Video Article

Expansion of Human Peripheral Blood $\gamma\delta$ T Cells using Zoledronate

Makoto Kondo^{1,2}, Takamichi Izumi^{1,2}, Nao Fujieda^{1,2}, Atsushi Kondo^{1,2}, Takeharu Morishita^{1,2}, Hirokazu Matsushita¹, Kazuhiro Kakimi¹

¹Department of Immunotherapeutics (Medinet), University of Tokyo Hospital

²MEDINET Co., Ltd

Correspondence to: Kazuhiro Kakimi at kakimi@m.u-tokyo.ac.jp

URL: <http://www.jove.com/details.php?id=3182>

DOI: 10.3791/3182

Keywords: Immunology, Issue 55, $\gamma\delta$ T Cell, zoledronate, PBMC, peripheral blood mononuclear cells,

Date Published: 9/9/2011

Citation: Kondo, M., Izumi, T., Fujieda, N., Kondo, A., Morishita, T., Matsushita, H., Kakimi, K. Expansion of Human Peripheral Blood $\gamma\delta$ T Cells using Zoledronate. J. Vis. Exp. (55), e3182, DOI : 10.3791/3182 (2011).

Abstract

Human $\gamma\delta$ 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 $\gamma\delta$ T cells in peripheral blood have the V γ 9V δ 2 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, $\gamma\delta$ T cells are attractive candidate effector cells for cancer immunotherapy. V γ 9V δ 2 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 $\gamma\delta$ T cells. Dysregulation of mevalonate pathway in tumor cells leads to accumulation of IPP and $\gamma\delta$ 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 sensitivity to $\gamma\delta$ T cells recognition can be therapeutically increased by aminobisphosphonates. IPP accumulation is less efficient in nontransformed cells than tumor cells with a pharmacologically relevant concentration of aminobisphosphonates, that allow us immunotherapy for cancer by activating $\gamma\delta$ 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 V γ 9V δ 2 T cells in the peripheral blood.⁶ Based on these mechanisms, we developed a technique for large-scale expansion of $\gamma\delta$ T cell cultures using zoledronate and interleukin-2 (IL-2).⁷ Other methods for expansion of $\gamma\delta$ 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 $\gamma\delta$ T cells for use in adoptive immunotherapy. However, only zoledronate is an FDA-approved commercially available reagent. Zoledronate-expanded $\gamma\delta$ T cells display CD27⁺CD45RA⁺ effector memory phenotype and their function can be evaluated by IFN- γ production assay.⁷

Video Link

The video component of this article can be found at <http://www.jove.com/details.php?id=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 x 10⁶ cells are recovered from 1 ml of whole blood.
7. Determine the frequency and phenotype of $\gamma\delta$ T cells in PBMC by flow cytometry (section 3) (**Fig. 2**).

2. Expansion of $\gamma\delta$ 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 μ M, respectively. ALyS203 (Cell Science & Technology Institute) or OpTmizer (Invitrogen) media support good expansion of $\gamma\delta$ T cells (more information in references 6 and 9). Zometa is provided in liquid form (4 mg/5-ml vial). To prepare a 5 μ M solution, add 50 μ l of Zometa to 30 ml of culture medium.
3. Resuspend cell pellet in culture medium and adjust to 1x10⁶ cells/ml.

- Pipet 1 ml of CM containing 1×10^6 cells into each well of a 24-well plate. For large-scale cultures, cells can be seeded at 0.5×10^6 cells/cm² according to the surface areas of plate wells, dish, or flask.
- 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 μ l for each well of a 24-well plate). Place the plates in a humidified 37°C, 5% CO₂ incubator for 24-48 hr.
- Maintain the culture at a cell density of $0.5\text{--}2 \times 10^6$ 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%.
- Harvest cells on day 12-14 and determine the frequency, phenotype, and functions of $\gamma\delta$ T cells by flow cytometry (see below).

