Video Article

Expansion of Human Peripheral Blood γδ T Cells using Zoledronate

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Abstract

Human γδ T cells can recognize and respond to a wide variety of stress-induced antigens, thereby developing innate broad anti-tumor and anti-infective activity.[1] The majority of γδ T cells in peripheral blood have the Vγ9Vδ2 T cell receptor. These cells recognize antigen in a major histocompatibility complex-independent manner and develop strong cytolytic and Th1-like effector functions.[1]Therefore, γδ T cells are attractive candidate effector cells for cancer immunotherapy. Vγ9Vδ2 T cells respond to phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which is synthesized in bacteria via isoprenoid biosynthesis;[2] and isopentenyl pyrophosphate (IPP), which is produced in eukaryotic cells through the mevalonate pathway.[3] In physiological condition, the generation of IPP in nontransformed cell is not sufficient for the activation of γδ T cells. Dysregulation of mevalonate pathway in tumor cells leads to accumulation of IPP and γδ T cells activation.[3] Because aminobisphosphonates (such as pamidronate or zoledronate) inhibit farnesyl pyrophosphate synthase (FPPS), the enzyme acting downstream of IPP in the mevalonate pathway, intracellular levels of IPP and sensitibity to γδ T cells recognition can be therapeutically increased by aminobisphosphonates. IPP accumulation is less efficient in nontransfomred cells than tumor cells with a pharmacologically relevant concentration of aminobisphosphonates, that allow us immunotherapy for cancer by activating γδ T cells with aminobisphosphonates. [4] Interestingly, IPP accumulates in monocytes when PBMC are treated with aminobisphosphonates, because of efficient drug uptake by these cells. [5] Monocytes that accumulate IPP become antigen-presenting cells and stimulate Vγ9Vδ2 T cells in the peripheral blood.[6] Based on these mechanisms, we developed a technique for large-scale expansion of γδ T cell cultures using zoledronate and interleukin-2 (IL-2).[7] Other methods for expansion of γδ T cells utilize the synthetic phosphoantigens bromohydrin pyrophosphate (BrHPP)[8] or 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP).[9] All of these methods allow ex vivo expansion, resulting in large numbers of γδ T cells for use in adoptive immunotherapy. However, only zoledronate is an FDA-approved commercially available reagent. Zoledronate-expanded γδ T cells display $CD27^{-}CD45RA^{-}$ effector memory phenotype and thier function can be evaluated by IFN-γ production assay. [7]

Video Link

The video component of this article can be found at https://www.jove.com/video/3182/

Protocol

1. Isolation of PBMC

1. Draw blood (7.5-8.0 ml) into a BD Vacutainer CPT Cell Preparation Tube with Sodium Heparin. The tube contains a sodium heparin anticoagulant and a Ficoll-Hypaque density fluid, plus a polyester gel barrier, which separates the two liquids. Centrifuge tube/blood sample at room temperature (18°C to 25°C) in a horizontal rotor (swing-out head) for 20 min at 1800 x *g*. Switch centrifuge brakes off.
2. After centrifugation, the sequence of layers occurs as follows (seen from top to bottom): a) plasma - b) peripheral blood mononuclear cells (PBMC) and platelets - c) density solution - d) polyester gel - e) granulocytes - f) red blood cells (**Fig. 1**).
3. Collect a fraction of the plasma layer, leaving 5 to 10 mm of plasma above the interphase without disturbing the cell layer. The plasma can be used for the culture (section 2.5).
4. Harvest the enriched fraction (PBMC) at the interphase with a pipette and transfer to a 15 ml conical tube.
5. Wash the PBMC with 10 ml of phosphate-buffered saline (PBS), by inverting the tube 5 times, and then centrifuge for 5 min at 400 x *g*.
6. Repeat washing steps twice, and then resuspend the cell pellet in 5 ml of PBS. Determine the cell number. Usually 1.3 $x10^6$ cells are recovered from 1 ml of whole blood.
7. Determine the frequency and phenotype of γδ T cells in PBMC by flow cytometry (section 3) (**Fig. 2**).

