Mouse Immunization and Hybridoma Screening Protocol using Membrane Proteins displayed on VLPs or Copolymer Nanodiscs

Materials

- 6-8 weeks female BALB/c mice
- Sp2/0 cells (product of ATCC)
- Antigen (VLPs or Nanodiscs)
- Control antigen (VLPs or Nanodiscs without the target membrane proteins)
- Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS)
- Dulbecco's Modified Eagle's Medium (DMEM) (Corning #10-013-CVRC)
- Polyethylene glycol #1500 (PEG) (Roche Diagnostics #10783641001)
- Fetal Bovine Serum (FBS) (MP Biomedicals #0929168-CF)
- HAT supplement 50X (Sigma Aldrich #H0262-10VL)
- HT supplement 100x (Gibco™ #11067-030)
- Minimal Essential Medium (MEM) (Sigma-Aldrich #M4655)
- Ethanol
- Hybridoma SFM (Gibco™ #12045076 or 12045084)
- DMEM 10% FBS Medium (see buffer formulas page 2)
- DMEM 10% FBS 1X HAT Medium (see buffer formulas page 2)
- DMEM 10% FBS 1X HT Medium (see buffer formulas page 2)
- PBS (see buffer formulas page 2)
- PBS-T (see buffer formulas page 2)
- Imject™ ALUM, Aluminium adjuvant (ThermoFisher #77161)

Equipment

- 75 cm^2 cell culture flask (T-75 flask) (ThermoFisher #156499))
- 15mL and 50mL plastic tube (Corning #352096 and 352070)
- 6cm and 10cm culture dish (Corning #430166 and 430167)
- 6-, 12-, 24-, 48- and 96-well cell culture plate (Corning #3506, 3512, 3526, 3548 and 3596)
- Light microscope
- Fluorescent microscope
- CO_2 incubator (5% CO_2, 37°C)
- Incubator
- Beaker
- Stirrer
- Stirrer bar
- Millex-HV Filter Unit 0.45 µm (Corning #431220 or 431225)
- 2.5mL syringe
- Water bath floating foam
- 21-G needle
- Centrifuge (Beckman Coulter, Model #V-15R)

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Buffer Formulas

1. DMEM-FBS medium (500 mL)
- DMEM 425 mL
- FBS 75 mL

2. DMEM-HAT medium (500 mL)
- DMEM 401 mL
- FBS 75 mL
- HAT supplement 8 mL
- HT supplement 1 mL

3. DMEM-HT medium (500 mL)
- DMEM 405 mL
- FBS 75 mL
- HT supplement 5 mL
- BM condimed 15 mL

4. PBS (1,000 mL)
- NaCl 8 g
- $Na_2HPO_4 \cdot 12H_2O$ 2.9 g
- KCl 0.2 g
- KH_2PO_4 0.2 g
- dH_2O 1,000 mL

5. PBS-T
- PBS with 0.05% Tween

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Immunization

→ VLP as Immunization Antigen

1. The total concentration of membrane protein VLP should be 0.5mg/mL with greater than 100ng membrane protein per 1mg of total membrane protein VLP. Mix 50μL-90μL of the membrane protein VLP with 50μL aluminum hydroxide adjuvant. Shake gently and then incubate on ice for 30 minutes.
2. Add VLP/adjuvant mixture into a 1mL syringe and remove excess air.
3. Inject the VLP/adjuvant mixture into mouse intradermal, subcutaneous, enterocoelia or muscle at 2 points.
4. In total, perform 4-5 immunizations at 10-day intervals.

→ Nanodisc as Immunization Antigen

1. Mix the membrane protein nanodisc (120 μg) with aluminum hydroxide adjuvant at a volume ratio of 2:1. Gently shake the mixture and incubate it on ice for 20 minutes.
2. Add the nanodisc/adjuvant mixture into a 1 mL syringe and carefully remove any excess air.
3. Inject the nanodisc/adjuvant mixture into the mouse at 2 points via intradermal, subcutaneous, intraperitoneal, or intramuscular routes.
4. In total, perform 4-5 immunizations at 10-day intervals.

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Anti-serum titer evaluation by ELISA

1. Coat the plate with 1.5μg/mL membrane protein VLP or 2.0μg/mL membrane protein nanodisc, 100uL/well. Store overnight at 4°C.
2. The next day, wash the plate once. Then block with 3% BSA PBS-T with 200μL/well at 37°C for 1 hr.
3. Dilute anti-serum 1:300. Then perform a 1:3 serial dilution and add 100μL/well of anti-serum to the plate. Incubate at 37°C for 1 hr.
4. Wash plate twice. Add 100μL/well of anti-mFC HRP. Incubate at 37°C for 1 hr.
5. Wash twice. Add 100μL/well of TMB substrate. Incubate at 37°C for 10 min. Add 50uL/well of 0.5M H_2SO_4 to terminate the reaction.

Note: Steps 3-5 are standard ELISA operations.

Example procedure for anti-serum titer evaluation by ELISA using CXCR4 VLP

30μL CXCR4 VLP (0.5mg/mL) was added to 10mL PBS and mixed gently. A 96-well plate was coated with 100μL/well of diluted CXCR4 VLP and stored at 4°C overnight. The plate was washed once the next day and blocked with 3% BSA PBS-T using 200μL/well. The plate was incubated at 37°C for 1 hr. 3μL anti-CXCR4 serum was added to 1mL 3% BSA PBS-T and mixed gently. Then, a 1:3 serial dilution was performed. 100μL/well of diluted anti-serum was added to the plate and incubated at 37°C for 1 hr. The plate was washed twice. Then, anti-mFC HRP was added at 100μL/well. The plate was incubated at 37°C for 1 hr. The plate was washed twice. TMB substrate was added at 100μL/well and incubated at 37°C for 10 min. The, 50μL/well of 0.5M H_2SO_4 was added to terminate the reaction.

