

Production of T-Cell Lines

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Abstract

Allergen-specific T-cell lines established from allergic patients provide the opportunity of investigating T-cell functions at the poly- or oligoclonal level. T-cell lines are useful in determining the presence or absence of antigen-specific T-cell reactivity. However, to obtain detailed knowledge of the action of T cells with clearly defined features, for example epitope specificity or phenotype, T-cell clones are necessary.

The frequency of allergen-specific T cells in peripheral blood mononuclear cells (PBMC) tends to be low and so stimulation of PBMC with single allergens often results in low allergen-specific reactivity or requires high doses of the allergen. In contrast, the stimulation of PBMC with whole allergen extract results in stronger reactivity because a greater spectrum of T-cell specificities is addressed. Therefore, for the investigation of polyclonal reactivity toward single allergens it is useful to establish T-cell lines, which represent an allergen-specific enrichment of T cells from the respective individual. These T cells are poly- or oligoclonal and might possess different epitope specificities. The method described here is based on experiences with human T-cell lines and clones specific for several allergens from grass pollens and tree pollens.

Key Words: T cell; oligoclonality; T-cell line; clone; allergen.

1. Introduction

Growing antigen-specific human T cells in vitro offers the possibility of investigating the function and characteristics of T cells and determining the influence of substances which may modulate T-cell functions. Allergen-specific T-cell lines established from allergic patients are useful for investigating T-cell functions at the poly- or oligoclonal level and for determining the presence or absence of antigen-specific T-cell reactivity. However, to obtain detailed knowledge of the action of T cells with clearly defined features, for example epitope specificity or phenotype, T-cell clones are necessary (*1*). Growing T-cell lines is less time consuming than growing T-cell clones (*1*).

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In general, T-cell response to allergens tends to be weak because of the low frequency of allergen-specific T cells in peripheral blood mononuclear cells (PBMC). Stimulation of PBMC with single major allergens often results in low allergen-specific reactivity or requires high doses of the allergen (2–5). In contrast, the stimulation of PBMC with whole allergen extract results in stronger reactivity because a greater spectrum of T-cell specificities is addressed (2,6). Therefore, for investigating polyclonal reactivity toward single allergens, it is useful to establish T-cell lines, which represent an allergen-specific enrichment of T cells from the respective individual. These T cells are poly- or oligoclonal and might possess different epitope specificities. It has been shown that single individuals can possess several T-cell specificities for one allergen (7–11).

The method described here is based on experiences with human T-cell lines and clones specific for several allergens from grass pollens and tree pollens. The method starts as usual with the separation of PBMC from the blood of the patients. The PBMC are seeded in a 96-well culture plate and stimulated with the desired allergen. During the culture period of 14 ± 2 d the cells are fed at defined intervals with fresh medium and interleukin-2 (IL-2). After 14 ± 2 d a proliferation test is set up with the respective allergen, using irradiated autologous PBMC as accessory cells. The antigen-specific stimulation of T cells requires autologous antigen-presenting cells as antigen presentation is restricted by the MHC class II molecules of the respective individual. B lymphocytes, monocytes, and a very low proportion of dendritic cells constitute the antigen-presenting cell fraction in PBMC. Positive cultures are either combined to represent a T-cell line or expanded separately to represent different T-cell lines. This procedure differs from the more frequently described procedure in the literature (3,7,12), where the initial stimulation with the antigen is performed in 24-well culture plates with a greater number of cells in the same well. The advantage of the method described here, which uses 96-well culture plates, is that T-cell lines with stronger reactivity for a certain major allergen could be obtained because wells including cells with no allergen-specific reactivity or unspecific reactivity can be excluded more efficiently right from the beginning. This is especially important when only a few patients with the respective allergy are available and when the frequency of T cells with specificity for a certain major allergen is low.

If T-cell clones are required then T-cell lines should be cloned immediately after selection. The expansion of T-cell lines involves sequential stimulation with allergen and autologous PBMC and unspecific stimulation with PHA and allogeneic PBMC.

2. Materials

2.1. General Equipment for Cell Culture

1. Laminar flow hood.
2. Cell incubator (37°C, 5% CO₂, humidified atmosphere).

3. Centrifuge with tube and microtiter plate carriers.
4. Microscope.
5. Single and 8-channel automatic pipets.
6. -80°C freezer and liquid nitrogen storage container for cryopreservation.

