TWMUJ Advance Publication

Original

Beta-Tricalcium Phosphate as a Possible Adjuvant in γδ T Cell-Based Immune Therapy for Human Disorders

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(Accepted July 26, 2022)
(Advance Publication by J-STAGE September 22, 2022)

Objective: γδ T cells can recognize and attack cancer cells even in the absence of information from dendritic cells. γδ T cells have been used in immune cell therapy because of their strong ability to kill cancer cells. However, the number of γδ T cells in peripheral blood is small; therefore, it is important to increase the number of γδ T cells via effective *ex vivo* culturing.
Methods: In this study, we analyzed the effects of beta-tricalcium phosphate (β-TCP) on γδ T cells derived from peripheral blood.
Results: We confirmed that the number of γδ T cells increased via β-TCP addition to the culture medium in 83.3% of the experiments (5/6 times).
Conclusion: As β-TCP has been widely used in medical therapy, it may be used as a possible adjuvant in γδ T-cell-based immune therapy for human disorders.

Keywords: immune therapy, γδ T cells, beta-tricalcium phosphate (β-TCP)

Introduction

Currently, surgery, chemotherapy, and radiation therapy are the three standard treatments for cancer.[1] Recently, immune cell therapy has attracted attention as a fourth cancer treatment method.[2] Immune cell therapy eliminates cancer cells by boosting the patients' immunity. The human immune system can potentially eliminate cancer cells. Among immune cells, T cells can attack cancer cells; however, if T cells are weakened, they may lose the ability to eliminate cancer cells. Most established immune cell therapies can maintain T cells or increase the number of T cells to attack cancer cells.

Although γδ T cells comprise a small population of T lymphocytes, they significantly contribute to a rapid and sustained immune response against cancer.[3] In particular, γδ T cells are known to be involved in tumor immunity because they show high cytotoxic activity against tumor cells.[4] Furthermore, because they have high antigen-presenting abilities and exhibit cross-presentation abilities, these cells also play a role in bridging natural and adaptive immunities.[5]

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doi: 10.24488/twmuj.2022012

Tanaka et al.[6] identified that human γδ T cells can recognize naturally occurring small non-peptidic antigens, as opposed to αβ T cells, which recognize major histocompatibility complex-bound peptide antigens. This allows the selective activation of γδ T cells *ex vivo*. Activated γδ T cells show high tumor cytotoxicity mediated by NK receptors such as NKG2D.[7] Efficient tumor growth inhibition is achieved when tumor-reactive γδ T cells are administered repeatedly.[8] Kato et al.[9] reported that certain human tumor cells can efficiently present non-peptide antigens to γδ T cells and suggested a strategic clue for developing a novel immunotherapy approach for human malignancies. Kobayashi et al.[10] conducted clinical immunotherapy trials using γδ T cells for various types of cancers, including kidney cancer, and showed a positive effect according to the RECIST standard. However, further research is required for its clinical application, including improving the culture methods.

Previously, a bisphosphonate prodrug was shown to expand human γδ T cells up to several thousand-fold in ten days and was considered ideal for preparing large numbers of highly homologous cells for use in adoptive immunotherapy for cancer.[11] However, more efficient proliferation methods are always desirable. In this study, we attempted to identify other materials that could support γδ T cell proliferation. Accordingly, the effect of beta-tricalcium phosphate (β-TCP) on γδ T cell proliferation was also analyzed in this study.

Materials and Methods

1. Ethics and subjects

This study was performed in accordance with the Declaration of Helsinki, and requisite permission was obtained from the ethical committee of Tokyo Women's Medical University (approval number: 2436). Peripheral blood samples were collected from five healthy volunteers after obtaining written informed consent.

2. Culturing Methods

Peripheral blood (10 mL) was obtained from healthy volunteers and collected in heparinised tubes. Nucleated cells were isolated from the blood samples using a lymphocyte separation solution (Lymphoprep; Cosmo Bio Co., Ltd., Tokyo, Japan). After adding the separator, the mixture was centrifuged at 25°C, 560 g, for 30 min with an accelerator brake slow, as described previously.[12, 13] The nucleated cell layer was collected in different tubes and used as samples.

