Video Article

An Efficient and Simple Method to Establish NK and T Cell Lines from Patients with Chronic Active Epstein-Barr Virus Infection

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URL: https://www.jove.com/video/56515
DOI: doi:10.3791/56515

Keywords: Immunology and Infection, Issue 133, NK cell lines, T cell lines, chronic active EBV infection, recombinant human IL-2, PBMC, whole blood

Date Published: 3/30/2018

Citation: Xu, C., Ai, J., Zhang, Q., Li, T., Wu, X., Xie, Z., Duan, Z. An Efficient and Simple Method to Establish NK and T Cell Lines from Patients with Chronic Active Epstein-Barr Virus Infection. *J. Vis. Exp.* (133), e56515, doi:10.3791/56515 (2018).

Abstract

A number of methods have been described to establish NK/T cell lines from patients with lymphoma or lymphoproliferative syndrome. These methods employed feeder cells, purified NK or T cells with as much as 10 mL of blood, or a high-dose of IL-2. This study presents a new method with a powerful and simple strategy to establish NK and T cell lines by culturing the peripheral blood mononuclear cells (PBMC) with the addition of recombinant human IL-2 (rhIL-2), and uses as little as 2 mL of whole blood. The cells can proliferate quickly in two weeks and be maintained for more than 3 months. With this method, 7 NK or T cell lines have been established with a high success rate. This method is simple, reliable, and applicable to establishing cell lines from more cases of CAEBV or NK/T cell lymphoma.

Video Link

The video component of this article can be found at https://www.jove.com/video/56515/

Introduction

Epstein-Barr virus (EBV) is ubiquitous and infects not only B cells, but also T and natural killer (NK) cells, which causes a number of EBV-associated NK/T lymphoproliferative diseases (LPD) and lymphoma/leukemia, such as EBV-associated hemophagocytic lymphohistiocytosis, hydroa vacciniforme-like lymphoma, extranodal NK/T-cell lymphoma, nasal type and aggressive NK cell leukemia[1,2,3]. Among these is severe chronic active EBV (SCAEBV) disease, which is incident mainly in East Asia, and which is now considered to be a LPD caused by clonal expansion of EBV-infected T or NK cells[4,5,6,7], but without the apparent immunodeficiency present in infectious mononucleosis (IM)-like symptoms including fever, hepatosplenomegaly, lymphadenopathy, and liver dysfunction persistently or recurrently, as well as high EBV-DNA load in the peripheral blood[8,9]. Patients with CAEBV have a poor prognosis[10,11], and its pathogenesis and the role of the EBV is unclear. Therefore, cell lines derived from EBV-associated NK/T lymphoproliferative diseases and lymphomas are very helpful as cell models for clarifying the mechanism of EBV induced NK or T cell proliferation and its relationship with high incidence of leukemia or lymphoma.

To date, several cell lines have been established with different techniques[12,13,14,15,16]. A human NK cell line, NK-YS, was established from NK cell lymphoma/leukemia, by co-culturing with a mouse stromal cell line as feeder, and in the presence of rhIL-2 at a concentration of 20U per mL[15]. KAI3 was another NK cell line established from patients with a severe mosquito allergy or SCAEBV with autologous lymphoblastoid cell line (LCL), B cells transformed by EBV, as feeder cells and addition of rhIL-2 at 100U/mL[16]. SNK6 and SNT8 were derived from tumor tissues of nasal NK/T cell lymphoma patients by adding high-dose of rhIL-2 (700U/mL)[12]. With similar technique, the SNK-1 cell was from CAEBV patients, cultured from PBMC by removing T cells and adding 700U/mL of rhIL-2[13,17]. SNT13 and SNT15 were established by removing CD4+ and CD8+ cells[18]. So far, other T cell and NK cell lines from EBV-NK/T LPD patients were all developed with this method[19].

The disadvantages of the existing methods mentioned above include the employment of feeder cells, the requirement of a high-dose of IL-2, the utilization of as much as 10 mL whole blood, or the purification of NK/T cells, which is very challenging in the clinic due to the necessity of verifying which types of cell that EBV latently infects before beginning to culture. As CAEBV mainly occurs in children in Asia, 10 mL blood is not easy to acquire in all regions. In this study, we developed a new simple method with a high success rate to establish NK and/or T cell lines by culturing PBMC from CAEBV patients using a low-dose of rhIL2 and a volume of 2 mL of whole blood without feeder cells. The results of this method have proven its high efficiency and time saving.

