

Production of Human T-Cell Clones

Adrienne Verhoef

Abstract

The study of monoclonal human T-cell populations has had a fundamental impact on our current knowledge of the function, specificity, and mechanisms of activation of these cells. The frequency of antigen-specific T cells in peripheral blood is low, necessitating several enrichment steps prior to the isolation of individual clones. Two different methods of limiting dilution cloning are described in this chapter, the choice of which depends on the availability of starting materials. After isolation, T-cell clones are expanded and then tested for specificity and cryopreserved, both of which are described.

Recently, time-saving variations to the above methods have emerged. However, they require the use of sophisticated equipment and reagents, making them less economical than the established techniques.

Key Words: T-cell clone; limiting dilution; antigen-presenting cells.

1. Introduction

The ability to study monoclonal human T-cell populations has had a fundamental impact on our current knowledge of the function, specificity, and mechanisms of activation of these cells. Several technological advances have facilitated the isolation and expansion of human T-cell clones. For example, the isolation and characterization of T-cell growth factor (interleukin-2 [IL-2]) represented a major step forward in generating T-cell clones *in vitro* (1,2). Furthermore, advances in determining the molecular structures of antigenic peptides, TCR, and MHC molecules have allowed the detailed analysis of the interactions among these molecules.

As the frequency of antigen-specific T cells in peripheral blood is low (for example, approximately 1:10,000 of peripheral T cells of a given individual recognizes house dust mite proteins [3]), several enrichment steps precede the isolation of individual clones. Unfractionated peripheral blood mononuclear cells

(PBMC) are incubated with the antigen of interest, allowing antigen-specific T cells to expand. This may be followed by several rounds of restimulation with the particular antigen and IL-2, resulting in an oligoclonal T-cell population (T-cell line). When satisfied that the T-cell line recognizes the antigen of interest, as measured in a proliferation assay, T-cell clones are isolated from the T-cell line by various methods. While ‘limiting dilution cloning’ was first described over 20 yr ago and has remained the method of choice, new technology has enabled more efficient cloning techniques to be developed. Two different methods of limiting dilution cloning are described in this chapter, the choice of which depends on the availability of starting materials. For both methods, a T-cell stimulatory or ‘feeding mixture’ is prepared consisting of irradiated, antigen-presenting cells (APC), antigen in a predetermined optimal concentration, IL-2, and a mitogen, depending on which method is used (*see below*). A set number of antigen-specific T cells are then added to the feeding mixture before plating out into wells of a tissue culture plate, aiming to dispense single T cells into individual wells.

Method A (4) is the preferred method when fresh, autologous (originating from the same donor as the T-cell line to be cloned) PBMC are readily available. This method has the added advantage of using significantly less in terms of reagents, as the cells are plated out into ‘Terasaki’ plates at 20 μ L/well. Method B (5) is used when autologous PBMC are in short supply. As APC, autologous Epstein–Barr virus (EBV) transformed B cells (EBV-B cells; *see Section 2.2.*) are used together with allogeneic PBMC from two donors, to ensure that appropriate costimulation by different types of APC is provided.

After T-cell clones have been isolated they are transferred to larger tissue culture wells and expanded during several rounds of restimulation (*see Section 2.1.*). After sufficiently large numbers have been generated, T cells are tested for specificity in a T-cell proliferation assay and cryopreserved, both of which are described under **Section 3**.

Recently, variations to the above methods have emerged. For example, Turcanu et al. (6) describe a system of obtaining peanut-allergen-specific T-cell clones, based on labeling PBMC with the fluorescent dye, carboxyfluorescein diacetate (CFSE), prior to antigen stimulation. When T cells divide on antigen recognition, CFSE-labeled proteins are equally divided among daughter cells, thus resulting in a reduction of fluorescence. CFSE^{low} T cells (representing antigen-specific cells) are subsequently isolated by cell-sorting on a flow cytometer and cloned by limiting dilution cloning, thus avoiding multiple rounds of antigen-specific stimulation.