The cells were counted using a OneCell counter (OneCell, Hiroshima, Japan). Thereafter, 2×10^6 nucleated cells were cultured in ALyS505N-0 medium (Cell Science & Technology Institute Inc., Miyagi, Japan) in a Falcon 6 multi-well plate (Corning, Glendale, AZ, USA) for 14 days at 37°C and 5% CO_2. Cultured nucleated cells were stimulated with 100 μmol/L 2-methyl-3-butenyl-1-diphosphate (2M3B1PP) as described previously.[12, 13]

β-TCP (Apatite β-TCP, triclinic; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was applied at 0, 30, and 100 μg in each well. The day after cell seeding, 30 μL (200 unit/mL) of recombinant human interleukin-2 (rhIL-2) and human serum derived from volunteers with AB blood type were added. During days 3-6, half of the culture medium was replaced with fresh medium and fresh IL-2 was added. During days 7-8, the cells were re-seeded into two separate wells; then, fresh IL-2 was added to the cells. From days 9-11, cells from two separate wells in a 24-well plate were re-seeded in a 6-well plate; then, fresh IL-2 was added to the cells. The cultured cells were analyzed on day 12.

3. Immunofluorescence analysis

γδ T cells were detected via immunofluorescence analysis using a BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Among the cultured nucleated cells, mononucleocytes were detected via forward scatter (FSC) and side scatter (SSC) measurements (**Figure 1A**). Then, T cell antigen receptor-expressing cells were selected using the CD3 antibody (**Figure 1B**). Among the selected cells, γδ T cells were screened using Vδ2 (Vd2) (**Figure 1C-F**). Although CD56 was measured for reference, all Vδ2 positive cells were counted as γδ T cells, regardless of whether CD56 was positive or negative. During all staining procedures, the cells were kept on ice.

The following monoclonal antibodies (mAbs) were used for the immunofluorescence analysis: FITC anti-human TCR Vδ2 antibody (BioLegend, San Diego, CA, USA) and CD3-PC5.5, CD56-PE, CD4-FITC, and CD8-