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Hybridoma cell fusion

1. Final immunization boost:

3 days before hybridoma cell fusion, take 60μL membrane protein VLP (0.5mg/mL) or 120μg membrane protein nanodisc and mix it with 300μL PBS, pH7.4. Intraperitoneally inject the mixture for a final immunization boost.

2. Feeder cell preparation:

One day before hybridoma cell fusion, use unimmunized BALB/c mice for feed cell preparation. Kill BALB/c mice (unimmunized) and soak in 75% alcohol for disinfection. Use surgical scissors to gently cut the skin of the mouse's abdomen. (Do not damage the peritoneum of mice). Add 10mL DMEM medium into a 50mL aseptic centrifuge tube. Take up 3-5mL of liquid with a 5mL syringe. Lift the peritoneum of the mouse with a surgical tweezer. Push 3-5mL medium into the abdomen of the mouse. Blow medium 3-4 times. Suck out medium containing abdominal cell suspension and put in a 50mL aseptic centrifuge tube. Take another fresh 5mL of medium and repeat one more time. Then remove mouse spleen under aseptic conditions. Grind spleen with a grinder and mix with DMEM. Use a strainer to obtain single spleen cell suspension. Combine single spleen cell suspension with abdominal cell suspension. Centrifuge at low speed (1000rpm for 10 min). Discard supernatant. Resuspend pellet in feed cell in HAT culture medium, with a cell density of 1E6 cells/mL, which can be evenly spread in a 96-well plate, with 100μL per well.

3. Pre-warm media and reagents including 50% PEG 1450 (1mL/tube), DMEM, FBS-DMEM, FBS-DMEM (HAT) in 37°C in water bath.

4. SP2/0 cell prep:

Revive SP2/0 cells one week before hybridoma cell fusion. Exchange media and maintain cells in an exponential growth phase.

Centrifuge SP2/0 cells at low speed (1000rpm for 5 min). Discard the supernatant. Wash with DMEM twice. Resuspend and keep at 37°C for use.

5. Immunized spleen cell suspension:

Take mouse blood from the eyeball of immunized BALB/c mice with a good immune response for anti-serum. Kill immunized BALB/c mice and soak in 75% alcohol for disinfection and sterilization. Remove the spleen under aseptic conditions. Grind the spleen with a grinder and mix with DMEM. Use a strainer to obtain immunized single spleen cell suspension. Centrifuge at low speed (1000rpm for 10 min). Discard supernatant. Wash with DMEM twice. Resuspend and store at 37°C for use.

Hybridoma cell fusion protocol continued on next page

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Hybridoma cell fusion cont.

6. Hybridoma cell fusion:

Mix the immunized spleen cells and SP2/0 at [spleen cells: SP2/0 = 2:1~3:1] in a 50mL centrifuge tube. Centrifuge at low speed (1000rpm for 10min). Discard supernatant with no liquid remaining in the tube. Gently tapping tubes, mix spleen cells & SP2/0 evenly and spread at the bottom of the tube. Preheat 50% 1mL PEG 1450 and add drop by drop over 1 min. Then, add 10~15mL of fusion termination solution (DMEM) over 1min. Keep cells at 37°C for 10 min.

7. Hybridoma polyclonal cell growth:

Centrifuge fusion cells from step 6 at low speed (1000rpm for 10min). Discard the supernatant. Add FBS-DMEM (HAT) (10mL/plate) and gently resuspend cells to get a hybridoma cell suspension. Take a cell culture plate that had been coated with feeding cells one day in advance. Remove supernatant in feeding cell coated 96-well culture plate and add hybridoma cell suspension at 100µL/well. Place in a constant temperature incubator with 5% CO_2 for culture.

8. Polyclone hybridoma cell culture:

Culture hybridoma cells in FBS-DMEM (HAT) for 3 to 7 days. Based on cell growth, exchange media with FBS-DMEM (HT), 200µL/well. Check the cell morphology after media exchange. Polyclone verification by ELISA can be performed after 4 to 7 days.

Example procedure hybridoma polyclones producing CXCR4-specific antibody evaluation by ELISA

30µL CXCR4 VLP (0.5mg/mL) was mixed with 10mL PBS and added to a 96-well plate with 100ul/well. The plate was stored at 4°C overnight. The plates were washed once the next day and blocked with 3% BSA PBS-T at 4°C for 2 hrs. The hybridoma supernatant was transferred from the fusion plate to the VLP plate at 100µL/well and incubated at 37°C for 1 hr. The plate was washed twice. Then, anti-mouse FC HRP was added at 100µL/well 37°C for 1 hr. The plate was washed twice. TMB substrate was added at 100µL/well and incubated at 37°C for 10 min. Then, 50µL/well of 0.5M H_2SO_4 was added to terminate the reaction. Polyclones with positive ELISA signals were selected for generating monoclonal hybridoma.

Note: Coat with the control VLP that doesn't contain the target membrane protein. Perform ELISA the same for counter selection.

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Hybridoma monoclones

1. Detach polyclone hybridoma cell by gentle pipetting.
2. Count the cell number.
3. Adjust the cell suspension to 1 cell per well in a 96-well plate with DMEM-HT (200μL per well).
4. Seed monoclonal cell onto a 96-well plate and incubated in a CO_2 incubator.
5. Exchange medium every 3-4 days with fresh DMEM-HT until the cells proliferate.
6. Perform ELISA the same as previous operation to verify the antigen-specific hybridoma monoclone.
7. Pass hybridoma monoclones to larger well plates (96-, 48-, 24-, 12- and 6-well plates and 6-cm culture dish). Finally, grow in a 10-cm culture dish.

References

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