2. Expansion of γδ T cells

1. Centrifuge cell suspensions in 15 ml conical tubes for 5 min at 400 x *g* at room temperature, and discard the supernatants.
2. Prepare culture medium (CM) by adding human IL-2 (IL-2) and zoledronate (Zometa) to final concentrations of 1000 IU/ml and 5 μM, respectively. ALyS203 (Cell Science & Technology Institute) or OpTmizer (Invitrogen) media support good expansion of γδ T cells (more

information in references 6 and 9). Zometa is provided in liquid form (4 mg/5-ml vial). To prepare a 5 μM solution, add 50 μl of Zometa to 30 ml of culture medium.
3. Resuspend cell pellet in culture medium and adjust to $1x10^6$ cells/ml.
4. Pipet 1 ml of CM containing $1x10^6$ cells into each well of a 24-well plate. For large-scale cultures, cells can be seeded at 0.5 x 10^6 cells/cm^2 according to the surface areas of plate wells, dish, or flask.
5. Add autologous plasma (section 1.3), pooled human AB sera, or FCS so that it is approximately 10% of the volume of the culture (100 μl for each well of a 24-well plate). Place the plates in a humidified 37°C, 5% CO_2 incubator for 24-48 hr.
6. Maintain the culture at a cell density of 0.5-2 x 10^6 cells/ml. Add fresh medium containing human IL-2 (1000 IU/ml) only (without Zometa) every 2-3 days, and transfer cultured cells into new wells or flasks as necessary, according to the degree of cell proliferation (**Fig. 3**). Supply plasma or serum to the medium so that the serum concentration can be maintained at least 1%.
7. Harvest cells on day 12-14 and determine the frequency, phenotype, and functions of γδ T cells by flow cytometry (see below).

3. Phenotypic analysis by flow cytometry

1. Transfer 200 μl samples containing 2 x 10^5 cells to fluorescence-activated cell sorting (FACS) tubes.
2. Add 2 ml of cold PBS and centrifuge for 5 min at 400 x *g*. Then, resuspend the pellets in 50 μl FACS buffer (PBS + 1% FCS + 0.1% sodium azide). Add 5 μl of each antibodies to the samples (the monoclonal antibodies are listed in **Table 1**).
3. Incubate on ice in the dark for 20 min.
4. Add 2 ml FACS buffer to each sample, and then vortex. Centrifuge samples for 5 min at 400 x *g* at 4°C. Carefully decant the supernatant.
5. Resuspend the cells in 300 μl FACS buffer and vortex. Analyze samples on a flow cytometer (**Fig. 2**).

4. IFN-γ production assay[10]

1. The day before assay, prepare stimulator cells by culturing 3-5 $x10^5$ Daudi cells/ml in RPMI 1640 medium plus 10% FCS (RPMI-10) overnight with Zometa (5 μM) (hereafter designated Z-Daudi).
2. Collect Z-Daudi and resuspend in RPMI-10 at $2x10^6$ cells/ml. Add 100 μl of Z-Daudi ($2x10^5$) to each well of a round-bottom 96-well plate.
3. Prepare γδ T cells at $2x10^6$ cells/ml in RPMI-10 containing Brefeldin A at 20 μg/ml. Transfer 100 μl of γδ T cell suspension ($2x10^5$) to each well containing Z-Daudi cells or to control wells (100 μl of RPMI-10 only, or RPMI-10 with 20 ng/ml of phorbol 12-myristate 13-acetate [PMA] plus 2 μg/ml of ionomycin).
4. Mix by pipetting up and down several times. Incubate for 4 hr in a 37°C, 5% CO_2 incubator.
5. Centrifuge the plate for 5 min at 400 x *g* at 4°C and resuspend the pellets in 200 μl of cold PBS.
6. Transfer the samples to FACS tubes. Add 4 ml of cold PBS and centrifuge the tubes for 5 min at 400 x *g* at 4°C.
7. Resuspend the pellets in 50 μl of FACS buffer with FITC-conjugated anti-TCRVγ9 (5 μl) and PE/Cy5-conjugated anti-CD3 mAb (2.5 μl). Incubate protected from light for 15 min at room temperature.
8. Add 100 μl of IntraPrep reagent 1 and incubate for 15 min at room temperature. Add 4 ml of PBS to each tube and centrifuge for 5 min at 400 x *g* at room temperature.
9. Remove the supernatant by aspiration and add 100 μl of IntraPrep reagent 2. Incubate for 5 min at room temperature without shaking.
10. Add 5 μl of PE-conjugated anti-IFN-γ mAb to the test tube. Incubate protected from light for 15 min at room temperature.
11. Add 4 ml of PBS to each tube and centrifuge for 5 min at 400 x *g* at room temperature. Remove the supernatant by aspiration and resuspend the cell pellet in 0.5 ml of FACS buffer.
12. Analyze the cells by flow cytometer. Gate on $CD3^+$ $TCRV\gamma9^+$ cells and examine the expression of IFN-γ (**Fig. 4**).