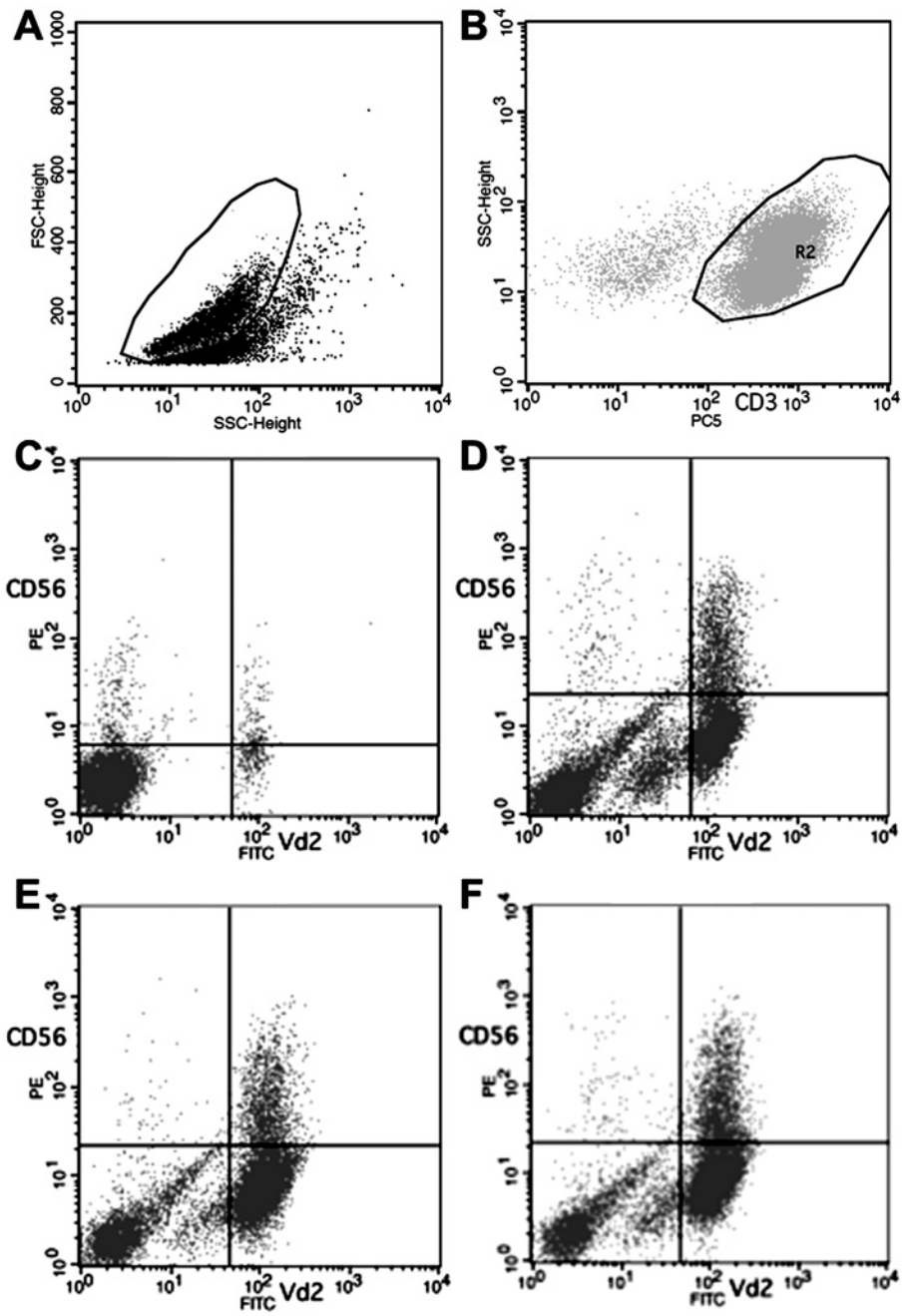

Figure 1. Representative flow cytometry results for control 1-1. Mononucleocytes were selected via forward scatter (FSC) and side scatter (SSC) (**A**). Then, T-cell antigen receptor-expressing cells were selected using CD3 antibody (**B**). Pre-culture γδT cells were measured using Vδ2 (Vd2) and CD56 at day 0 (**C**). Vδ2-expressing cells increased during 12-day culturing with 0 μg β-TCP (**D**). Further expansion of Vδ2-expressing cells via 30- (**E**) and 100-μg (**F**) β-TCP stimulation is shown. All Vd2 positive cells were counted as γδ T cells regardless of whether CD56 was positive or negative.

PE conjugated antibodies (Beckman Coulter, Brea, CA, USA).

Results

First, we added different β-TCP amounts to the medium at the start of the culture to investigate the impact of β-TCP amount on γδ T cell expansion. Six analyses were performed for the five healthy subjects, and the results are summarized in **Table 1**. For subject 1 (control 1; C1), the analyses were performed twice, with both analyses exhibiting a similar tendency, as shown in **Fig-**