2.2. Separation of PBMC

1. Leucosep tubes, 50 mL (Greiner, Frickenhausen, Germany) (*see Note 1*).
2. Lymphocyte separation medium (density 1.077; PAA, Cölbe, Germany) (*see Note 2*).
3. Heparin as anticoagulant (Liquemin N 20.000; Hoffman-La Roche, Grenzach-Whylen, Germany), 1 mL containing in aqueous solution 20,000 I.E. Na-heparin (*see Note 3*).
4. 30–50 mL syringes and appropriate cannulas.
5. Medium for cell washing: RPMI 1640 (PAA) either with 10% fetal calf serum (FCS) ($\Rightarrow$ RPMI*) or without it ($\Rightarrow$ RPMI $\emptyset$). Shelf life: 4 wk when stored at 4°C under sterile conditions.

2.3. Generation of Allergen-Specific T-Cell Lines

2.3.1. Patients

For the generation of allergen-specific T-cell lines, it is necessary to use PBMC from subjects with a positive clinical history of the respective allergy and high allergen-specific IgE level in blood (> 3.5 kU IgE/L). The availability of patients for subsequent blood donations must be confirmed before bleeding the patients to ensure a source of autologous PBMC (*see Notes 4 and 5*). The amount of blood taken from patients should in the first instance be a minimum of 100 mL; however, up to a maximum of 450 mL blood enables you to restimulate the T cells without having to subsequently bleed the patient for fresh cells.

2.3.2. Culture Medium

Grow the T-cell culture under serum-free conditions in Ultraculture medium (Cambrex, Verviers, Belgium) (*see Note 6*) supplemented with the following ingredients:

1. UltraGlutamine I (Cambrex) at a final concentration of 2 mM. Aliquot the stock solution of UltraGlutamine I (100 $\times$) to 5 mL and store at -20°C (*see Note 7*).
2. Antibiotic–antimycotic solution (Sigma, Taufkirchen, Germany) at a final concentration of 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin sulfate, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B. Aliquot the 100-fold stock solution to 5 mL and store at -20°C .
3. Mercaptoethanol (Invitrogen, Karlsruhe, Germany) at a final concentration of 20 μM . Aliquot 50 mM stock solution to 250 μL and store at -20°C .
4. For preparation of the culture medium thaw an aliquot of each, UltraGlutamine I, antibiotic–antimycotic solution, and mercaptoethanol (200 μL), mix and add to 500 mL Ultraculture medium under sterile filtration through a 0.22- μm filter (Millex GV13; Millipore, Eschborn, Germany).
5. The prepared medium is stored at 4°C until use. The shelf life is 4 wk.

2.3.3. T-Cell Stimulation

1. Allergens are obtained from either purified natural extracts or recombinant technologies. Dilute allergen to a concentration of 100 $\mu\text{g/mL}$ with Ultraculture medium without supplements and then filter through 0.22- μm filter (Millex GV4; Millipore) (The filter should be suitable for sterile filtration of aqueous solutions with very low protein-binding capacity and with small diameters.) Aliquot the allergen solution to 200 μL or any other desired volume and store at -20°C . Dilute the allergen extract to a final concentration of 1 mg/mL with Ultraculture medium and sterile filter in the same way as described above, aliquot, and store at -20°C (see **Note 8**).
2. Recombinant IL-2 may be obtained from different sources. The material used by the author (IL-2CC) was obtained from Strathmann Biotec (Hamburg, Germany). Reconstitute the 50,000 units/vial in Ultraculture medium without supplements to a concentration of 2500 U/mL, aliquot at 200–500 $\mu\text{L/vial}$ or any other desired volume, and store at -20°C .
3. 96-well round-bottom culture plates (Greiner).

