2.1. Cell Culture

1. Swiss mouse 3T3 cells

Random-bred Swiss mouse 3T3 cells provide optimal feeder support of epithelial cells (4). The cells are maintained by weekly passage at 1:10 to 1:20. Use fresh cells within 6 months after thawing, since the cells start to senesce or undergo spontaneous transformation with prolonged passages.

2. DMEM culture medium for Swiss mouse 3T3 cells and TECs.

Five hundred mL of Dulbecco's Modified Eagle's Medium (DMEM) (D5796, SIGMA) is supplemented with 1.2 g of HEPES, 0.375 g of L-Glutamine (final 0.750 g/L), 25 mg of Gentamicin, 2 μL of 2-mercaptoethanol, and 25 - 50 mL of fetal bovine serum (5.0 - 10% fetal bovine serum).

3. SMEM culture medium for TECs (note 1)

Five hundred mL of SMEM (SMEM, 11380, Gibco) is supplemented with 1.2 g of HEPES, 0.375 g of L-Glutamine (final 0.750 g/L), 25 mg of Gentamicin, 2 μL of 2-mercaptoethanol, 5 μL of 50 mg/mL-hydrocortisone hemisuccinate stock solution and 37.5 - 50 mL of fetal bovine serum (7.5-10 %).

4. 50 mg/mL-hydrocortisone hemisuccinate stock solution

100 mg of Hydrocortisone 21-hemisuccinate sodium salt (H2270, SIGMA) is dissolved in 2mL of H_2O.

5. Typsin solution (0.25%)

Solutions of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1mM) are purchased from Gibco/BRL.

You may use TrypLE™ Exress (12604, Gibco) instead of Typsin solution (0.25%).

6. IFN-γ

IFN-γ (#315-05, PeproTech EC Ltd) is dissolved at 10^6 units/mL in 5.0% FCS-DMEM and stored in 50 μL of aliquots at -20°C. To enhance the expression of MHC class II molecules on TECs, IFN-γ is added to the 5.0% FCS-DMEM at 500 units/mL (note 2).

7. Plastic Wares for cultivation of TECs

The use of Falcon Primaria (60 mm dish, #353802 and 6-well multi-well plate, #353846) or gelatin coated wares (60 mm dish, #4010-020, IWAKI) warrants both the growth and the colony formation of TECs in both the isolating procedure and the cloning procedure. After the cloning, any other plastic wares for tissue culture as well as the above wares can be utilized to culture TECs.

8. Redcently, the follows reagents from Gibco are available.

GlutaMax™-1, 35050: Add 1/40 volumes of this solution into 500 ml of SMEM.

1M HEPES, 15630: Add 1/100 volumes of this solution into 500 mL of SMEM

50mg/mL of Gentamicin, 15750-060: Add 1/1000 volumes of this solution into 500 mL of SMEM.

2.2. Immunofluorescence with Confocal Microscope

1. Confocal microscope

A Zeiss LSM 510 confocal microscope is equipped with a x64/1.4 NA Plan-Apochromat oil-immersion lens. Ar-laser liner at 488 nm is used for excitation of FITC or Alexa 488. On the other hand, He-Ne laser liner at 545 nm is used for excitation of Rhodamin or Alexa 543. Emission wavelengths are separated by band pass (505-530 nm) and long pass (585 nm) filters, respectively.

2. Preparation of slide glasses.

1) Eight-hole heavy Teflon-coated slides (Bokusui Brown, New York, NY) are washed extensively with a neutral detergent , rinsed well with hot water, and rinsed once with acetone.

2) Slides are sterilized by rinse with 70% ethanol and dried in a clean bench.

3) Put one drop of 10%FCS-DMEM on each hole of the slides and incubate them in a CO_2 incubator for over 24 hr.

3. Phosphate buffered saline (PBS)

Dissolve 9.6 g of the PBS powder (cat. No 05913, Nissui, Japan) in milli-Q grade water to make 1000 mL. Sterilize PBS by autoclave.