5. Representative Results:

It is important to determine the percentage of γδ T cells in PBMC at the initiation of culture. As shown in **Fig. 2 A**, the percentage of $CD3^+TCRV\gamma9^+$ γδ T cells in PBMC was 1.6% on day 0. The dominant populations were $CD27^+CD45RA^+$ naive or $CD27^+CD45RA^-$ central memory phenotypes. When γδ T cells were efficiently stimulated, they formed clusters on days 3-5 (**Fig. 3 A and B**). When cluster formation was delayed, the growth of other cell types, such as $CD4^+$ or $CD8^+$ αβ T cells or NK cells could dominate the growth of γδ T cells (**Fig. 3 C and D**). After 14 days of culture, the frequency of γδ T cells increased to more than 93.8% of the cultured cells in successful γδ T cell cultures (**Fig. 2 E**). The cultured γδ T cells upregulated NKG2D and CD69 expression (**Fig. 2 G and H**). They displayed $CD27^-CD45RA^-$ effector memory phenotype (**Fig. 2 F**). The functions of γδ T cells were evaluated with regard to cytokine production and cytotoxicity. The intracellular IFN-γ staining demonstrated that γδ T cells produced IFN-γ in response to PMA/ionomycin treatment or Z-Daudi cells that accumulated IPP after zoledronate treatment (**Fig. 4**). These results indicate that zoledronate can efficiently stimulate and expand functional γδ T cells.

tube	FITC	PE	ECD	PE/Cy5
1	CD3	CD19	CD45	CD14
2	CD3	TCRαβ	CD4	CD8
3	CD3			CD56
4	TCR Vγ9	TCRαβ	CD45	CD3
5	TCR Vγ9	NKG2D		
6	TCR Vγ9	CD69		
7	TCR Vγ9	mouse IgG1		
8	TCR Vγ9		CD45RA	CD27

9	TCR Vγ9		mouse IgG1	mouse IgG1

Table 1. Monoclonal antibodies used in multicolor staining of γδ T cells. An example of the phenotypic analysis of γδ T cells performed in our laboratory is shown in **Fig. 2**.

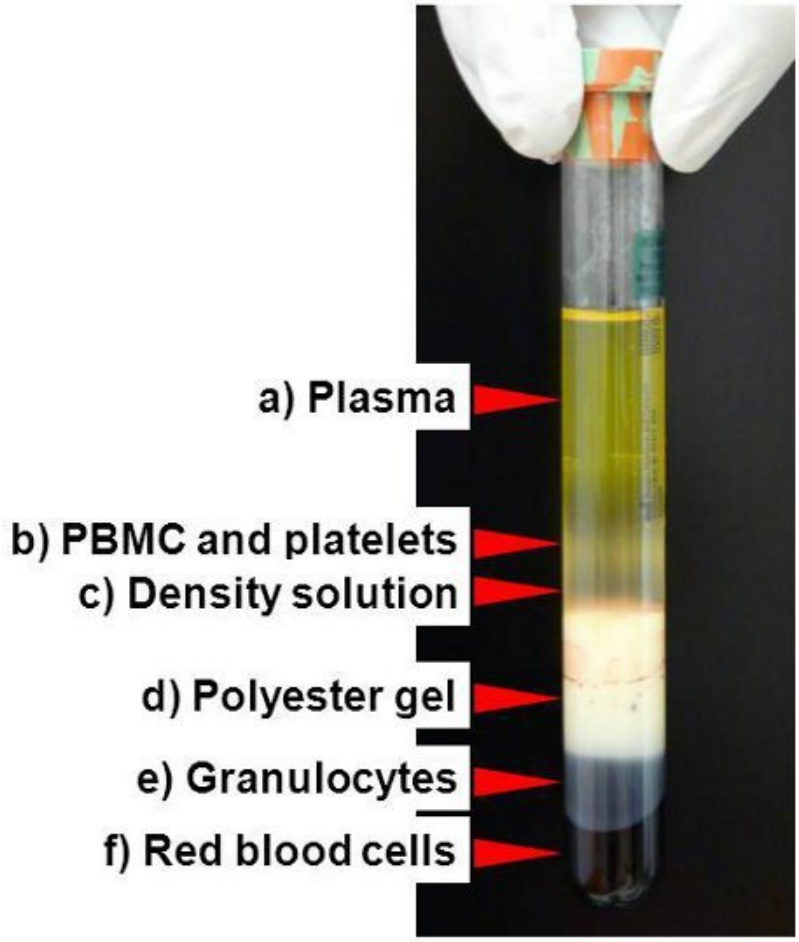

Figure 1. Separation of PBMC. Blood (7.5-8.0 ml) is drawn into a BD Vacutainer CPT Cell Preparation Tube with Sodium Heparin and directly centrifuged for 20 min at 1800 x *g*. After centrifugation, the resulting layers as seen from top to bottom: a) Plasma - b) PBMC and platelets - c) Density solution - d) Polyester gel - e) Granulocytes - f) Red blood cells.

