Hooke Laboratories

Cytokine Production Induced By T Cell Recall Response In Vitro

Recommended protocol for use with:

Hooke Immunization Kits:

- Hooke Kit™ MOG_{35-55}/CFA Emulsion (EK-0111),
- Hooke Kit™ [Ser^{140}]-$PLP_{139-151}$/CFA Emulsion (EK-0120),
- Hooke Kit™ $PLP_{139-151}$ (native)/CFA Emulsion (EK-0230),
- Hooke Kit™ MBP_{1-17}/CFA Emulsion (EK-0130), or,
- Hooke Kit™ $OVA_{323-339}$/CFA Emulsion (EK-0132)

Hooke Antigens in TC media:

- MOG_{35-55} in TC Media, 100x (cat. no. DS-0111),
- [Ser^{140}]-$PLP_{139-151}$ in TC Media, 100x (cat. no. DS-0121),
- $PLP_{139-151}$ (native) in TC Media, 100x (cat. no. DS-0161)
- MBP_{1-17} in TC Media, 100x (cat. no. DS-0131), or,
- $OVA_{323-339}$ in TC Media, 100x (cat. no. DS-0141)

Introduction

This protocol describes antigen-induced in vitro activation of spleen and lymph node cells from immunized mice.

This activation causes T cells (and other cells) to produce cytokines. The amount and type of cytokines produced are indicative of the strength and type of immune response which mice developed in vivo after immunization.

The cytokines present in the resulting supernatants may therefore be measured for evaluation of immune responses.

Either spleen or lymph node cells of immunized mice can be used to evaluate cytokine production. Lymph node cell analysis often gives more consistent results. It is important to collect enlarged, draining lymph nodes for this analysis.

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Method overview

Mice are immunized with antigen/CFA emulsion.

Ten (10) to fourteen (14) days later mice are then euthanized, spleens and/or lymph nodes collected, and cell suspensions are prepared.

Cells are plated with antigen into 96 well plates, cultured for approximately 3 days, and supernatants collected.

Materials needed

Hooke Immunization Kit(s) MOG_{35-55}/CFA Emulsion (Hooke #EK-0111), [Ser^{140}]-$PLP_{139-151}$/CFA Emulsion (Hooke #EK-0120), MBP_{1-17}/CFA Emulsion (Hooke #EK-0130), $OVA_{323-339}$/CFA Emulsion (Hooke #EK-0132), or, $PLP_{139-151}$ (native)/CFA Emulsion (Hooke #EK-0230)
Antigen in TC Media, 100x MOG_{35-55} in TC Media, 100x (Hooke #DS-0111), [Ser^{140}]-$PLP_{139-151}$ in TC Media, 100x (Hooke #DS-0121), MBP_{1-17} in TC Media, 100x (Hooke #DS-0131), $OVA_{323-339}$ in TC Media, 100x (Hooke #DS-0141), or, $PLP_{139-151}$ (native) in TC Media, 100x (Hooke #DS-0161)
Fetal Bovine Serum for T cell culture
Mice of suitable strain, age, and gender (see immunization kit for recommendation)
96-well tissue culture plates, flat bottom
RPMI 1640
1 M HEPES (Life Technologies #15630080)
L-Glutamine–Penicillin–Streptomycin solution (Sigma #G6784)
MEM Non-Essential Amino Acids, 100x (Life Technologies #11140-050)
Sodium Pyruvate, 100 mM (Life Technologies #11360-070)
2-Mercaptoethanol, 55 mM (Life Technologies #21985-023)
Red Blood Cell Lysing Buffer (Sigma #R7757)
Trypan Blue solution (Sigma #T8154)
70% alcohol in spray bottle
50 mL sterile polypropylene tubes
5 mL pipette
Media bottles
Petri dishes

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Protocol

Acclimate and immunize mice

1. Acclimate mice of suitable strain, age, and gender (see details of each immunization kit for recommendation) to your facility for at least 7 days before immunization.