4. Stock PBS (2X)

Dissolve 9.6 g of the PBS powder in milli-Q grade water to make 500 mL. Sterilize stock PBS (2X) by autoclave.

5. Fixation solution

Add 50 mL of milli-Q grade water and 40 μL of 1N NaOH to 4.0 g of paraformaldehyde (P001, TAAB Lab. Equip. Ltd.). The suspension is swirled and then carefully warmed with a microwave oven at intervals of 10 seconds. These procedures are repeated until the suspension solution turns clear (note 3). Cool the solution down to room temperature and then add 50 ml of 2X stock PBS. Check that the pH value of the solution is approximately 7.0, by using a pH-test paper (note 4).

6. Tris-buffered saline (TBS)

Dissolve one package of the TBS powder (T903, TAKARA, Japan) with 1000 mL of milli-Q grade water.

7. Quenching solution (0.1M Glycine solution (pH 7.0))

Dissolve 3.75 g of Glycine with 500 mL of milli-Q grade water. Adjust the pH of the solution to pH 7.0 with 1M Tris-solution.

8. Permeabilization solution (0.05% saponin-TBS)

Dissolve 25 mg of saponin (S-7900, SIGMA) with 50 mL of TBS.

9. Antibody dilution buffer (1% BSA-0.02% NaN_3-TBS): Dissolve 1.0 g of BSA powder (Fraction V) and 20 mg of NaN_3 with 100 mL of TBS.

10.Mounting medium

Mix one volume of PBS with nine volumes of glycerin (for fluorescence microscopy, cat. no. 1.04095. MERCK, Germany).

2.3. Antibodies used in the Immunofluorescence Analyses.

1. Anti-pan keratin antibody: A rabbit anti-human keratin antibody (A0575) is purchased from DAKO Co, CA.

2. Anti-keratin 5 antibody: A rabbit anti-mouse cytokeratin 5 (AF138) antibody is purchased from CONVANCE, Berkeley, CA.

3. Anti-keratin 8 antibody: A mouse anti-human cytokeratin 8 monoclonal antibody is purchased from PROGEN Biotechnik GmbH, Heiderberg, Germany.

4. M5/114: M5/114 is a rat monoclonal antibody against mouse I-A^b (5). M5/114 has been purified from the culture supernatant of the hybridoma with the protein A-Sepharose column according to the manufacturer's protocol (Amersham Biosciences AB, Uppsala, Sweden). Determine the concentration of this antibody by the Bradford method according to the manufacturer's manual (BIO-RAD Lab., CA). .

5. In1.1: In1.1 is a rat monoclonal antibody against mouse invariant chains (6). In1.1 is supplied by Dr. G. J. Hämmerling (German Cancer Research Center, Germany). Add 326 g of ammonium sulfate (55% of ammonium sulfate solution at 0^{o}C) into 1000 mL of the culture supernatant. After centrifugation at 15,000g x 15 min, the precipitate is dissolved in a small volume of PBS and dialyzed against 100 times volume of PBS containing 0.02%NaN_3 three times at intervals of more than 4 hours. Determine the antibody concentration by the Bradford method.

6. 30-2: 30-2 is a mouse monoclonal antibody that reacts with the I-A^b molecules associated with CLIP (7). 30-2 is supplied by Dr. A. Rudensky (Howard Hughes Medical Institute, Univ. of Washington School of Med., Seattle). 30-2 has been purified from the culture supernatant of the hybridoma with a protein A-Sepharose column. Determine the antibody concentration by the Bradford method.

7. Anti-H2-DM antibody: Rabbit antisera to H2-DMβ2 chain has been raised against a KLH-coupled synthetic peptide derived from the cytoplasmic tail of H2-DMβ2 chain (CRKSHSSSYTPLPGSTYPEGRH) (8). As shown in the sequence of the synthetic peptide, the cystein residue is added to the N-terminal end. This cystein is available for the conjugation of the synthetic peptide with both KLH for immunization and with epoxy-activated Sepharose 6B for affinity purification of the antisera. The antisera were purified with the above synthetic peptide-conjugated epoxy-activated Sepharose 6B, which is made according to the manufacturer's protocol (Amersham Biosciences AB, Uppsala, Sweden). Determine the antibody concentration by the Bradford method. The antibody is designated as anti-H2-DM antibody by immunofluorescent staining and western blotting.

