1.

Immunization test

Timing: 3 days

The presence of antibodies directed against ACBP is tested by immunoblotting the recombinant target protein with plasma. The presence of anti-ACBP antibodies is revealed by means of a secondary anti-mouse immunoglobulin G (IgG) antibody coupled to horseradish peroxidase (HRP).

15. Plasma "primary" solution
    a. After the fourth week of immunization, take 100–150 μL samples of blood from the submandibular vein in lithium-heparinized tubes. Centrifuge them 10 min at 2000 *g* to recover plasma.
    b. Dilute 50 μL plasma in 450 μL of TTBS-BSA. Place this solution in a non-treated 12-wells plate, one well per mouse, and incubate 12–15 h at 4°C.

Pause point: Remaining plasma can be stored at −80°C for subsequent analysis, such as ELISA quantification of circulating ACBP.

Overnight step

16. Recombinant protein immunoblotting
    a. Load 4–12% Bis-Tris acrylamide precast gels with one in two wells containing 2 μg of recombinant ACBP and the alternates wells with protein ladder. Run one pair of ladder wells + ACBP for each vaccinated mouse.
    b. Transfer the proteins in the gel to 0.2 μm PVDF membranes, then use Ponceau S staining to cut 1.2 cm-wide squares including the ladder and the ACBP protein.
    c. Take a photography of the Ponceau S staining for quantification of protein content.
    d. Rinse the Ponceau S out and place each square in TTBS-BSA for 1–2 h of blocking. Use numbered 12-wells plates with 500 μL of TTBS-BSA in each well to keep track of the correspondence between Ponceau S pictures and final results.
    e. Transfer the squares to their corresponding plasma solution in the 12-wells plates. Incubate 12–15 h at 4°C.

Overnight step

17. Rinse each well for 5 min with 500 μL TTBS. Repeat this operation three times.
18. Incubate 1 h at 18°C–25°C with 500 μL anti-mouse IgG HRP-conjugated secondary antibody (1:5000 dilution in TTBS-BSA).
19. Rinse each well for 5 min with 500 μL TTBS. Repeat this operation three times.
20. Reveal the squares with ECL substrate and an adequate chemiluminescence camera.

ELISA quantification of circulating ACBP

Timing: 2 days

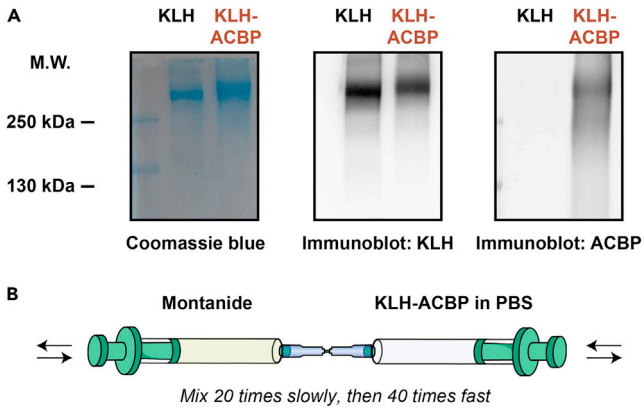

Figure 1. Verification of the KLH-ACBP conjugation after glutaraldehyde crosslinking

(A) Molecular weights were compared by SDS-PAGE followed by Coomassie blue staining while the presence of ACBP in the conjugate was confirmed by immunoblotting against KLH and ACBP. A polyclonal primary antibody directed against ACBP was chosen to detect the various epitopes present on the KLH-ACBP conjugate.

(B) Schematic view of the emulsification protocol.

The neutralization of ACBP by the autoantibodies can be quantified by measuring the concentration of free ACBP in the plasma after immunization.

21. Capture antibody coating
    a. Fill the adequate number of wells in 96-wells high-binding plates with 200 µL anti-ACBP capture antibody, diluted in PBS to a final concentration of 0.5 µg/mL.