2. Inject mice subcutaneously with antigen emulsified in CFA at two sites, either at the base of the tail, or in the lower back.

   If injecting at the base of the tail, administer 0.05 mL on each side of the tail base, at the hairline (0.1 mL total).

   If injecting in the lower back, administer 0.1 mL over each hip (0.2 mL total).

Media preparation and tissue harvest

Tissues may be harvested any time after 7 days from immunization. We recommend tissue harvest 10 to 14 days after immunization (cells collected earlier than 7 days after immunization produce very little Th1 and Th17 cytokines).

Perform all cell preparations aseptically in a biosafety cabinet. Keep cells cold (0 to 4 °C). Use cold media and keep cells on ice when practical.

Any FBS lot may be used for wash media, but the quality of FBS for the tissue culture media is critical, as all FBS lots are not equally able to support cytokine production in T cell cultures.

Use a refrigerated centrifuge at 0 to 4 °C.

1. Prepare wash media by supplementing RPMI 1640 to reach the following concentrations:
   - 2% FBS (lot of FBS is not critical in this preparation)
   - 10 mM HEPES

   Keep cold (0 to 4 °C).

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2. Prepare tissue culture media by supplementing RPMI 1640 to reach the following concentrations:
    - 10% FBS
    - 2 mM L-glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin (1% of 100x solution)
    - 1x MEM non-essential amino acids solution (1% of 100x solution)
    - 1 mM sodium pyruvate (1% of 100 mM solution)
    - 5.5×10^{-5} M 2-mercaptoethanol (0.1% of 55 mM solution)
    - 10 mM HEPES (1% of 1 M solution)

    Keep cold (0 to 4 °C).

3. Processing 2 to 5 mice at a time, euthanize mice, spray them with 70% isopropyl alcohol, and remove inguinal lymph nodes and/or spleens – keep them separate. Place spleens and lymph nodes in separate Petri dishes containing 10-15 mL of wash media.

Spleen cell suspension preparation

1. Squash spleens in each Petri dish by pressing several times with the hard end of a 10 mL syringe plunger.

    For each Petri dish, place a 70 μm cell strainer in a 50 mL tube. Collect all media and squashed tissue from the Petri dish into the cell strainer.

    Using the soft end of a clean 10 mL syringe plunger, press the tissue through the strainer.

    Carefully rinse the cell strainer into each tube with ~10 mL of wash media. (Lift one end of strainer off tube to let air escape; this helps avoid spilling material on the outside of the tube, losing cells.)

    Repeat for all mice or groups.

    Keep cell suspension cold on ice until all tissues have been processed.

2. Spin down cells in the 50 mL tubes for 10 minutes at approximately 300 g (do not put cells from more than 5 mice in one tube).

3. Carefully discard supernatant (avoid losing cells).

4. Resuspend the cell pellet in 2.5 to 3 mL per mouse of cold red blood cell lysing buffer, and keep cold for 4 to 5 minutes while red blood cells lyse.

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5. While red blood cells are lysing, preload 35 mL of cold wash media into each of another set of 50 mL tubes, for use in the next step.

   Keep cell suspension cold until cell lysis is complete. This is indicated by the solution becoming clear, bright red (before the lysis is complete, the cell suspension will be opaque red). This typically this takes 4 to 5 minutes. Watch carefully for color change in order to start next step immediately (if unsure, stop lysis after 5 minutes).

6. Immediately after cell lysis is complete (within 10 seconds) add the previously prepared 35 mL of cold wash media to the cell suspension in each tube.

7. Spin down cells again for 10 minutes at approximately 300 g, discard supernatant.

8. Resuspend the cells in ~5 mL wash media per mouse. Filter suspension through a fresh 70 µm cell strainer to remove clumps of dead cells.