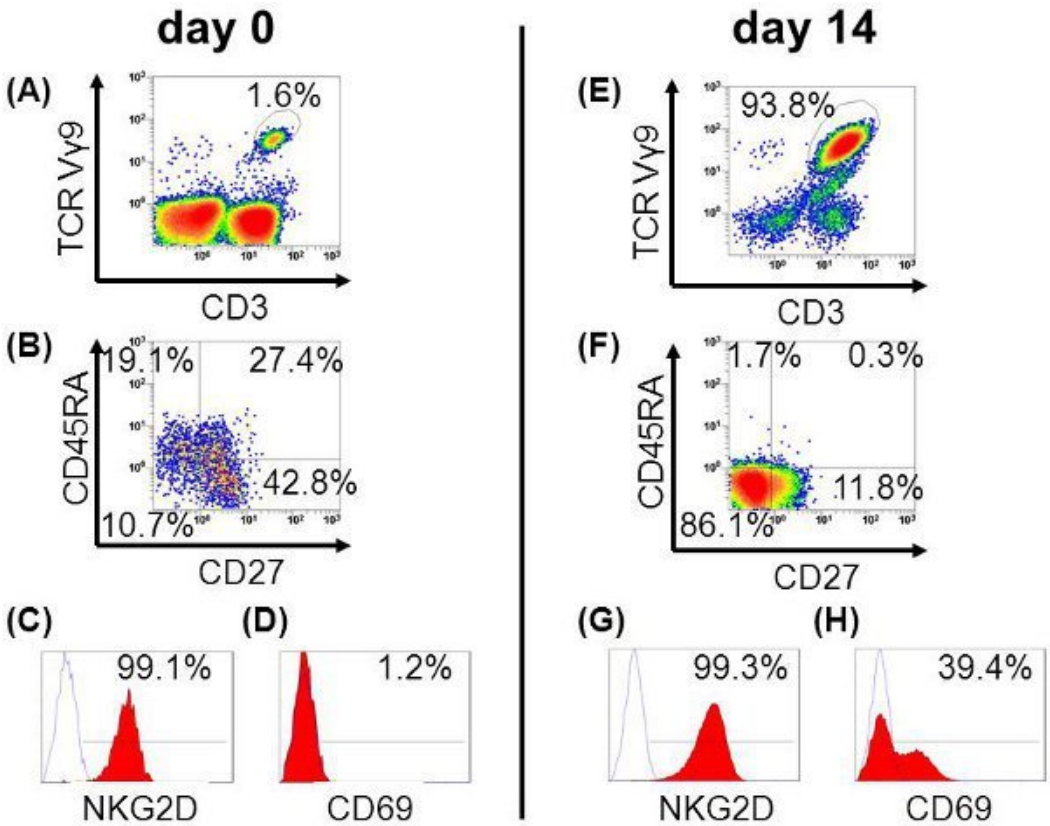

Figure 2. Typical surface phenotype of γδ T cells. PBMC were stimulated with zoledronate and IL-2 for 14 days. Cells were stained with FITC-labeled anti-TCR Vγ9 and PE/Cy5-labeled anti-CD3 to monitor the expansion of γδ T cells (A and E). γδ T cells were identified by their expression of TCRVγ9, and their expression of CD27 and CD45RA (B and F), NKG2D (C and G), or CD69 (D and H) was examined.