Protocol

This study was approved by the Ethics Committee of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, and the protocol follows the institutional guidelines for human welfare.

NOTE: See **Figure 1** for a schematic of the workflow.

1. Isolation of PBMC from CAEBV Patients

1. Purify primary PBMC from 2 mL of whole blood of CAEBV patients by gradient (*e.g.*, Ficoll-plaque) centrifugation following the manufacturer's instructions. Alternatively, thaw cryopreserved PBMC from liquid nitrogen with RPMI 1640 medium.
2. Add 2 µL of trypan blue (0.4%) to 18 µL cell suspension, wait for 3 min to stain cells, transfer the suspension to the cell counter plate, and measure the cells' concentration and viability with an automated cell counter.
3. Prepare the cell suspension at a density of 2-3×10^6 cells/mL supplemented with complete RPMI 1640 culture medium containing 20% human serum (heat inactivated at 56 °C), 2 mM L-glutamine, and antibiotics (100 µg/mL streptomycin, 100 U/mL penicillin). Seed 1 mL of PBMC suspension per well into 24-well cell culture plates.
4. Add 150 U rhIL-2 per well.
5. Place the culture plates in a 5% CO_2 incubator at 37 °C.
6. On day 2, observe the cell morphology under a microscope (200X magnification); cells are in good condition when they are bright and round.

2. Expansion of Cells and Numbering the Viable Cells

1. Observe the cell morphology under a microscope (200X magnification), and monitor the condition of cell growth by numbering the cells before changing medium: add 2 µL of trypan blue (0.4%) to 18 µL cell suspension, and calculate the cells' concentration (see 1.2, **Figure 2**).
2. Discard 500 µL of the medium in a 24-well plate, and add 500 µL fresh complete culture medium. Add 150U rhIL-2 per well.
3. Change the medium twice a week as in step 2.2.
4. Draw the cell growth curve (**Figure 3**).
5. When the viable cells' concentration exceeds 5×10^6/mL, divide cells into new wells at a concentration of 1-2×10^6 cells/mL in each well. This process takes 2-4 weeks.

3. Cell Phenotyping by Flow Cytometry

1. When cells have expanded, collect 1×10^6 cells to determine the phenotypes of cell lines. Centrifuge cells at 240 x *g* for 5 min at room temperature (NK/T cells will precipitate at the bottom of the tube).
2. Discard the supernatant, wash the precipitation by adding 7 mL PBS. Centrifuge for 5 min at 240 x *g* at room temperature. Repeat once.
3. Add 200 µL human serum to each tube, mix well and incubate for 10 min at room temperature.
4. Centrifuge cells at 240 x g for 5 min at room temperature. Discard the supernatant and re-suspend the cells at 1×10^6/mL with cold PBSA (PBS+0.2%BSA). Divide cells into 5 tubes, each tube containing 2×10^5 cells.
5. Centrifuge cells at 240 x g for 5 min at 4 °C. Discard the supernatant and re-suspend the cells with PBSA buffer or PBSA containing 10 µL PE (or PE-Cy7) and 10 µL FITC labeled antibodies to stain cell receptors of T or NK cells on ice as is indicated by **Figure 4**. Cells suspended with PBSA buffer are used as a negative control.
6. Incubate the cells with antibodies for 20-30 min on ice in the dark.
7. Wash the cells twice with cold PBSA, re-suspend the cells with 300 µL cold PBSA.
8. Analyze the cell phenotype with a flow cytometer.
    1. Set up the Flow Cytometer in "Create worksheet" condition. Set up the experimental template with a dot plot that displays forward scatter (FSC) versus side scatter (SSC).
    2. Load the isotype control tube to optimize the FSC and SSC voltages, and optimize the FSC threshold value to eliminate debris without interfering with the cell population of interest. Delete all parameters except FSC, SSC, FITC, PE and PE-Cy7.
    3. Perform compensation using the isotype control and a single positive control in each 2-color analysis group.
    4. Load samples and create HLA-DR VS CD19, CD4 VS CD8, CD56 VS CD16 and CD3 VS CD16 dot plots showing different population of cells.
    NOTE: PBMC were used to perform compensation using the negative/isotype control and the single positive control. 2-color immunofluorescence with flow cytometer was used routinely to analyze the expression of surface markers. The following antibodies are included: anti-HLA-DR, anti-CD4, anti-CD16 conjugated with fluorescein isothiocyanate (FITC), anti-CD8, anti-CD56, CD3 conjugated with phycoerythrin (PE), and anti-CD19 conjugated with PE-Cy7.