9. Spin down cells again, 10 minutes at approximately 300 g, discard supernatant.

10. Resuspend the cells in ~5 mL tissue culture media per mouse. Again, filter suspension through a fresh 70 µm cell strainer to remove clumps of cells.

11. Count cells using Trypan Blue solution. Expected cell number is approximately 150-200 million cells from each mouse.

12. Adjust cell number to 8 million/mL and keep on ice until ready to plate into 96 well plate.

Lymph node cell suspension preparation

1. Squash lymph nodes in each Petri dish by pressing several times with the hard end of a 10 mL syringe plunger.

   For each Petri dish, place a 70 µm cell strainer in a 50 mL tube. Collect all media and squashed tissue from the Petri dish into the cell strainer.

   Using the soft end of a clean 3 mL syringe plunger, press the tissue through the strainer.

   Carefully rinse the cell strainer into each tube with ~10 mL of wash media. (Lift one end of strainer off tube to let air escape; this helps avoid spilling material on the outside of the tube, losing cells.)

   Repeat for all mice or groups.

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Keep cell suspension cold on ice until all tissues have been processed.

2. Spin down cells in the 50 mL tubes for 10 minutes at approximately 300 g.

3. Carefully discard supernatant.

4. Resuspend the cells in ~5 mL wash media per mouse. Filter suspension through a 70 µm cell strainer to remove clumps of dead cells.

5. Spin down cells again, 10 minutes at approximately 300 g, discard supernatant.

6. Resuspend the cells in ~3 mL tissue culture media per mouse. Again, filter suspension through a fresh 70 µm cell strainer to remove clumps of cells.

7. Count cells using Trypan Blue solution. Expected cell number is approximately 20-40 million cells per mouse.

8. Adjust cell number to 8 million/mL and keep on ice until ready to plate into 96 well plate.

Cell culture

1. Dilute the stock 2 mg/mL antigen in TC media (Hooke #DS-0111, DS-0121, DS-0131, or DS-0141) to 40 µg/mL with tissue culture media (a 50-fold dilution).

2. Make 6 to 7 serial 3-fold dilutions starting with the 40 µg/mL solution (these are 2x of the final concentrations that will be in the cultures). Use TC media alone (without antigen) as a negative control.

3. Add 100 µL of the cell suspension into each well of the 96 well plate. In order to have a uniform number of cells in each well, gentle but continuous mixing of the cell suspension is necessary while plating the cells into 96 well plates.

4. Add 100 µL of each peptide dilution (as well as TC media only negative control) into appropriate wells of the 96 well plate. (Plating in triplicates is recommended.)

5. Culture for 70-72 hours, 37 °C, 5% CO_2, humidified.

6. Collect supernatants.

Measure cytokine concentration.

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Expected results

Supernatants from the top concentration of antigen (20 μg/mL) will produce large amounts of interferon gamma (IFNγ), IL-17A, IL-6 and TNF, and little or no IL-2, IL-4 and IL-10.

Typical amounts of each cytokine expected from supernatants cultured with 20 μg/mL of antigen are shown below.

Cytokine	Spleen supernatant (pg/mL)	Lymph node supernatant (pg/mL)	Notes
IFNγ	800-8000	700-11,000	Spleen within 2 fold of lymph node
IL-17A	150-650	800-2500	Always higher in lymph node
IL-6	100-900	10-150	Always lower in lymph node
TNF	100-400	80-500	Spleen usually within 2 fold of lymph node
IL-2	0-70	0-10	Reverse dose response often observed; highest concentrations sometimes in negative control cultures
IL-4	0	0	IL-4 is never detected in these cultures
IL-10	0-30	0-30	IL-10 traces (typically less than 10 pg/mL) detected

IL-2 is consumed by the proliferating T cells in these cultures. IL-2 is more easily detected if culture supernatants are analyzed after only 18-24 hours of culture.

References

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Carter LL et al, J Neuroimmunol 182:124 (2007)
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