2.4. Testing of T-Cell Lines for Allergen Specificity

1. Centrifuge capable for centrifugation of 96-well culture plates, 96-well round-bottom culture plates (Greiner, Germany).
2. Cell harvester. The method described here uses a 96-channel harvester (PerkinElmer, Rodgau-Jügesheim, Germany).
3. Beta-Counter. The method described here uses a Microbeta scintillation counter (PerkinElmer).
4. Microplate heat sealer (PerkinElmer) (see **Note 9**).
5. [6-3H]-Thymidine, 37 MBq/mL (Amersham Biosciences, Freiburg, Germany).
6. Allergen (see **Section 2.3.3.** and **Note 8**).
7. Autologous PBMC from respective patients should be prepared for the presentation of allergen-derived peptides to T cells in the proliferation assays (see **Note 4**).
8. Harvest PBMC from the blood as described under **Section 3.1**. After counting of PBMC with a hematocytometer, freeze the cells in aliquots of 5×10^6 cells/mL freezing medium in 2-mL cryovials (Greiner), and store in liquid nitrogen (see **Section 3.5**). Count cells following thawing to ensure that only viable cells are considered. Adjust cells to a maximum of 100×10^6 PBMC/10 mL RPMI* tissue culture tubes (Greiner) and irradiate with 30 Gy (see **Note 10**).

2.5. Expansion of T-Cell Lines

1. Reconstitute phytohemagglutinin (PHA) (Sigma) in Ultraculture medium without supplements to a final concentration of 240 $\mu\text{g/mL}$, aliquot, and store at -20°C .
2. For nonspecific stimulation with PHA, use a mixture of irradiated (30 Gy) allogeneic PBMC from at least five healthy individuals. Harvest and freeze the cells in the same way as described for autologous PBMC (see **Sections 2.4.** and **3.1.**).
3. 24-well culture plates (Nunc, Wiesbaden, Germany).

2.6. Cryopreservation of T-Cell Lines

1. Freezing medium: RPMI 1640 with 10% DMSO (Merck, Darmstadt, Germany) and 20% FCS. Mix all the components, aliquot (5–10 mL), and store at -20°C until use.
2. Cryovials (Greiner).

3. Methods

The whole procedure is summarized in **Fig. 1**.

3.1. Separation of PBMC

1. Draw blood from the patients by using heparin as anticoagulant. Prepare the 50-mL syringes with approximately 100 μL heparin (≈ 2000 I.E. heparin).
2. Centrifuge (1 min, 1000g) 15 mL of lymphocyte separation medium in Leucosep tubes. Following centrifugation, the lymphocyte separation medium is found under the filter disc of the Leucosep tubes.
3. Transfer 30 mL heparinized undiluted blood on the filter disc and centrifuge for 20 min at 800g.
4. Harvest the cells from the interface using a pasteur pipet.
5. Wash cells with RPMI $\emptyset$ (1 volume cell suspension:2 volumes of RPMI $\emptyset$) and centrifuge for 10 min at 800g.
6. Wash cells twice with RPMI* (10 min, 200g) and count the cells with a hemacytometer (*see Notes 11 and 12*). 1 mL of blood yields approximately 1×10^6 PBMC.
7. Freeze remaining cells as a supply of autologous PBMC (*see Sections 2.4. and 2.6.*).

3.2. Generation of Allergen-Specific T-Cell Lines

1. Incubate freshly isolated PBMC from allergic patients at a concentration of 1×10^5 per well in a 96-well round-bottom culture plate in a total volume of 100 μL Ultraculture medium with the desired concentration of the respective allergen (1–25 $\mu\text{g/mL}$) (*see Note 8*).
2. Incubate for 5–7 d. Allergen-stimulated cells develop to form clusters of proliferation and increase in number.
3. Add 100 μL of fresh culture medium with a final concentration of 5–10 units IL-2/mL (*see Note 13*).
4. After 3–4 d of culture, exchange half of the medium with fresh medium containing 5–10 U IL-2/mL and incubate for another 4 d.
5. Fourteen days (± 2 d) after setting up the culture, perform a proliferation assay as described under **Section 3.3.** and check for antigen specificity.

3.3. Testing of T-Cell Lines for Allergen Specificity

1. Sediment the cells of the 96-well culture plate by centrifugation at 200g for 5 min. Remove the supernatant and wash the cells with 100 μL RPMI*.
2. Repeat the washing step once with 100 μL RPMI* and then once with 100 μL Ultraculture medium.