4. Expansion and Cryopreservation of NK/T Cells

1. Change the medium when the cell phenotype analysis is completed. Carefully remove half of the supernatant and avoid touching the cells at the bottom of the plate. Add the same volume of fresh culture medium containing 300 U/mL rhIL-2 to the cell plates.
2. Change half of the medium every 3 days (as 2.2) until the cell clusters are clearly visible under the microscope (**Figure 2**). Typically, this process takes 2-3 weeks.
3. Transfer the cells from 24-well plates to T25 culture flasks after mixing different wells of the same lineage. Double the volume of culture medium as it turns yellow until the volume of the medium expands to 10-15 mL. Add rhIL-2 with the concentration of 150 U/mL.

4. Change medium 24 h before freezing after 2-3 weeks growing, when the cell mass can be observed with the naked eye. Measure cell concentration with a cell counter.
5. Centrifuge the cell suspension for 5 min at 240 x g, and re-suspend cell pellet at a density of 5-10 x 10^6 cells/mL with the frozen stock solution which contains 90% fetal bovine serum and 10% dimethylsulfoxide (DMSO). Freeze at a rate of 1 °C per min to -80 °C and then transfer directly into liquid nitrogen.

Representative Results

After 3 days culture during the establishment of cell lines, the polymorphic cells begin to appear (**Figure 2**). After 7 days, cells grow quickly, as both the number and viability of cells are increased at a high rate (**Figure 3**). Small clusters of cells are clearly visible after 10-14 days growing, when the cell concentration can exceed 3-6 × 10^6. In this period, cells should be expanded by division into two or three wells of the culture plate. About a month later, once the cell number reaches 3-5×10^7, the count is high enough for preservation.

Another important issue is to determine the phenotypes when cells have been cultured successfully. Our results indicate that T (L196) and NK (M296) cells can be cultured by the method described above (**Figure 4**), and cell lines could be established with this technique, as the cells grew more than 3 months in good condition.

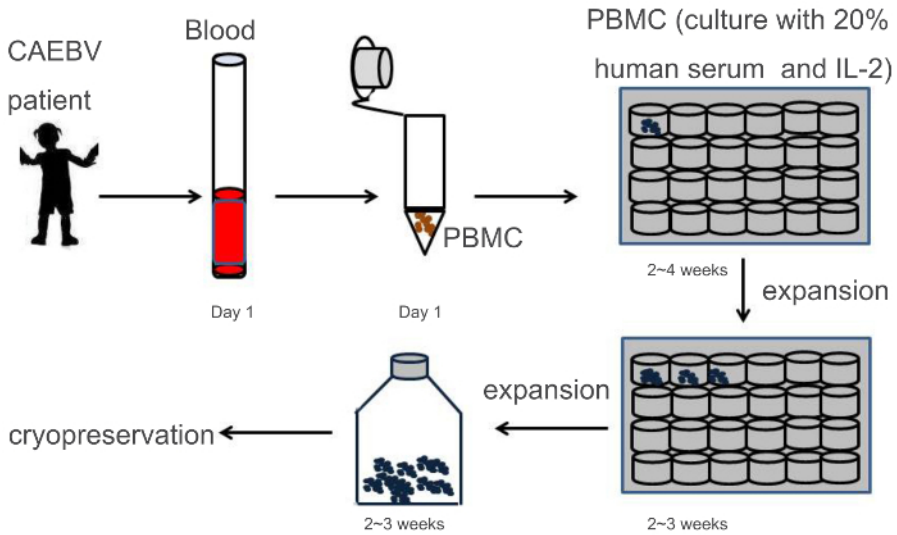

Figure 1: Schematic representation of workflow. On the first day, anticoagulant blood was collected from CAEBV patients, then PBMC were isolated and cultured with 1640 medium containing 20% human serum and 150U/mL of rhIL-2. The NK/T cells were grown at 37 °C in the presence of 5% CO_2. After 2-4 weeks culturing, the cells began to grow quickly. When the concentration exceeded 5 × 10^6 /mL, we divided cells into 2-3 wells at concentration of 2×10^6. Continuing the culture for 2-3 weeks, cells were transferred from the 24-well plate into T25 flask when cell clusters were clearly visible under the microscope. Cryopreserve cells when the cell mass can be observed with the naked eye. Please click here to view a larger version of this figure.