8. Anti-rab5 antibody: A mouse monoclonal antibody to rab 5 for immunofluorescent study is purchased from Transduction Lab. (Lexington, KY).

9. Anti-rab5 antibody: A rabbit antibody to rab 5A for western blot analysis is purchased from Santa Cruz Biotech.Inc (Santa Cruz, California).

10. Anti-cathepsin B antibody, anti-cathepsin D antibody, and anti-cathepsin L antibody: These antibodies are provided by Dr. E. Kominami (Department of Biochemistry, School of Medicine, Juntendo University, Tokyo, Japan).

11. Anti-cathepsin S antibody: A goat antibody raised against a peptide corresponding to an amino acid sequence at the carboxy terminus of rat cathepsin S is purchased from Santa Cruz Biotech.Inc (cat. No sc-6505, Santa Cruz, California).

12. anti-LC3 antibody: A rabbit antibody to LC3, a peculiar marker molecule to the autophagy (9) is provided by Dr. T. Ueno (Department of Biochemistry, School of Medicine, Juntendo University, Tokyo, Japan).

13. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD107a (LAMP-1) monoclonal antibody is purchased from BD PharMingen.

14. Fluorescent conjugated anti-immunoglobulin (IgGs) antibody: On single-color immunofluorescent study, use an anti-IgGs antibody conjugated with Alexa 543 (Molecular Probes, Inc, Eugene, OR) as usual. On two-color immunofluorescent study, use an anti-rabbit IgG antibody conjugated with TRITC (R 156, DAKO A/S, Denmark) or Alexa 543 (A11034, Molecular Probes, Inc.) to probe the first antibody and an anti-IgGs antibody conjugated with Alexa 488 (Molecular Probes, Inc,) to probe the secondary antibody. Upon reaction with the biotin-conjugated second antibody, use avidin-conjugated Alexa 488 (Molecular Probes, Inc,) to probe it.

3. Methods

3.1 Establishment and Maintenance of TEC Lines

3.1.1. Preparation of Feeder Layer

1. Swiss mouse 3T3 cells is selected to support optimal growth of TECs isolated from mouse thymus. The cells are cultured with 5% FCS-DMEM at 37°C in 5% of CO_2, and maintained by weekly passage at 1:10 dilution.

2. Upon the confluent condition of the cells, remove the medium and rinse the cells once with 0.02% EDTA-PBS, and then cells are harvested with 0.25% trypsin-0.02% EDTA-PBS. After centrifugation at 500g for 10 min, suspend 1 x 10^7 cells in 10 ml of 5%FCS-DMEM.

3. To irreversibly inhibit proliferation of the cells, irradiate the cell suspension at 40 Gy.

4. Plate 7 x 10^5 irradiated cells per 4 ml of 7.5% FCS-DMEM on a 60-mm dish, and culture for 1-2 days.

3.1.2. Establishment of TEC Lines from Mouse Thymus

1. Isolate thymic lobes from embryos at 15 days of gestation or newborn mice and disperse them in PBS containing 0.25% trypsin and 0.02% EDTA with gentle pipetting.

2. Add an equal volume of 10% FCS-DMEM into the cell suspension. After centrifugation at 500g for 10 min, resuspend the cell pellets in 2 mL of 10% FCS DMEM. Repeat this procedure once.

3. After centrifugation, resuspend the cell pellets at 10^5 per 1 mL of 10% FCS-DMEM. Plate 1 mL of the cell suspension on Swiss 3T3 cells that are pre-irradiated at 40Gy, plated at 7 x 10^5 per 2 mL of 10% DMEM in a 60-mm dish, and culture for 2 days in 5%CO_2 at 37°C.