    Note: Reliable quantification can be achieved by performing duplicate measurements and two standard curves per plate (total 16 wells + 2 x number of samples).

    b. Incubate 12–15 h at 4°C.
    Overnight step.

22. Blocking
    a. Wash the plate twice with 300 µL washing buffer.

    Note: Tape the plate dry on absorbent paper between each step and make sure to eliminate any drops or bubbles at the bottom of the plate.

    b. Add 200 µL blocking buffer per well.
    c. Incubate 2 h at 18°C–25°C under gentle agitation.
23. Samples loading
    a. Wash 4 times with 300 µL washing buffer.
    b. Add 100 µL of sample or standard, diluted in the ELISA reaction buffer.
    c. Incubate 2 h at 18°C–25°C under gentle agitation.

    Note: Plasma dilution ranges should be optimized in-house, but we recommend using a 1:20 dilution in PBS as a start. The recommended standard dilution range is 0–10 ng/mL, use the same recombinant protein as the one used in the vaccine preparation.

24. Detection with biotin-conjugated antibody
    a. Wash 4 times with 300 µL washing buffer.
    b. Add 100 µL biotin-conjugated anti-ACBP antibody, diluted to 1 µg/mL in ELISA reaction buffer.

c. Incubate 2 h at 18°C–25°C under gentle agitation

25. HRP-Avidin conjugation
    a. Wash 4 times with 300 µL washing buffer.
    b. Add 100 µL HRP-avidin, diluted to 1:1000 in ELISA reaction buffer.
    c. Incubate 30 min at 18°C–25°C under gentle agitation
26. Substrate addition
    a. Wash 5 times with 300 µL washing buffer.
    b. Add 100 µL of 1-Step Ultra TMB-ELISA.
    c. Incubate 5–20 min at 18°C–25°C in the dark, until coloration.
27. Stop reaction
    a. Add 50 µL of stop solution, i.e., 2 M H_2SO_4.
    b. Measure absorbance at 450 nm using a microplate reader.

EXPECTED OUTCOMES

KLH-ACBP conjugation

The expected final concentration of conjugated KLH solutions should be greater than 5 mg/mL for the presented initial quantities. This amount is sufficient to vaccinate 25 mice. To verify the coupling of the target protein, two strategies are used. Figure 1A shows on the left panel a SDS-PAGE gel of KLH versus KLH-ACBP (2 µg per well) stained with Coomassie blue to illustrate the -small- difference in molecular weight between the coupled and uncoupled proteins. The middle and right panels present a similar gel transferred to a PVDF membrane and revealed with anti-KLH and anti-ACBP antibody, respectively. In this case, the ACBP protein is present in the KLH-ACBP lanes only, which show a slightly higher molecular weight, and the emulsion step described in Figure 1B can be performed.

Immunization

With the injection plan presented in Table 1, the vaccination rates obtained in C57Bl/6J mice is close to 100 % after the fourth injection. The intensity of the detected immunity can slightly vary from mouse to mouse, as presented in Figure 2A, and can be quantified by normalization to the Ponceau S-stained ACBP protein. As shown in Figure 2B, the immune response induced by vaccination is specific to the protein used in the KLH-ACBP construct (murine ACBP isoform 1). Indeed, the signal revealed by immunoblotting human ACBP isoforms with blood if vaccinated mice is much weaker than the signal obtained on murine ACBP. Moreover, mouse ACBP single mutants that reduced the affinity of ACBP for acyl-CoA does not interfere with the recognition of ACBP by autoantibodies. Neutralization of ACBP by self-antibodies decreases the circulating level of free ACBP in the plasma of mice, as indicated by an ELISA developed for this purpose (Figure 3A). Of note, this ELISA test provides accurate results irrespective of repeated freeze-thawing of the plasma from mice, although plasma should preferentially be kept at a low temperature to avoid degradation of the analyte (Figure 3B).