Additionally, with the advances of tetramer technology, antigen-specific T cells within unfractionated PBMC populations may be directly identified and isolated by labeling with antigen-specific tetramers (7). However, although saving

time, these new methods require the use of sophisticated equipment and reagents, making them less economical than the established techniques.

2. Materials

2.1. T-Cell Cloning, Expansion, and Maintenance (see Note 1)

1. Basic medium: RPMI-1640 supplemented with 2 mM L-glutamine (Invitrogen, Paisley, UK) (see **Note 2**).
2. AB medium: Basic medium with the addition of 5–10% screened, heat-inactivated human AB serum (Sigma, Poole, UK) (see **Notes 2 and 3**).
3. FCS medium: Basic medium with the addition of 10% heat-inactivated fetal calf serum (FCS; Invitrogen) (see **Notes 2 and 3**). Serum should be stored at -70°C .
4. Freezing mix: 85% FCS and 15% DMSO (Sigma).
5. Ficoll-Hypaque (Sigma).
6. Unfractionated PBMC, freshly obtained or stored in liquid nitrogen (see **Note 4**).
7. Recombinant IL-2 (R&D Systems, Abingdon; Peprotech, London).
8. Antigens (storage depends on manufacturer's instructions) (see **Note 5**).
9. Use of a γ -cell irradiator.
10. Phytohemagglutinin (PHA; Sigma).

2.2. EBV Transformation of B Cells

1. B95-8 EBV producing cell line (ECACC, Salisbury, UK).
2. Cyclosporin A (Sigma) or PHA (Sigma).

2.3. T-Cell Proliferation Assay for Testing T-Cell Antigen Specificity

1. $^3\text{[H]}$ -Thymidine (Amersham).
2. Cell harvester, β -plate counter.
3. Plastics: Terasaki plates, 96-well and 24-well plates, cryovials, tissue culture flasks (all Nunc, Fisher Scientific, Loughborough, UK).

3. Methods

3.1. EBV Transformation of B Cells (8)

3.1.1. Generating EBV Supernatant

1. Culture B95-8 cells according to the supplier's instructions. Harvest supernatant and filter through a $0.45\text{-}\mu\text{m}$ filter.
2. Store in 1 mL aliquots at -70°C until needed.

3.1.2. Transformation of Human B Cells with EBV Virus

1. Incubate 1×10^7 unfractionated fresh or frozen human PBMC with 1 mL of EBV supernatant in a 15-mL conical tube with loose cap, for 4 h in a 37°C , 5% CO_2 humidified incubator.
2. Add 8 mL of FCS medium to the tube, as well as 1 mL of cyclosporin A ($1\text{ }\mu\text{g/mL}$ final concentration) to prevent T-cell proliferation. Mix well and dispense into five wells of a 24-well tissue culture plate at 2 mL/well.

3. Feed cultures weekly by removing 1 mL of supernatant from each well, without disturbing the cells, and replacing this with 1 mL of fresh FCS medium, and leave until foci of proliferating transformed B cells appear (usually after 2–4 wk).
4. Transfer growing cultures to a 75-cm² flask in 30 mL of FCS medium; when growth medium turns yellow, expand by transferring 15 mL of the growing culture to a new flask followed by addition of 15 mL of FCS medium and cryopreserve (*see Section 3.5.*).
5. For use as APC, the cells should be irradiated at 6000 rad.

3.2. T-Cell Cloning

Prior to cloning, T-cell lines are generated by incubating PBMC with the antigen of interest (1° line) followed by at least one round of restimulation with irradiated, autologous PBMC, antigen, and IL-2 to generate 2° and 3° lines, with the aim of increasing the precursor frequency of antigen-specific T cells. It is important to test the antigen-specific nature of the T-cell pool as soon as enough T cells have been generated (usually at the 3° stage), by using a range of antigen concentrations. This will also provide information on optimal antigen concentration to be used in the subsequent cloning procedure. Always include a negative control (APC + T cells) as it is not unusual for autoreactive T cells present in PBMC to be activated as a consequence of *in vitro* culture conditions. When antigen specificity has been determined (3° stage or beyond), the T-cell line is ready to be cloned. The T-cell line should be ‘resting’, i.e., 7 d after antigen-specific restimulation and 3–4 d after the last addition of IL-2.

