Protocol

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

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.
Protocol

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

Léa Montégut,1,2,3,6 Hui Chen,1,2,3,6 José Manuel Bravo-San Pedro,1,2,4,6 Omar Motiño,1,2 Isabelle Martins,1,2,7 and Guido Kroemer1,2,5,8,*

1Centre de Recherche des Cordeliers, Equipe labellisée par la Ligue Contre le Cancer, Université de Paris, Sorbonne Université, INSERM U1138, Institut Universitaire de France, Paris, France
2Metabolomics and Cell Biology Platforms, Institut Gustave Roussy, Villejuif, France
3Université Paris Saclay, Faculty of Medicine Kremlin Bicêtre, Le Kremlin Bicêtre, France
4Complutense University of Madrid, Faculty of Medicine, Department Section of Physiology, Madrid, Spain
5Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France
6These authors contributed equally
7Technical contact
8Lead contact
*Correspondence: kroemer@orange.fr
https://doi.org/10.1016/j.xpro.2021.101095

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 (Champilias 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; Monteégu 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# MAS-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 |
| Montanide ISA 51 VG sterile and endotoxin free | Thermo Scientific | Cat# 77649 |

**Experimental models: Organisms/strains**

| C57BL/6JolaHsd mice | Envigo | Cat# S704F |
| Acbp<sup>fl/fl</sup> 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# DIDRACD1LFT |
| 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 Micravette C8 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 |
| ddH₂O                 | 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 |
| ddH₂O                            | 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  |
| ddH₂O                            | 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 10x   | 1 x                 | 100 mL |
| ddH₂O     | 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.
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.
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.
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

### 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        |
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 H₂SO₄.
   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; Montégu 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.
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 R390kDa band, check 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 phase.
syringe (Figure 1B). Also, respect the slow speed steps before the faster ones to ensure gradual homogenization.

RESOURCES 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.

ACKNOWLEDGMENTS

G.K. is supported by the Ligue contre le Cancer (équipe labellisée); Agence National de la Recherche (ANR) – Projets blancs; AMMica US23/CNRS UMS3655; Association pour la recherche sur le cancer (ARC); Association “Ruban Rose”; Cancéropôle Ile-de-France; Fondation pour la Recherche Médicale (FRM); a donation by Elior; Equipex Onco-Pheno-Screen; European Joint Programme on Rare Diseases (EJP-RD); Gustave Roussy Odyssea, the European Union Horizon 2020 Projects Oncobiome and Crimson; Fondation Carrefour; Institut National du Cancer (INCa); Institut Universitaire de France; LabEx Immuno-Oncology (ANR-18-IDEX-0001); the RHU Torino Lumière; Seerave Foundation; SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); and SIRIC Cancer Research and Personalized Medicine (CARPEM). This study contributes to the IdEx Université de Paris ANR-18-IDEX-0001. J.M.B.-S.P. is funded by “Ramon y Cajal Program” (RYC-2018-025099-I) and supported by Spain’s Ministerio de Ciencia e Innovacion (PID2019-108827RA-I00).
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.

REFERENCES
Ascarateil, S., Puget, A., and Koziol, M.-E. (2015). Safety data of Montanide ISA 51 VG and Montanide ISA 720 VG, two adjuvants dedicated to human therapeutic vaccines. J. Immunotherapy Cancer 3, 5428.

Bandivdekar, A.H. (2014). Development of antifertility vaccine using sperm specific proteins. Indian J. Med. Res. 140, 373–377.

Bravo-San Pedro, J.M., Sica, V., and Kroemer, G. (2019a). The elusive “hunger protein”: an appetite-stimulatory factor that is overabundant in human obesity. Mol. Cell Oncol 6, e1667193.

Bravo-San Pedro, J.M., Sica, V., Martins, I., Anagnostopoulos, F., Mauri, C., and Kroemer, G. (2019b). Cell-autonomous, paracrine and neuroendocrine feedback regulation of autophagy by DBI/ACBP (diazepam binding inhibitor, acyl-CoA binding protein): the obesity factor. Autophagy 15, 2036–2038.