3. Phenotypic analysis by flow cytometry

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

4. IFN- γ production assay¹⁰

- The day before assay, prepare stimulator cells by culturing $3\text{--}5 \times 10^5$ Daudi cells/ml in RPMI 1640 medium plus 10% FCS (RPMI-10) overnight with Zometa (5 μ M) (hereafter designated Z-Daudi).
- Collect Z-Daudi and resuspend in RPMI-10 at 2×10^6 cells/ml. Add 100 μ l of Z-Daudi (2×10^5) to each well of a round-bottom 96-well plate.
- Prepare $\gamma\delta$ T cells at 2×10^6 cells/ml in RPMI-10 containing Brefeldin A at 20 μ g/ml. Transfer 100 μ l of $\gamma\delta$ T cell suspension (2×10^5) to each well containing Z-Daudi cells or to control wells (100 μ l of RPMI-10 only, or RPMI-10 with 20 ng/ml of phorbol 12-myristate 13-acetate [PMA] plus 2 μ g/ml of ionomycin).
- Mix by pipetting up and down several times. Incubate for 4 hr in a 37°C, 5% CO₂ incubator.
- Centrifuge the plate for 5 min at 400 x g at 4°C and resuspend the pellets in 200 μ l of cold PBS.
- 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.
- Resuspend the pellets in 50 μ l of FACS buffer with FITC-conjugated anti-TCRV γ 9 (5 μ l) and PE/Cy5-conjugated anti-CD3 mAb (2.5 μ l). Incubate protected from light for 15 min at room temperature.
- Add 100 μ l 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.
- Remove the supernatant by aspiration and add 100 μ l of IntraPrep reagent 2. Incubate for 5 min at room temperature without shaking.
- Add 5 μ l of PE-conjugated anti-IFN- γ mAb to the test tube. Incubate protected from light 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. Remove the supernatant by aspiration and resuspend the cell pellet in 0.5 ml of FACS buffer.
- Analyze the cells by flow cytometer. Gate on CD3⁺ TCRV γ 9⁺ cells and examine the expression of IFN- γ (**Fig. 4**).

5. Representative Results:

It is important to determine the percentage of $\gamma\delta$ T cells in PBMC at the initiation of culture. As shown in **Fig. 2 A**, the percentage of CD3⁺TCRV γ 9⁺ $\gamma\delta$ T cells in PBMC was 1.6% on day 0. The dominant populations were CD27⁺CD45RA⁺ naive or CD27⁺CD45RA⁻ central memory phenotypes. When $\gamma\delta$ 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⁺ $\alpha\beta$ T cells or NK cells could dominate the growth of $\gamma\delta$ T cells (**Fig. 3 C and D**). After 14 days of culture, the frequency of $\gamma\delta$ T cells increased to more than 93.8% of the cultured cells in successful $\gamma\delta$ T cell cultures (**Fig. 2 E**). The cultured $\gamma\delta$ 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 $\gamma\delta$ T cells were evaluated with regard to cytokine production and cytotoxicity. The intracellular IFN- γ staining demonstrated that $\gamma\delta$ 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 $\gamma\delta$ T cells.

tube	FITC	PE	ECD	PE/Cy5
1	CD3	CD19	CD45	CD14
2	CD3	TCR $\alpha\beta$	CD4	CD8
3	CD3			CD56
4	TCR V γ 9	TCR $\alpha\beta$	CD45	CD3
5	TCR V γ 9	NKG2D		
6	TCR V γ 9	CD69		
7	TCR V γ 9	mouse IgG1		
8	TCR V γ 9		CD45RA	CD27
9	TCR V γ 9		mouse IgG1	mouse IgG1

Table 1. Monoclonal antibodies used in multicolor staining of $\gamma\delta$ T cells. An example of the phenotypic analysis of $\gamma\delta$ 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 $\gamma\delta$ T cells. PBMC were stimulated with zoledronate and IL-2 for 14 days. Cells were stained with FITC-labeled anti-TCR V γ 9 and PE/Cy5-labeled anti-CD3 to monitor the expansion of $\gamma\delta$ T cells (A and E). $\gamma\delta$ T cells were identified by their expression of TCRV γ 9, and their expression of CD27 and CD45RA (B and F), NKG2D (C and G), or CD69 (D and H) was examined.