3.2.1. Method A

Prepare feeding mixture in a total of 23 mL AB medium as follows:

1. Fresh, irradiated (3000 rad), autologous PBMC (0.5×10^6 per mL), antigen at pre-determined optimal concentration, 10 ng/mL IL-2.
2. Prepare T cells as follows: recover T cells into suitable container and wash cells by filling up the tube with RPMI. Centrifuge (160g, 21°C, 10 min), remove medium, loosen the cell pellet, fill up the tube with RPMI, and centrifuge again.
3. Resuspend T cells in 5 mL AB medium at 1×10^6 – 2×10^6 per mL. Carefully layer the T-cell suspension onto an equal volume of Ficoll, in a 15-mL conical tube. Centrifuge (280g, 21°C, 10 min) and wash 2× with RPMI (as for step 2).
4. Recover the T cells from the interface of Ficoll gradient and wash 2× with RPMI (as for step 2).
5. Accurately count live T cells and add 3.6×10^5 cells to 1 mL AB medium (dilution 1). Make three subsequent serial dilutions of the T cells by adding 0.1 mL of dilution 1 to 0.9 mL of AB medium, resulting in a final dilution of 3.6×10^2 T cells in 1 mL. Add this to the feeder mixture, giving a final volume of 24 mL cloning mixture, containing 15 T cells/mL, resulting in a final concentration of 0.3 T cells/well.
6. Dispense mixture into ‘Terasaki’ plates at 20 µL/well and incubate for 7–10 d in a humidified 37°C, 5% CO₂ incubator.

7. Transfer contents of wells with expanding T-cell clones to 96-well flat bottom plates in the presence of autologous, irradiated PBMC (fresh or frozen; 1×10^5 cells/200 μ L per well), antigen at optimal concentration and 10 ng/mL IL-2. Add 10 ng/mL IL-2 after 3–4 d; rapidly expanding clones (recognized by yellowing of AB medium) can be ‘split’ into two wells by transferring 100 μ L of cell suspension into a new well and adding 100 μ L of fresh AB medium to both wells.
8. Seven days after transfer to 96-well plates, the expanding T cells are transferred to 24-well plates, in the presence of a feeder mixture as described above, with 1×10^6 APC per well in a total volume of 2 mL/well.

3.2.2. Method B

1. Prepare feeding mixture as follows: irradiated allogeneic PBMC from two donors (1×10^6 total/mL), irradiated autologous EBV-B cells (1×10^5 per mL), antigen at optimal concentration, 1 μ g/mL PHA in a total volume of 49 mL AB medium.
2. Prepare T-cell mixture as for method A with the following variations: add 1.67×10^5 T cells to AB medium in a final volume of 1 mL. Make three serial dilutions as for method A and add final dilution (167 T cells) to the feeding mixture, resulting in a final concentration of 3.33 T cells/mL of cloning mix.
3. Dispense cloning mixture into 96-well round bottom plates at 100 μ L/well. Incubate in 5% CO₂ incubator at 37°C.
4. After 3–4 d, add 10 ng/mL IL-2, and make volume of wells up to 200 μ L with AB medium.
5. Seven days after cloning, transfer expanding T cells to 24-well plates in the presence of a feeder mixture consisting of irradiated, allogeneic PBMC from two donors (1×10^6 per well), autologous irradiated EBV-B cells (1×10^5 per well), antigen at optimal concentration, PHA (1 μ g/mL), and IL-2 (10 ng/mL), in a total volume of 2 mL/well.