Bravo-San Pedro, J.M., Sica, V., Martins, I., Pol, J., Loos, F., Mauri, M.C., Durand, S., Bossut, N., Aparahamian, F., Anagnostopoulos, G., et al. (2019c). Acyl-CoA binding protein is a lipogenic factor that triggers food intake and obesity. Cell Metab 30, 754–767.e9.

Charmillas, N., Ruckenstuhl, C., Sica, V., Bütter, S., Habernig, L., Dichtinger, S., Madeo, F., Tavernarakis, N., Bravo-San Pedro, J.M., and Kroemer, G. (2020). Acyl-CoA binding protein (ACBP): a phylogenetically conserved appetite stimulator. Cell Death Dis 11, 7.

Ding, L., Meng, Y., Zhang, H.Y., Yin, W.C., Yan, Y., and Cao, Y.P. (2016). Active immunization with the peptide epitope vaccine AB3-10-KLH induces a Th2-polarized anti-Ab antibody response and decreases amyloid plaques in APP/PS1 transgenic mice. Neurosci. Lett. 634, 1–6.

Haba, S., and Nisonoff, A. (1995). Prolongation of the responsiveness of newborn mice to syngeneic IgE by inhibition of IgE synthesis. Immunol. Lett. 47, 205–206.

Harris, J.R., and Markl, J. (1999). Keyhole limpet haemocyanin (KLH): a biomedical review. Micron 30, 597–623.

Joseph, A., Chen, H., Anagnostopoulos, G., Montégué, L., Lafarge, A., Motriño, O., Castedo, M., Mauri, M.C., Clément, K., Terrisse, S., et al. (2021). Effects of acyl-coenzyme A binding protein (ACBP)/diazepam binding inhibitor (DBI) on body mass index. Cell Death Dis 12, 599.

Joseph, A., Moriceau, S., Sica, V., Anagnostopoulos, G., Pol, J., Martins, I., Lafarge, A., Mauri, M.C., Leboyer, M., Loftus, J., et al. (2020). Metabolic and psychiatric effects of acyl coenzyme A binding protein A binding protein (ACBP)/diazepam binding inhibitor (DBI) on body mass index. Cell Stress Dis 11, 502.

Li, M., Yan, Z., Han, W., and Zhang, Y. (2006). Mimotope vaccination for epitope-specific induction of anti-CD20 antibodies. Cell Immunol. 239, 136–143.

Li, S., Joseph, A., Martins, I., and Kroemer, G. (2021). Elevated plasma levels of the appetite-stimulator ACBP/DBI in fasting and obese subjects. Cell Stress 5, 89.

Madeo, F., Tavernarakis, N., Pedro, J.M.B., and Kroemer, G. (2020). ACBP is an appetite stimulator across phylogenetic barriers. Cell Stress 4, 27–29.

Montégué, L., Lopez-Otin, C., Magnan, C., and Kroemer, G. (2021). Old paradoxes and new opportunities for appetite control in obesity. Trends Endocrinol. Metab. 32, 264–294.

Sica, V., Martins, I., Motriño, O., Bravo-San Pedro, J.M., and Kroemer, G. (2020). Antibody-mediated neutralization of ACBP/DBI has anorexigenic and lipolytic effects. Adipocyte 9, 116–119.

Swaminathan, A., Lucas, R.M., Dear, K., and Memetchia, A.J. (2014). Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies. Br. J. Clin. Pharmacol. 78, 1135–1142.

van Doorn, E., Liu, H., Huckriede, A., and Hak, E. (2016). Safety and tolerability evaluation of the use of Montanide ISA51 VG as vaccine adjuvant: a systematic review. Hum. Vaccin. Immunother. 12, 159–169.

Zhang, S., Wang, M., Li, J., Li, Y., Zhou, J., Tian, Z., Liu, C., and Yao, Q. (2020). Vaccine of RANKL mutant conjugated with KLH effectively stabilizing bone metabolism and preventing trabecular microstructural degeneration in osteoporotic rats. J. Orthop. Res. 39, 2465–2473.