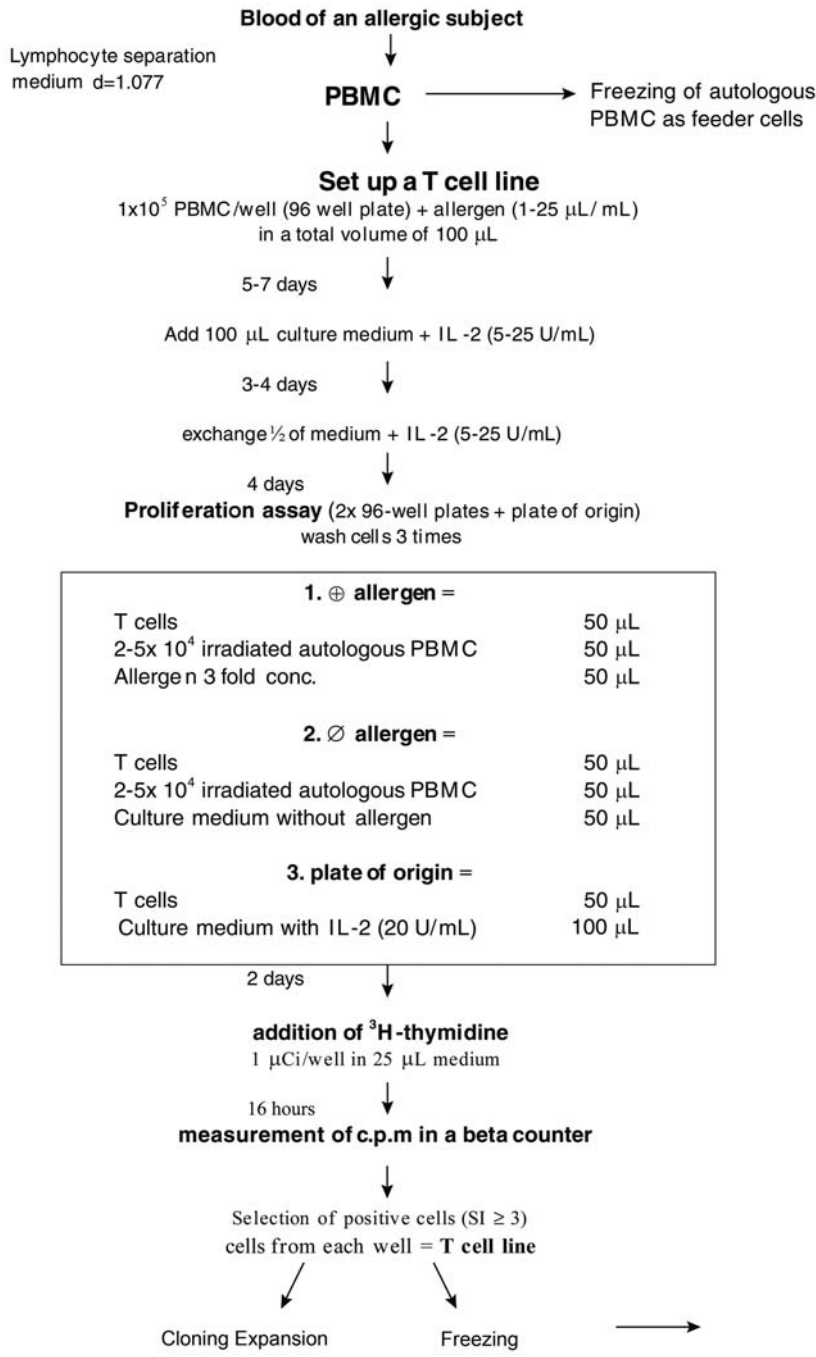


Fig. 1. Generation of allergen-specific T-cell lines.