3.3. T-Cell Expansion and Maintenance (see Note 6)

When T-cell clones have reached the 24-well stage, they can be maintained and expanded. T-cell clones should be maintained on a weekly feeding cycle, with irradiated autologous APC (1×10^6 per well for method A), antigen, and IL-2 (10 ng/mL), or with irradiated allogeneic APC (1×10^6 PBMC, 1×10^5 EBV-B), PHA, antigen, and IL-2 for method B, in a total volume of 2 mL/well of a 24-well plate; 3–4 d after each antigenic stimulation, 10 ng/mL IL-2 should be added to all wells, and rapidly expanding T-cell clones can be split at this stage. At the end of the 7-d feeding cycle when T cells are fully resting, they may be used for cryopreservation and functional assays.

3.4. T-Cell Proliferation Assay for Testing T-Cell Antigen Specificity

When sufficient numbers of T cells have been generated (at least 5×10^6), they should be tested for antigen specificity in a proliferation assay, with T cells incubated with APC in the absence of antigen as background control.

The T cells should be used at the end of the 7-d feeding cycle and be fully rested (see **Notes 7** and **8**).

1. Prepare and irradiate APC (autologous PBMC or EBV-B cells) and plate out in triplicate in a round bottom, 96-well plate at 2.5×10^4 per well.
2. Add antigen to appropriate wells at optimal concentration.
3. Add T cells at 2.5×10^4 cells/well.
4. Make volume of each well up to 200 μ L with AB medium.
5. Incubate for 72 h with the addition of ^3H -thymidine (1 μCi /well) for the last 8–16 h.
6. Harvest and count plate.
7. Discard those T-cell clones that do not proliferate significantly as compared to background control.

3.5. Cryopreservation (see **Note 9**)

For long-term storage, cells are kept at -180°C , in the vapor phase of a liquid nitrogen freezer. To freeze cells:

1. Add cold freezing mix dropwise to an equal volume of cells suspended in complete medium (2×10^6 – 20×10^6 cells/mL, kept on ice).
2. Dispense 1 mL aliquots into cryovials and store at -70°C in a polystyrene container.
3. After 24 h, transfer the vials to a liquid nitrogen container, where they can remain viable for at least 10 yr.

4. Notes

1. As T-cell work requires long-term culture, contamination is an ever-present threat. Therefore, measures must be taken to prevent loss of valuable material. When there is uncertainty about the sterility of materials, they should be filter-sterilized (0.02- μM filter). Antibiotics (penicillin/streptomycin; 100 IU/mL) may be added to the tissue culture medium, but this does not prevent fungus or mycoplasma infection. The latter can also alter functional characteristics of T cells and thus lead to invalid experimental data. Routine mycoplasma testing, for which different kits are commercially available, is therefore recommended.
2. All media may be stored at 4°C for up to 2 wk.
3. Before embarking on T-cell cloning, some parameters should be established. Human serum should be tested for its ability to support T-cell growth (without causing nonspecific proliferation) at different concentrations, by measuring proliferation of PHA-stimulated PBMC in the presence of 2.5, 5, 7.5, and 10% serum containing RPMI, using serum from different batches as they can vary. This is not necessary for FCS, as this is not used for culturing human T cells. Alternatively, autologous plasma may be used to supplement media as a (low-cost) alternative to commercially available human serum. The preparation procedure is as follows: do not dilute donor blood before layering on Ficoll gradient, and after centrifugation, carefully transfer the top yellow layer (plasma) to a separate tube, taking care not to disturb the PBMC layer. Centrifuge plasma (1000g, 21°C , 15 min) and transfer plasma to new tube. Discard the pellet. Heat inactivate (55°C for 25–30 min)

and centrifuge again (1000g, 21°C, 15 min). Aliquot plasma and store at -20°C. Autologous plasma is generally used at a concentration ranging from 1 to 5%, but if important, optimal concentration should be assessed in a proliferation assay.