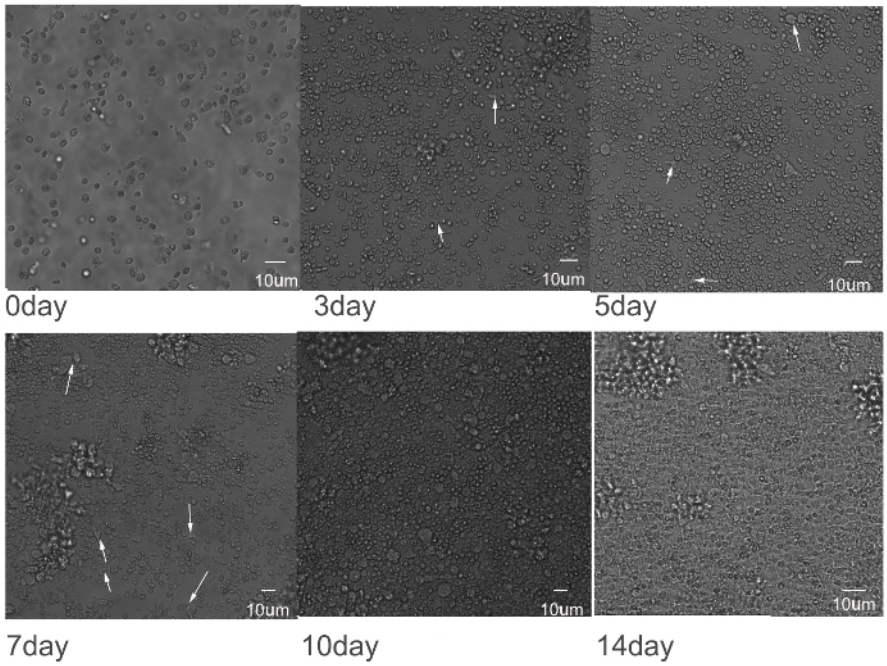

Figure 2: Cellular morphological changes in the process of culturing. Cells are cultured and observed for the indicated number of days. Polymorphic cells (the arrows pointed) were visible under the microscope clearly after 3-7 days, and cells began to grow quickly after 7-14 days culture. The original magnification for light microscopy was 200X. Please click here to view a larger version of this figure.