4. Culture the cells in 5% CO_2 at 35°C (note 5). Suck out a half volume of the medium and add the same volume of 10%FCS-Jolik MEM (note 1) at intervals of 4-5 days.
5. The epithelial cells push aside feeder cells and form a colony (Fig. 1). When the colony grows up to have a diameter of approximately 10 mm, the epithelial cells are cloned by the following procedures.
6. Wash the dish twice with 4 ml of PBS containing 0.02% EDTA to remove Swiss 3T3 cells and thymic non-epithelial cells. Add 2 ml of PBS containing 0.25% trypsin and 0.02% EDTA into the dish and incubate the solution to remove the cells from the dish.
7. Add an equal volume of 10%FCS DMEM into the dish, and centrifuge the cell suspension at 500g for 10 min. Resuspend the cell pellet in 2 mL of 10% FCS DMEM. Repeat this procedure once.
8. Suspend the cells at 10^4 per 1mL of 10% FCS-DMEM in a 60 mm dish. Under an inverted microscope, pick up a single cell or a cluster of cells from the cell suspension by using a micropipette and transfer them to each hole of 24-well plates (Falcon, 3847), which is coated with 10^5 of 40Gy-irradiated Swiss 3T3 cells in 1 mL of 10% FCS DMEM. The culture is done in 5%CO_2 at 35°C with replacing the cultured medium with 0.5 mL of 10%FCS-Jolik MEM at intervals of 4 days. The cloning procedure is repeated once to obtain TEC lines.

3.1.3. Maintenance of TEC Lines

1. After establishment of TEC lines, culture TEC lines with 10% FCS-JMEM at 35°C ~ 37°C in 5% CO_2. Replace the cultured medium with the flesh medium at intervals of 4 days. If the growth of the TECs is getting slow or arrested, add 10^5 of 40Gy-irradiated Swiss 3T3 cells per 1 mL of 10% FCS DMEM.
2. When the cell growth reaches the confluent condition, cells are washed with PBS(-) once and treated with PBS containing 0.25% trypsin and 0.02% EDTA at 37°C. Add 10% FCS DMEM to inhibit the residual enzymatic activity of trypsin and then centrifuge at 500g for 10min. Suspend the cell pellet in 10%FCS-JMEM. Replace the one-fourth portion of the cell suspension in a new dish supplemented with the fresh medium and cultured at 35°C ~ 37°C in 5% CO_2

3.1.4. Preservation of TEC Lines

Cells are washed with PBS(-) once, treated with 0.25% trypsin-PBS(-) in 37°C, and centrifuged at 500g for 10min. Suspend approximately 5 x 10^6 cells in 1ml of FCS containing 10% DMSO. The stock is first stored at -70°C, and then transferred into liquid nitrogen 2 days later.

3.2. Immunofluorescence Staining with Antibodies against Keratins and Epithelial Cell Markers for Characterization of TEC Lines

1. Put one drop (approximately 50 μL) of 10^5 TEC line cells in 5% FCS-DMEM on eight-hole heavy Teflon-coated slide prepared as described in chapter 2.2.2, and incubate the slides at 35°C in 5%CO_2 for several days.
2. To enhance the expression of molecules, including class II MHC molecules, involved in the class II MHC-restricted antigen presentation, TEC line cells are cultured in 5% FCS-DMEM containing 400 IU/mL

of IFN-γ at 37°C in 5%CO_2 for 72 hr.

3. Wash the cells twice with FCS-free MEM for 10 min at room temperature

4. Fix the cells with cold acetone (kept at 4°C) for 10 min on ice.

5. Wash the cells three times with cold TBS for 10 min.

6. Incubate the cells with 5 μg/mL of the primary antibody in 1%BSA-TBS overnight in a refrigerator with gentle swinging.

7. Wash the cells three times with cold TBS for 10 min.

8. Incubate the cells with 5 μg/mL of the secondary antibody in 1%BSA-TBS for 3 hr at room temperature.

9. After washing the cells three times with cold TBS for 10 min, seal the slides with PBS (-)-glycerin solution (1: 9). The sealed slides can be stored at -20°C for at least one month.