4. Initially, when generating antigen-specific lines, the following numbers of PBMC are needed: 1×10^7 for each T-cell line and an additional 1×10^6 – 5×10^6 PBMC for each round of restimulation. EBV transformation of B cells requires 1×10^7 PBMC; therefore, 50–100 mL of blood would provide sufficient starting material (as a rule of thumb: 1 mL of blood yields 1×10^6 PBMC on average). When cloning, an additional 50–100 mL of blood would ensure sufficient PBMC for fresh feeders and PBMC storage for subsequent stimulation of T-cell clones.
5. The optimal antigen dose should be determined by measuring the proliferation of PBMC incubated with increasing concentrations of the antigen. The dose which results in maximal proliferation should be used in subsequent procedures.
6. Once T-cell clones have been established by either method A or B, they may be restimulated for expansion and maintenance by method A or B depending on the continuous availability of materials. For example, if clones were generated using method A, but autologous PBMC are no longer available, method B can be used for further expansion. It is, however, not recommended to exclusively use method B for stimulation as this can lead to nonresponsiveness. Therefore, it is important to determine the MHC restriction of the T-cell clones so that PBMC from an HLA-matched donor may be used to replace autologous PBMC. Method A is the preferred method for several reasons: first, the T cells are stimulated in an antigen-specific way (in the absence of mitogenic stimulation as in method B), which results in a higher percentage of antigen-specific T-cell clones. Secondly, this method makes use of Terasaki plates which hold only 20 μ L. Therefore, far less starting material is needed than in method B. The slight disadvantage of this method is the fact that the procedure only reaches the 96-well stage after 7–10 d, thus prolonging the procedure.
7. When identifying positive wells, it may be difficult to identify slow-growing clones and one may be tempted to select only fast-growing ones, thus introducing a bias. Therefore, it may be better to score clones 10 d after incubation.
8. T-cell cloning is not guaranteed to be successful first time around and may have to be repeated several times. When clones are obtained, low cloning efficiencies could result in very few clones, with even fewer antigen-specific ones. Another major problem is the inability to expand clones.
9. Early and continuous cryopreservation of T-cell clones is advisable because of the finite life span of T-cell clones in culture, with their potential to expand decreasing with time in culture.

References

1. Morgan, D. A., Ruscetti, F. W., and Gallo, R. (1976) Selective *in vitro* growth of T lymphocytes from normal human bone marrows. *Science* **193**, 1007–1008.
2. *Human T Cell Clones: A New Approach to Immune Regulation*, Eds: Feldmann, M.; Lamb, J. R.; Woody, J. N., Humana Press, New York, 1985.

3. Halvorsen, R., Bosnes, V., and Thorsby, E. (1986) T cell responses to a *Derмато-phagoides farinae* allergen preparation in allergics and healthy controls. *Int. Archs. Allergy Appl. Immunol.* **80**, 62–69.
4. Lamb, J. R., Eckels, D. D., Lake, P., Johnson, A. H., Hartzman, R. J., and Woody, J. N. (1982) Antigen-specific human T lymphocyte clones: induction, antigen specificity and MHC restriction of influenza virus-immune clones. *J. Immunol.* **128**, 233–238.
5. van Schooten, W. C. A., Ottenhof, T. H. M., Klatser, P. R., Thole, J., de Vries, R. R. P., and Kolk, A. H. J. (1988) T cell epitopes on the 36K and 65K *Mycobacterium leprea* antigens defined by human T cell clones. *Eur. J. Immunol.* **18**, 849–854.
6. Turcanu, V., Maleki, S. J., and Lack, G. (2003). Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J. Clin. Investig.* **111**, 1065–1072.
7. Reijonen, H. and Kwok, W. W. (2003). Use of HLA class II tetramers in tracking antigen-specific T cells and mapping T-cell epitopes. *Methods* **29**, 282–288.
8. Miller, G. and Lipman, M. (1973) Release of infectious Epstein-Barr virus by transformed marmoset leucocytes. *Proc. Natl Acad. Sci. USA* **69**, 383–387.