LIMITATIONS

Since KLH and Montanide are strong non-specific immunoactivators (Harris and Markl, 1999, van Doorn et al., 2016), the immunization procedure may affect the immune tonus of mice. Therefore, we recommend running all experiments with a control group in which mice are immunized with unconjugated KLH emulsified in Montanide. However, if the purpose of the experiment is the exploration of immune responses, it may be necessary to add further vehicle control groups receiving (i) injection of Montanide emulsified with PBS and (ii) injection of PBS alone. If the purpose of the experiment is the long-term neutralization of ACBP by autoantibodies over several months, the use of just two experimental groups (immunization with ACBP-KLH conjugate versus KLH alone) is acceptable. This procedure has revealed the obesogenic activity of ACBP that was confirmed by other methods including the knockout of the gene coding for ACBP (*Dbi*) or the injection of suitable neutralizing monoclonal antibodies (Bravo-San Pedro et al., 2019c; Joseph et al., 2020; Montégut et al., 2021; Sica et al., 2020). At this point, however, the maximal duration of the humoral anti-ACBP response elicited by KLH-ACBP conjugates has not been explored.

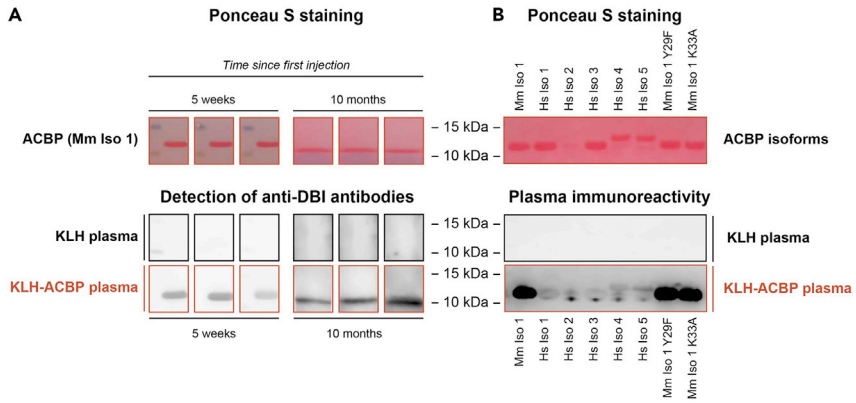

Figure 2. Detection of autoantibodies directed against ACBP in vaccinated mice plasma

(A) Mice were immunized with KLH alone or KLH-ACBP conjugates. Recombinant ACBP separated by SDS-PAGE was transferred to PVDF membranes followed by the detection of anti-ACBP autoantibodies in the plasma of each individual mouse. Ponceau S staining was performed prior to plasma incubation to confirm the presence of recombinant protein and allow for comparison of auto-immunity levels between mice, and representative images of the Ponceau S-stained membranes are shown (KLH-ACBP membranes).

(B) Plasma-revealed immunoblots were repeated with pooled plasma samples from non-vaccinated (KLH) vs. vaccinated (KLH-ACBP) mice to compare the responses against different isoforms of murine and human isoforms of the protein. Representative images of the Ponceau S-stained membranes are shown (KLH-ACBP membrane). Mm stands for Mus musculus (mouse). Hs stands for Homo sapiens (human). Isoforms of human and mouse ACBP/DBI are numbered from 1 to 5 following official nomenclature (Uniprot). Y29F and K33A are single amino acid-substituted mutants of mouse ACBP isoform 1 that have lost their interaction with the natural intracellular ligand of ACBP, acyl-coenzyme A.

TROUBLESHOOTING

Problem 1

The sizes of the conjugated and unconjugated proteins look identical when running the SDS-PAGE of Conjugation control.

Potential solution

Multiple factors can explain that the bands appear at the same height:

The pore size and running time were not adapted to high molecular weights.KLH subunits are 390 kDa and the conjugation will reduce their electrophoretic mobility. If your bands stay at the top of the gel, choose a gel with a larger pore size, or increase the running time.