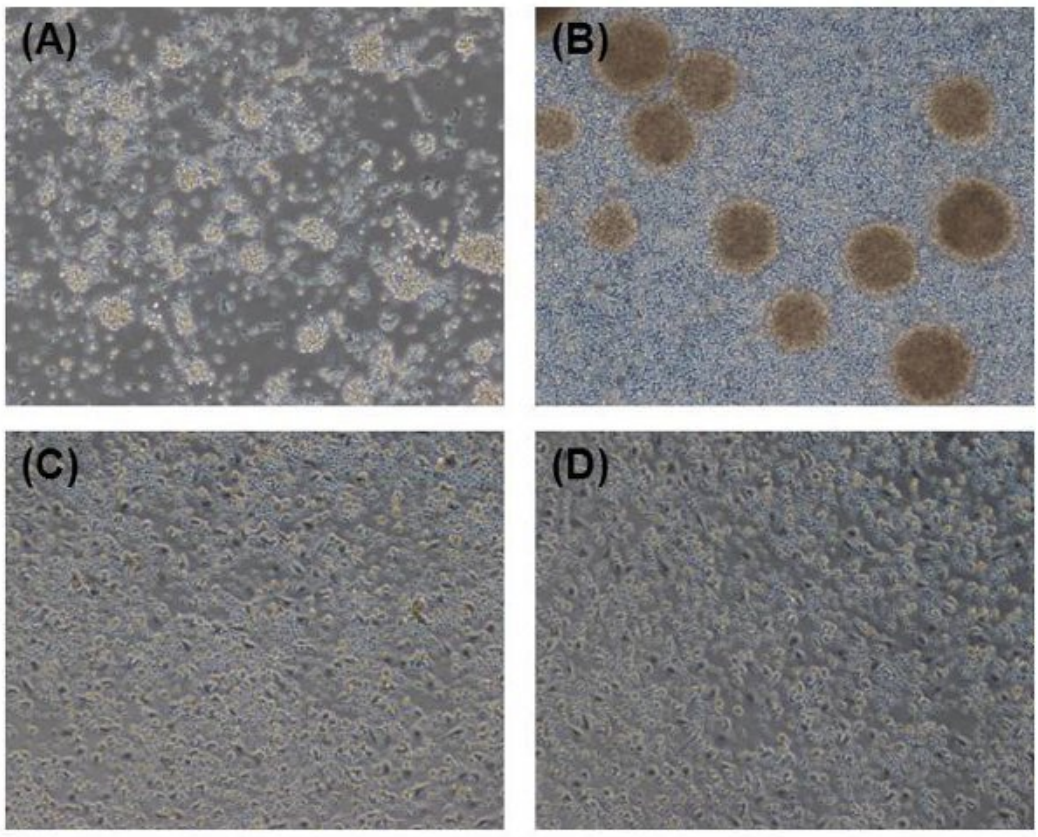

Figure 3. Representative γδ T cell cultures. PBMC were stimulated with IL-2 (1000 IU/ml) and zoledronate (5 μM). Representative fields are shown (IX71 inverted microscope [Olympus] x 200). Clusters and aggregates of γδ T cells can be observed on day 3 (A) and day 5 (B), when γδ T cells were successfully expanded. In contrast, no clusters or aggregates were observed when γδ T cell growth was not adequate (C and D).

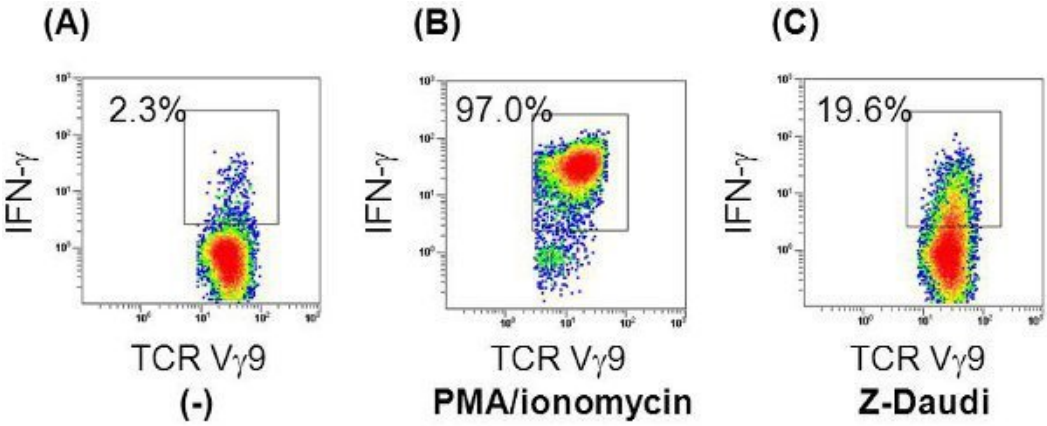

Figure 4. IFN-γ production. γδ T cells were incubated with RPMI-10 only (A) or PMA/ionomycin (B) or Z-Daudi (C) for 4 hr. First, surface expression of TCRVγ9 was stained and then IFN-γ production was examined by intracellular IFN-γ staining.