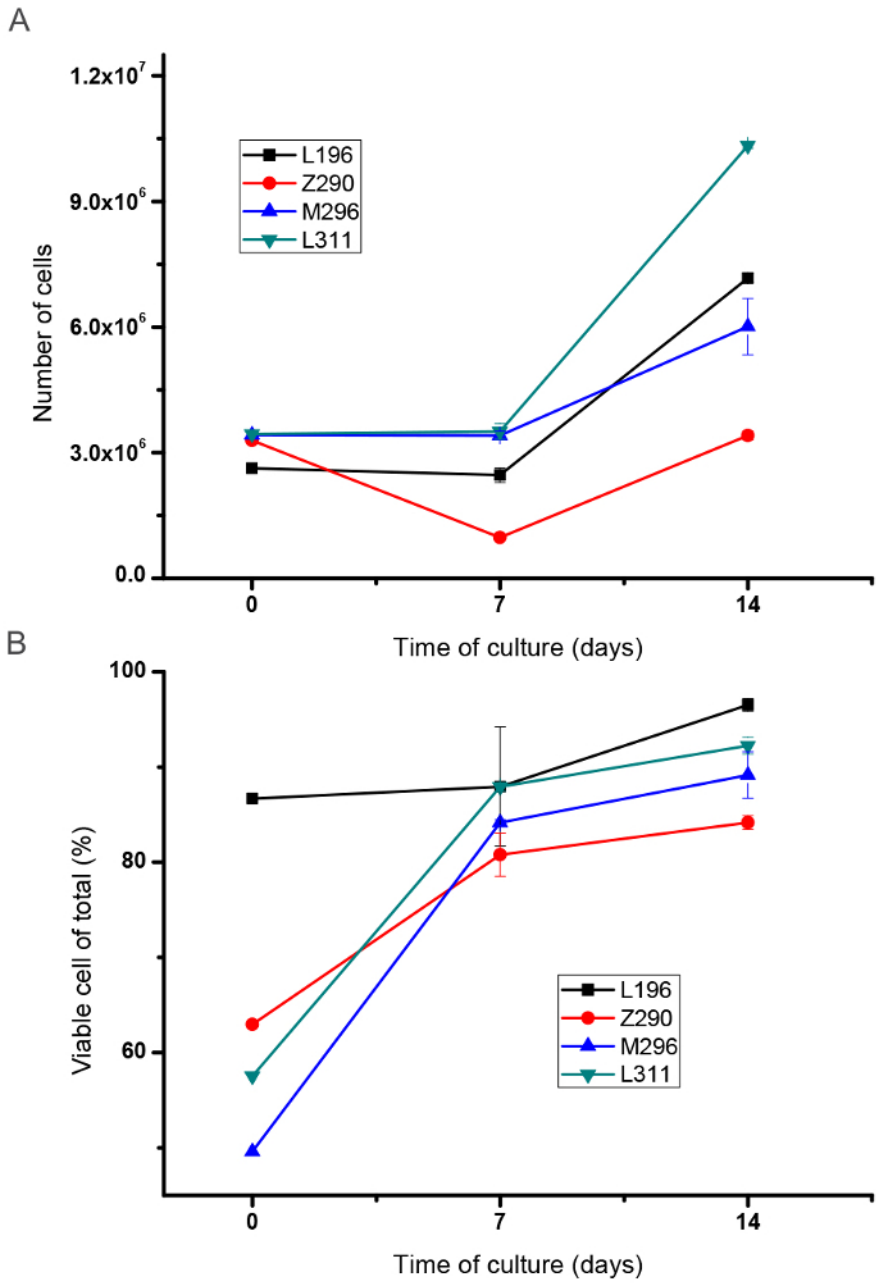

Figure 3: The growth curves of cell proliferation. After 7 days of culturing, cells began to grow quickly **(A)** and with high viability **(B)**. The cells' concentration and viability were measured by an automated cell counter with the following procedure: add 2 µL of trypan blue (0.4%) to 18 µL of cell suspension for staining, wait 3 min, transfer the suspension to the cell counter plate, and measure the cells' concentration and the cell viability with an automated cell counter. The error bars are the standard deviations of three replicas. L196, Z290, M296, L311 are the names of four cell lines. The starting concentration to measure growth curve is about 3×10^6. Please click here to view a larger version of this figure.

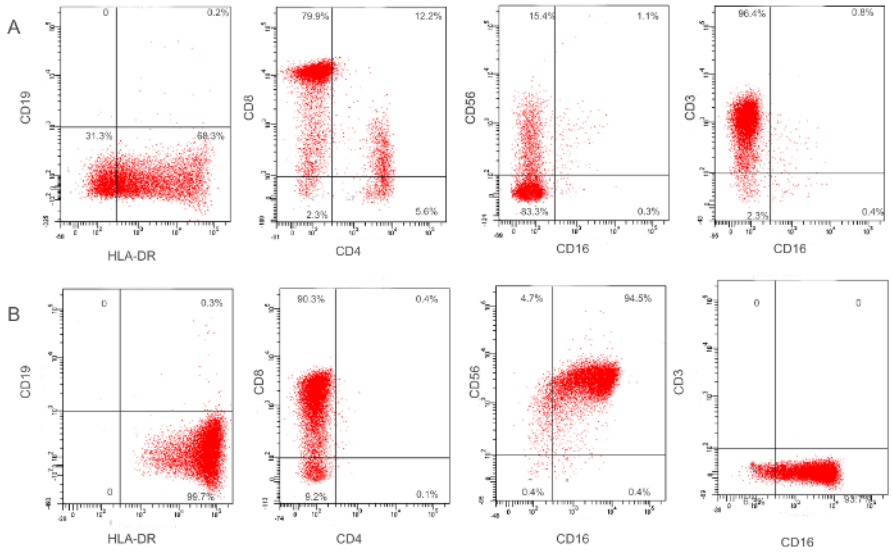

Figure 4: Representative gating strategy for flow cytometry analysis of cell lines L196 and M296. PBMC were used for adjusting FSC and SSC voltages. The cluster of live and single lymphocytes was gated for P1 in the FSC and SSC plot. The isotype control and single positive controls were used to perform compensation. Four pairs of 2-color immunofluorescence conjugated antibody staining were used to analyze the expression of surface markers. Anti-HLA-DR and anti-CD19 were used to detect B cells, whereas anti-CD3, anti-CD4, and anti-CD8 were used to detect T cells. Anti-CD16 and anti-CD56 were used to define NK cells. **(A)** The phenotype of L196 is CD19-HLA-DR +CD16-CD56+CD3+CD4+CD8+, the main cell type is CD8+. **(B)** M296 is CD19-HLA-DR+CD16+CD56+CD3-CD4-CD8+, the main cell type is CD16+CD56+. Please click here to view a larger version of this figure.