8. The immunofluorescence photographs are taken on a Zeiss confocal laser scanning inverted-microscope, LSM 510.

3.3. Immunofluorescence staining for Localization and Characterization of H2-DM-positive compartments in TEC Lines

1. Put one drop of 10^5 TEC line cells in 10% FCS-DMEM on eight-hole heavy Teflon-coated slides prepared as described in chapter 2.2.2, and incubate the slides at 35°C in 5%CO_2 for several days.

2. To enhance the expression of molecules involved in the class II MHC-restricted antigen presentation including class II MHC molecules, TEC line cells are cultured in 5% FCS-DMEM containing 400 IU/mL of IFN-γ at 37°C in 5%CO_2 for 72 hr.

3. A half portion of the cells is further cultured in 5% FCS-DMEM containing 100 IU/mL of IFN-γ, 6 μg/ml of pepstatinA, and 6 μg/mL of E64d at 37°C for 8hr (note 6). Another half portion of cells is further cultured in 5% FCS-DMEM containing 100 IU/mL of IFN-γ, without protease inhibitors.

4. Wash the cells once with FCS-free MEM for 10 min at room temperature.

5. Fix the cells with PBS (pH7.0) containing 4% of paraformaldehyde (PFA) for 10 min at room temperature, and then incubate them in 0.1 M glycine solution (adjusted to pH 7.0 with 1M Tris) to stop the reaction of PFA. .

6. After washing the cells twice with TBS for 10 min, permeabilize the cells with TBS containing 0.05%w/v of saponin for 10 min at room temperature.

7. Wash the cells three times with TBS for 10 min, apply 5 μg/mL of rabbit anti-H2-DM antibody or anti-LC3 antibody in 1%BSA-TBS onto the cells, and incubate the cells with the antibody overnight in a refrigerator with gentle swinging.

8. Wash the cells three times with TBS for 10 min, incubate the cells with 5ug/ml of Alexa 543-conjugated anti-rabbit immunoglobulins in 1%BSA-TBS for 3 hr at room temperature with gentle swinging.

9. Wash the cells three times with TBS for 10 min, incubate the cells with 5 μg/mL of the secondary antibody in 1%BSA-TBS-0.02%NaN_3 overnight in a refrigerator with gentle swinging.

10. Wash the cells three times with TBS for 10 min, incubate the cells with 5 μg/mL of Alexa488-conjugated anti-IgGs antibody or Alexa488-conjugated avidin in 1%BSA-TBS-0.02%NaN_3 for 3 hr at room temperature with gentle swinging.

11. Wash the cells three times with TBS for 10 min, the stained samples were sealed with PBS/glycerin (1:9) and imaged on a Zeiss LSM 510 confocal microscope.

Notes

1. This medium (JMEM) is a Ca^{2+}-free MEM. The low concentration of Ca^{2+} is recommended for establishment and maintenance of TEC lines.
2. The addition of IFN-γ to the 5%FCS-DMEM is necessary for the expression of MHC class II molecules on TEC lines. To fully express the MHC class II molecules, TEC lines must be cultured for 2 days in 5% FCS-DMEM with 500IU/mL of IFN-γ.
3. Paraformaldehyde (PFA) is toxic. Wear gloves, face mask, and safety glasses. Do not boil the PFA solution with a microwave oven. If so, you must immediately bring the solution into a chemical fume hood.
4. It is better to fix cells with the PFA solution indicating the value of pH 6.8-7.2. Upon fixation with the acidic PFA solution (below pH 6.5), antibodies could not react to the fixed cells efficiently. On the other hand, upon fixation with the basic PFA solution (over pH 7.5), the extent of background in immunofluorescent staining slides could increase.
5. The irradiated Swiss mouse 3T3 cells secret growth factors to support the growth of TECs and oppose the adherence of fibroblast cells derived from thymus. The culture of cells at 35°C does not affect the growth rate of TECs but diminish the growth rate of the fibroblast cells.

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