3. Suspend the cells in 150 μ L Ultraculture medium and divide the initial culture among three 96-well plates. Resuspend the cells in the well of the original plate and transfer 50 μ L to corresponding wells of the two new culture plates using a multi-channel pipet. These two plates represent the two test plates.
4. 50 μ L of the cell suspension remains in the original plate for the continuation of the culture. Feed these cells with 100 μ L Ultraculture medium containing IL-2 at a final concentration of 20 U/mL.
5. The proliferation test is performed with the two test plates. Add 5×10^4 irradiated autologous PBMC in a volume of 50 μ L Ultraculture to each well of the two test plates.
6. Add the antigen in the optimal concentration in a volume of 50 μ L to the wells of the antigen stimulation plate and 50 μ L of culture medium without antigen to the control plate (*see Note 14*).
7. Incubate the plates for 48 h in the incubator with 5% CO₂, 37°C, and humidified atmosphere.
8. Add 1 μ Ci ³H-thymidine (= 37 kBq) in 25 μ L Ultraculture medium and incubate for another 16 h.
9. Harvest the cells onto a filter. The following steps of the method used by the author involves cell harvesting with a 96-channel cell harvester (PerkinElmer) on filter mats in 96-well plate format and the subsequent counting in a Microbeta scintillation counter (PerkinElmer).
10. Dry the filter mats (*see Note 15*) and apply a solid scintillator sheet on the filter, put it in a Microbeta sample bag (PerkinElmer), and allow the scintillator to melt into the filter mat using a microplate heat sealer (PerkinElmer).
11. Transfer the prepared filter into a 96-well filter mat cassette and then the cassette into the Microbeta counter for the measurement of the cpm (counts per minute).
12. Results are expressed as a stimulation index (SI), calculated as the quotient of the cpm of the allergen-stimulated wells and the cpm of the corresponding non-stimulated wells.
13. The cells from 10–16 wells with $SI \geq 3$ should be combined to form a T-cell line. Several different T-cell lines can be collected from one 96-well plate depending on the stimulation indices for the individual.
14. Alternatively, if the amount of wells yielding appropriate SI is low or if a large panel of different T-cell lines is desired, the cells of individual wells may be expanded separately (*see Note 16*).

3.4. Expansion of Established T-Cell Lines

1. Transfer the T cells from the selected wells into a centrifugation tube and spin them down (10 min, 200g). Resuspend the cells in 500 μ L Ultraculture medium and transfer them into a well of a 24-well culture plate.
2. Add 1×10^6 – 2×10^6 irradiated autologous PBMC. The ratio of T cells to PBMC should be approximately 2:5.
3. Add antigen in the desired concentration and start the expansion in a total volume of 1 mL.

4. After 3–4 d incubation, prepare a feeding mixture of 1×10^6 – 2×10^6 irradiated allogeneic PBMC, 2.5 μ g PHA/mL, and 25 U IL-2/mL and add Ultraculture medium to make a total volume of 1 mL/well in a 24-well culture plate.
5. After 3–4 further days, exchange half of the medium with fresh Ultraculture medium containing 25 U IL-2/mL.
6. Continue the culture by exchanging half of the medium every third or fourth day with fresh medium and IL-2. When the medium becomes yellow and the cells are confluent, split the culture by dividing the cells between two wells with addition of fresh medium and IL-2.
7. Freeze as many cells as possible. This can be performed at any time-point of the culture, but a proliferation assay can only be set up 12–14 d after the last feeding with PBMC (*see Note 17*).
8. When the T cells are maintained in culture, antigenic stimulation with autologous PBMC is necessary every 28 d (*see Note 18*).

3.5. Cryopreservation of T-Cell Lines

1. Centrifuge the cells from the desired wells at 200g for 10 min. One to two fully grown wells of a 24-well plate are required for one cryovial.
2. Discard the supernatant, resuspend the cells in 1 mL cold (4°C) freezing medium, and transfer them to a cryovial.
3. Close the vial and transfer it immediately into a styropor box with approximately 2-cm-thick walls and place in a –80°C freezer or use temperature-controlled cell freezer when available.
4. Transfer the cryovials to liquid nitrogen after at least 1 d.