Discussion

In this protocol, a novel technique for establishing NK/T cell lines from whole blood of CAEBV patients has been developed. Compared with the existing methods, the major advantage of this method is its simplicity and its requirement of only a small volume of blood, while it exhibits a high success rate and good conditions of cell viability. Furthermore, NK/T cell lines can be established by culturing PBMC without determining the cell types the EBV latently infected in advance, as the determination would consume more blood and time before culturing. Alternatively, cell lines can be analyzed and different phenotypes of cells can be purified with flow cytometry after cell line establishment. Besides, the method uses a low dose of rhIL-2 and needs no feeder cells. With this method, we have developed 7 cell lines from 8 CAEBV patients and cryopreserved them within a month. The one which has not been developed into a cell line can be maintained alive for more than 5 months without significant proliferation, and the reasons behind this need further exploration.

It is necessary to note that cell growth is strictly dependent on the presence of recombinant human IL-2, which is consistent with previous reports[20,21]; with no human IL-2, the cells will die in 3 weeks. Evidently, a high quality of IL-2 is essential for cell proliferation, though the factors that determine the proliferative capacity of cells *in vitro* still need to be clarified. The dose of IL-2 required is variable among different cell lines, for instance, 50U/mL is satisfactory to maintain the proliferation of M296. However, the most economical and reliable concentration for establishing NK/T cell lines has not been presented in the study; indeed, 150 U/mL is sufficient for success.

In this process of cell culturing, of the elements affecting cell growth, the viability of primary PBMC is the most important. To ensure success, the PBMC should be as fresh as possible. Undoubtedly, cell concentration is an important factor affecting successful culturing, therefore the threshold value should be no lower than 10^5 per mL. If the total cell number isolated from the sample is less than 10^6, using a mini-well cell plate to maintain an adequate cell concentration is an alternative choice in initial culture. Furthermore, NK and T cell line could be established with even a limited amount of peripheral blood when using the hemolytic reaction to enrich PBMC as we reported previously[22]. As described in the protocol, the human serum is another key factor for cell survival at the beginning of the culture. We speculate that some unknown ingredients exist in the serum which are absent or different in fetal bovine serum and are necessary for T/NK cell proliferation.

There are two limitations of the method. First, there are several unanswered questions about this method, such as how and why EBV infects the NK or T cells *in vivo*, the mechanisms of the NK/T cell growth and proliferation *in vitro*, and what types of cell can be established into cell lines in one patient. Though we could not control the cell type and purity, which are not determined by the method, these factors might be dependent on the cells EBV infects in patients. With this protocol, NK and T cells could be established into cell lines, though this method could not preferentially culture one type. Nevertheless, there is a dominant group of these cells. Second, there was a report that NK and T cell clones from patients with LPD or NK/T lymphoma could be established to cell lines, while others could be maintained for several months[17]. Cells obtained in present study can proliferate well over 3 months, however whether they could proliferate indefinitely needs to be validated.

In summary, using this method, more cell lines from CAEBV or NK/T lymphomas can be easily obtained with a limited amount of blood and a low-dose of IL-2 without feeder cells. These cell lines will contribute to the study of EBV persistency in NK/T cells and pathogenesis of EBV associated leukemia or lymphoma.

Disclosures

D.Z., X.Z., and X.C. are co-inventors on pending patent applications covering potential uses of this method for establishing NK/T cell lines and the cell lines established in this study. D.Z., X.Z., and X.C. declare no competing financial interests in this work. The remaining authors declare they have no competing financial interests.

Acknowledgements

This work was supported by the Key Projects of Chinese Academy of Sciences (KFZD-SW-205), Strategic Biological Resources Technology Support System of Chinese Academy of Sciences (CZBZX-1 and ZSSB-004), and by Grants from National Science Foundation of China (81401640) and Shanghai Natural Science Fund (14ZR1434800).

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