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

Figure 4. IFN- γ production. $\gamma\delta$ T cells were incubated with RPMI-10 only (A) or PMA/ionomycin (B) or Z-Daudi (C) for 4 hr. First, surface expression of TCRV γ 9 was stained and then IFN- γ production was examined by intracellular IFN- γ staining.

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

Discussion

The method presented here enables efficient expansion of $\gamma\delta$ T cells from PBMC. $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cell expansion.¹¹ Verify that autologous plasma, pooled human AB sera or FCS can support $\gamma\delta$ 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 $\gamma\delta$ T cells was achieved relatively early; almost 80 % of cultured cells were $\gamma\delta$ T cells by day 7. $\gamma\delta$ T cells continued to proliferate up to 12-14 days (Fig. 5). Approximately 2.2×10^8 $\gamma\delta$ T cells can be obtained from 1×10^6

PBMC containing 1.6×10^4 $\gamma\delta$ T cells. This culture method has been used in phase I clinical trials evaluating the safety and feasibility of Zoledronate-expanded $\gamma\delta$ T cell transfer therapy in patients with multiple myeloma or lung cancer.^{12,13}

Disclosures

No conflicts of interest declared.

References

1. Bonneville, M., O'Brien, R.L., & Born, W.K. $\gamma\delta$ T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol.* **10**, 467-478 (2010).
2. Hintz, M. *et al.* Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human $\gamma\delta$ T cells in *Escherichia coli*. *FEBS Lett.* **509**, 317-322 (2001).
3. Gober, H.J., *et al.* Human T cell receptor $\gamma\delta$ cells recognize endogenous mevalonate metabolites in tumor cells. *J Exp Med.* **197**, 163-168 (2003).
4. Kabelitz, D., Wesch, D., & He, W. Perspectives of gammadelta T cells in tumor immunology. *Cancer Res.* **67**, 5-8 (2007).
5. Roelofs, A.J., *et al.* Peripheral blood monocytes are responsible for gammadelta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. *Br J Haematol.* **144**, 245-250 (2009).
6. Dieli, F., *et al.* Induction of $\gamma\delta$ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo. *Blood.* **102**, 2310-2311 (2003).
7. Kondo, M., *et al.* Zoledronate facilitates large-scale ex vivo expansion of functional $\gamma\delta$ T cells from cancer patients for use in adoptive immunotherapy. *Cytotherapy.* **10**, 842-856 (2008).

8. Espinosa, E., *et al.* Chemical synthesis and biological activity of bromohydrin pyrophosphate, a potent stimulator of human $\gamma\delta$ T cells. *J Biol Chem.* **276**, 18337-18344 (2001).
9. Kobayashi, H., *et al.* Safety profile and anti-tumor effects of adoptive immunotherapy using $\gamma\delta$ T cells against advanced renal cell carcinoma: a pilot study. *Cancer Immunol Immunother.* **56**, 469-476 (2007).
10. Murali-Krishna, K., *et al.* Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity.* **8**, 177-187 (1998).
11. Sato, K., *et al.* Impact of culture medium on the expansion of T cells for immunotherapy. *Cytotherapy.* **11**, 936-946 (2009).
12. Abe, Y., *et al.* Clinical and immunological evaluation of zoledronate-activated V γ 9 $\gamma\delta$ T-cell-based immunotherapy for patients with multiple myeloma. *Exp Hematol.* **37**, 956-968 (2009).
13. Nakajima, J., *et al.* A phase I study of adoptive immunotherapy for recurrent non-small-cell lung cancer patients with autologous $\gamma\delta$ T cells. *Eur J Cardiothorac Surg.* **37**, 1191-1197 (2010).