4. Notes

1. The advantage of Leucosep tubes is that the blood can be decanted directly onto the filter disc in the tube. This prevents the undesired mixing of the blood with the separation medium. Alternatively, the blood can be overlaid carefully over the separation medium in tubes without filter discs.
2. Any other lymphocyte separation medium with a density of 1.077 can be used as an alternative to the one used here.
3. When using heparin from other sources, it is wise to consider whether the heparin is free of preservatives, which may be harmful to the cells.
4. It is possible to establish Epstein-Barr virus (EBV)-transformed B-cell lines in order to have an unlimited supply of autologous MHC class II matched feeder cells for antigen presentation (for details *see ref. 13*). The use of EBV-transformed B cells leads to higher backgrounds in proliferation assays and needs antigen preincubation before irradiation. Their advantage, however, is that they are available in unlimited amounts.
5. The status of the patient is important for the outcome of the T-cell culture: Patients who are or were treated with allergen-specific immunotherapy will be increasingly unresponsive to the allergen because of tolerance induction (*3,14*). Furthermore, it is necessary to make sure that the patients have not used any symptomatic medication around the time-point of blood donation or in case of glucocorticoids approximately

- 6 wk before blood donation. When blood is drawn during an acute allergic phase (for example during the pollen season if grass pollen allergen-specific TCL are wanted) it has to be considered that the T cells might already be stimulated and that no difference between allergen and control stimulation might be observed in vitro.
6. It is also possible to use a medium supplemented with 5% human AB serum. The advantage of serum-free medium is that variations in background level because of components in AB serum are avoided. Because AB serum may vary from batch to batch serum-free medium offers the opportunity of more defined and stable culture conditions.
 7. Glutamine may be used as an alternative. The advantage of using dipeptides such as UltraGlutamine I is that toxic degradation products of glutamine in medium are avoided.
 8. The optimal concentration of the allergens for stimulation is generally in a range of 1–25 $\mu\text{g/mL}$ (for grass or birch pollen major allergens) and has to be evaluated individually.
 9. A microplate heat sealer is necessary when scintillation counting is performed with a Microbeta counter and when it is desirable to limit the amount of radioactive waste.
 10. If irradiation facilities are not available, mitomycin C (Sigma) treatment of the PBMC can be used as an alternative (12). In this case, PBMC should be adjusted to 5×10^6 cells/mL in sterile phosphate-buffered saline (PBS). Add 100 μL of a solution containing 0.5 mg/mL mitomycin C in PBS to 1 mL of the cell suspension and incubate for 20 min in the incubator at 37°C. Add RPMI* to a final volume of approximately 12 mL and centrifuge for 10 min at 200g. Repeat the washing procedure twice in order to remove any residual mitomycin C which might interfere with the T-cell proliferation. The solution should be prepared freshly each day prior to use. It should be considered that mitomycin C is very toxic and light sensitive.
 11. Recommended dilution of freshly harvested PBMC is 1:20 in 3% acetic acid, to eliminate any remaining red blood cells, which makes counting easier.
 12. In the last washing step, the same medium as needed for the T-cell stimulation should be used.
 13. The low IL-2 concentration at this time-point in the culture setup is not capable of activating resting T cells and can only be utilized by T lymphocytes expressing large numbers of IL-2 receptors, which is typical for activated T cells. The activation under these conditions is induced mainly by the allergen.
 14. The three components, T cells, autologous PBMC, and antigen, are added separately to the wells of the test plate. Therefore, the allergen solution has to be prepared threefold concentrated in order to achieve the final desired dilution in the plate. Always pipet in the following order: (1) T cells, (2) PBMC, (3) antigen.
 15. It is important, for correct measurement, that the filters are completely dry. This can be achieved either with an electrical hair dryer or with a drying cabinet.
 16. When expansion of single wells from the 96-well plate is desired, then first transfer the cells into wells of a 48-well plate and stimulate with 0.5×10^6 – 1×10^6 autologous or allogeneic PBMC/well and add allergen or PHA in the indicated concentration. Transfer to 24-well plate when cells are confluent and the medium is turning yellow.

17. It is recommended that detailed documentation about the culture status of a certain T-cell line is maintained. When freezing is performed 14 ± 2 d after the last stimulation with autologous/allogeneic PBMC, the cells could be used in a proliferation assay immediately after thawing.
18. Because the cells of a T-cell line are polyclonal, strong clones might overgrow weak clones and the feature of a T-cell line might change during the expansion process. Thus T-cell lines sometimes lose their specificity or at least their preliminary strong reactivity with the desired antigen during the expansion procedure. Other T-cell lines reveal progressively stronger antigen-specific proliferative responses with each expansion round.

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