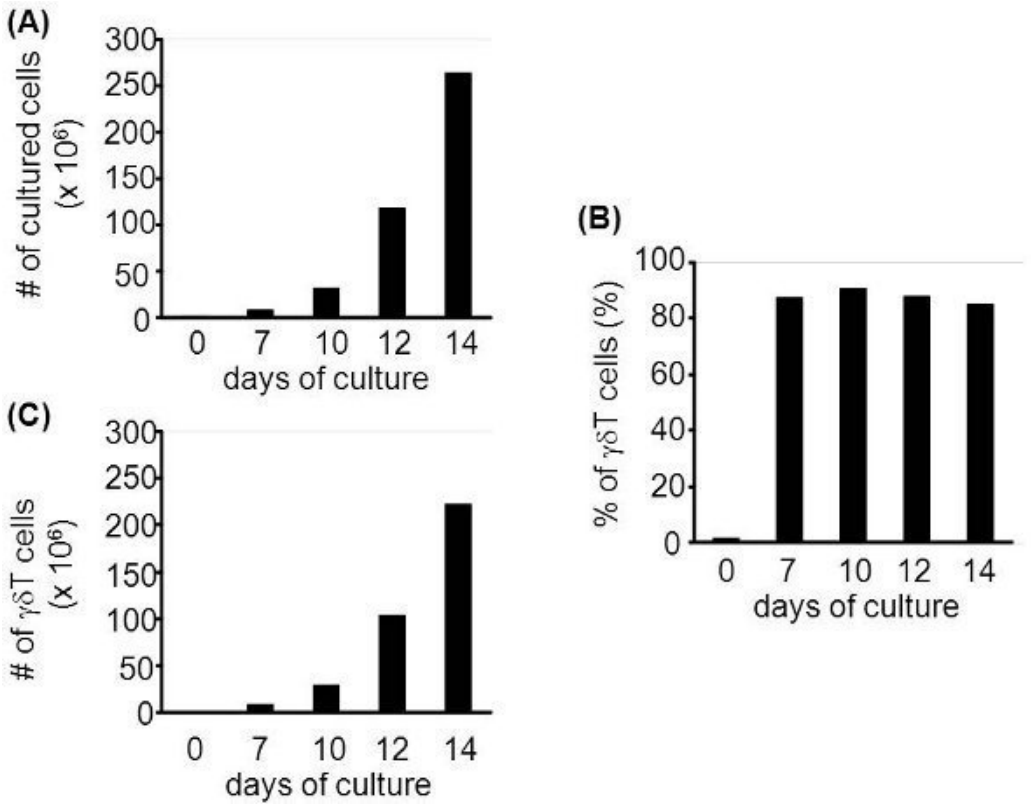

Figure 5. Kinetics of γδ T cell culture. (A) Absolute number of cultured cells, (B) percentage of γδ T cells, and (C) absolute number of γδ T cells at the indicated time points.

Discussion

The method presented here enables efficient expansion of γδ T cells from PBMC. γδ T cells activated and expanded by zoledronate and IL-2 develop complete effector functions, reflected by cytokine production and cytotoxicity. It has been reported that the synthetic phosphoantigens bromohydrin pyrophosphate (BrHPP) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP) also expand γδ T cells; however, they are not commercially available. In contrast, zoledronate is already licensed for clinical applications as Zometa. Therefore, a reliable reagent is easily available.

The selection of culture media and serum is critical. Use appropriate culture media such as ALyS203 (Cell Science & Technology Institute) or OpTmizer (Invitrogen) for successful γδ T cell expansion.[11] Verify that autologous plasma, pooled human AB sera or FCS can support γδ T

cell culture. Also remember that PBMC from some donors fail to respond to zoledronate stimulation regardless of other culture reagents. If that happens, the only option is to change the donor.

As we have demonstrated, enrichment of γδ T cells was achieved relatively early; almost 80 % of cultured cells were γδ T cells by day 7. γδ T cells continued to proliferate up to 12-14 days (**Fig. 5**). Approximately 2.2 x 10^8 γδ T cells can be obtained from 1 x 10^6 PBMC containing 1.6 x 10^4 γδ T cells. This culture method has been used in phase I clinical trials evaluating the safety and feasibility of Zoledronate-expanded γδ T cell transfer therapy in patients with multiple myeloma or lung cancer.[12,13]

Disclosures

No conflicts of interest declared.

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