The bands look blurred.This is normal when using glutaraldehyde cross-linking. Increase the running time to detect differences in the size of conjugates despite smearing bands.

The cross-linking did not happen.Verify that the crosslinking did not work by immunoblotting. If ACBP is not detected in the $\geq$390 kDa bands, check the pH of the borate buffer (which should be 10), that the proteins used were well purified and that the glutaraldehyde stock solution is well preserved. If all these factors are correctly controlled, the amount of lysine and arginine residues in the proteins can affect glutaraldehyde crosslinking: the duration, temperature and concentration of glutaraldehyde can be optimized to account for this parameter.

Problem 2

The aqueous and oily phases separate before injection during Vaccination.

Potential solution

The emulsion protocol is critical for the stability of the solution. Make sure that no air gets trapped in the system by filling the connector with one of the two phases before connecting it to the second

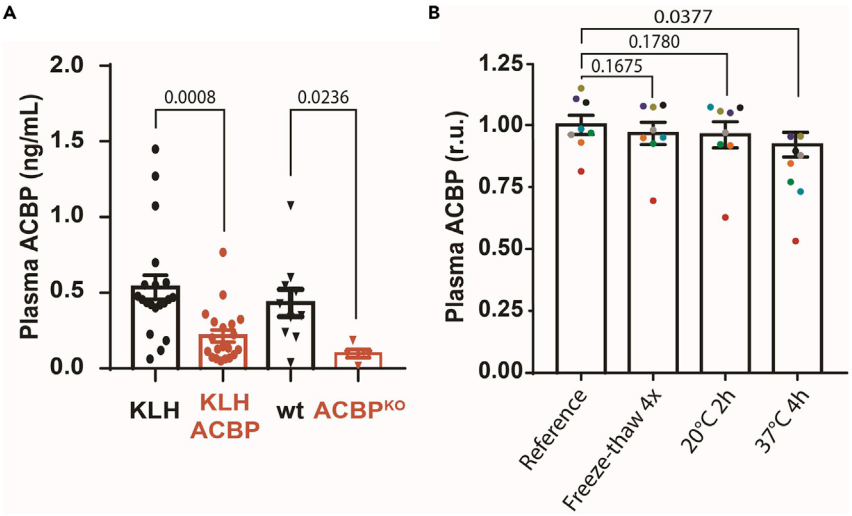

Figure 3. ELISA measurement of free ACBP plasma levels

(A) Reduced circulating levels of ACBP after autovaccination with KLH-ACBP. Self-immunity results in neutralization of circulating ACBP, with a decrease of bioavailable plasma ACBP comparable to the levels observed in animals after a tamoxifen-induced whole-body knockout of the protein (daily 75 mg/kg *i.p.* injection for 5 consecutive days).

(B) Stability of the ACBP concentration in eight plasma samples measured by ELISA after successive freeze-thaw cycles (four cycles), a 2-h incubation at 20°C or a 4-h incubation at 37°C. Results are displayed as means ± standard error of the mean. Statistical differences were calculated by unpaired t-test or one-way NOVA with Dunnett's correction for multiple comparisons.

syringe (Figure 1B). Also, respect the slow speed steps before the faster ones to ensure gradual homogenization.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pr. Guido Kroemer (kroemer@orange.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

The protocol was originally set up by J.M.B.-S.P. and O.M. and then validated by I.M. and L.M. H.C. optimized the ELISA protocol. L.M. wrote this protocol with edits by G.K.

DECLARATION OF INTERESTS

G.K. and J.M.B.-S.P. filed a patent application dealing with targeting the ACBP/DBI system in anorexia, obesity, and co-morbidities. G.K. has filed patent applications dealing with caloric restriction mimetics (autophagy inducers) for the treatment of aging, age-related diseases, cancer, obesity, and co-morbidities. G.K. is a scientific co-founder of everImmune, Samsara Therapeutics, and Therafast Bio. All the other authors declare no conflicts of interest.

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