Table 1. Analysis results

		β-TCP dose	Cell count*	CD3 positive		Vd2 positive		CD4 positive		CD8 positive	
				Cell count	Ratio (%)	Cell count	Ratio (%)	Cell count	Ratio (%)	Cell count	Ratio (%)
C1-1	Pre-culture		20.00	9.22	46.11	0.50	5.38	5.99	64.90	2.43	26.40
	Post-culture	0 μg	18.00	17.03	94.62	8.98	52.74	2.86	16.80	1.69	9.91
		30 μg	17.20	16.77	97.50	12.48	74.42	1.54	9.19	0.83	4.98
		100 μg	19.40	18.99	97.89	15.07	79.36	1.18	6.19	0.65	3.44
C1-2	Pre-culture		80.00	9.05	11.32	0.40	4.44	6.15	67.89	2.21	24.44
	Post-culture	0 μg	70.00	66.30	94.72	44.15	66.58	14.41	21.74	6.44	9.71
		30 μg	80.00	78.03	97.54	64.58	82.76	7.36	9.43	4.58	5.88
		100 μg	100.00	98.22	98.22	84.70	86.24	5.56	5.66	4.42	4.50
C2	Pre-culture		20.00	12.06	60.31	0.17	1.37	8.05	66.73	3.33	27.61
	Post-culture	0 μg	10.30	8.98	87.14	1.66	18.47	3.30	36.77	1.73	19.24
		30 μg	17.15	15.68	91.44	6.03	38.46	3.57	22.74	1.87	11.94
		100 μg	18.10	17.01	93.96	8.19	48.17	3.12	18.35	1.53	8.97
C3	Pre-culture		20.00	11.32	56.60	0.02	0.16	8.40	74.17	2.26	20.00
	Post-culture	0 μg	24.60	21.92	89.09	0.68	3.10	16.59	75.70	1.05	4.79
		30 μg	26.50	22.80	86.03	2.20	9.65	14.06	61.68	1.74	7.62
		100 μg	13.10	10.69	81.59	1.43	13.35	6.41	59.93	0.75	7.04
C4	Pre-culture		20.00	12.82	64.12	0.72	5.58	9.07	70.71	2.73	21.31
	Post-culture	0 μg	6.30	5.77	91.55	3.79	65.72	1.42	24.64	0.36	6.31
		30 μg	6.80	6.37	93.68	4.52	70.91	1.66	26.11	0.49	7.69
		100 μg	8.60	8.07	93.80	5.11	63.36	1.80	22.37	0.55	6.79
C5	Pre-culture		20.00	11.56	57.82	0.11	0.98	5.62	48.62	5.03	43.51
	Post-culture	0 μg	14.30	12.60	88.10	4.35	34.50	3.68	29.23	2.13	16.90
		30 μg	10.20	8.92	87.45	2.95	33.04	2.32	25.99	1.96	21.92
		100 μg	7.80	6.62	84.88	1.73	26.15	1.44	21.79	1.54	23.30

* Cell count unit: 1×10^5.

The positive ratios for Vd2, CD4, and CD8 are obtained versus CD3 positives.

ures 2A and 2B. The number of γδ T cells is shown in **Figure 2**. In the samples from three of the five healthy subjects (C1, C2, and C4), the γδ T cell expansion increased in a dose-dependent manner as the β-TCP amount increased. In one heakthy subject (C5), 30 μg of β-TCP, rather than 100 μg, affected the expansion. Furthermore, in only one healthy subject (C5), β-TCP inhibited γδ T cell expansion.

Next, we examined the impact of large β-TCP particles on the formation of expanding γδ T cell colonies. The β-TCP used in this study was granular, and its granule size varied from 1 to 50 μm. We investigated whether larger β-TCP could accelerate colony formation as scaffolds. γδ T cells proliferated and formed colonies 12 days after starting the culture. Larger colonies were observed via light microscopy in a dose-dependent manner after β-TCP addition (**Figure 3**). Interestingly, β-TCP particles were found in the nuclei of the colonies.

Discussion

To the best of our knowledge, this is the first study to investigate the effect of β-TCP on γδ T cell culture. This study aimed to establish an effective *in vitro* culturing system for γδ T cells. Accordingly, β-TCP was selected as the additive in the culture medium.

β-TCP has been widely used for bone substitution in orthopedic, maxillofacial, and dental surgeries.[14] Naito et al.[15] reported the antitumor effect of β-TCP in a xenograft tumor model of athymic mice, suggesting a novel potential role of β-TCP as an adjuvant for cancer treatment. It has also been shown that macrophage accumulation around tumor cells is important for tumor shrinkage. Tai et al.[16] analyzed the *in vitro* effects of β-TCP on primary cultured murine dendric cells. β-TCP particles were phagocytosed into macrophages and dendritic cells, which enhanced the expression of co-stimulatory surface molecules and induced phenotypic and functional matu-

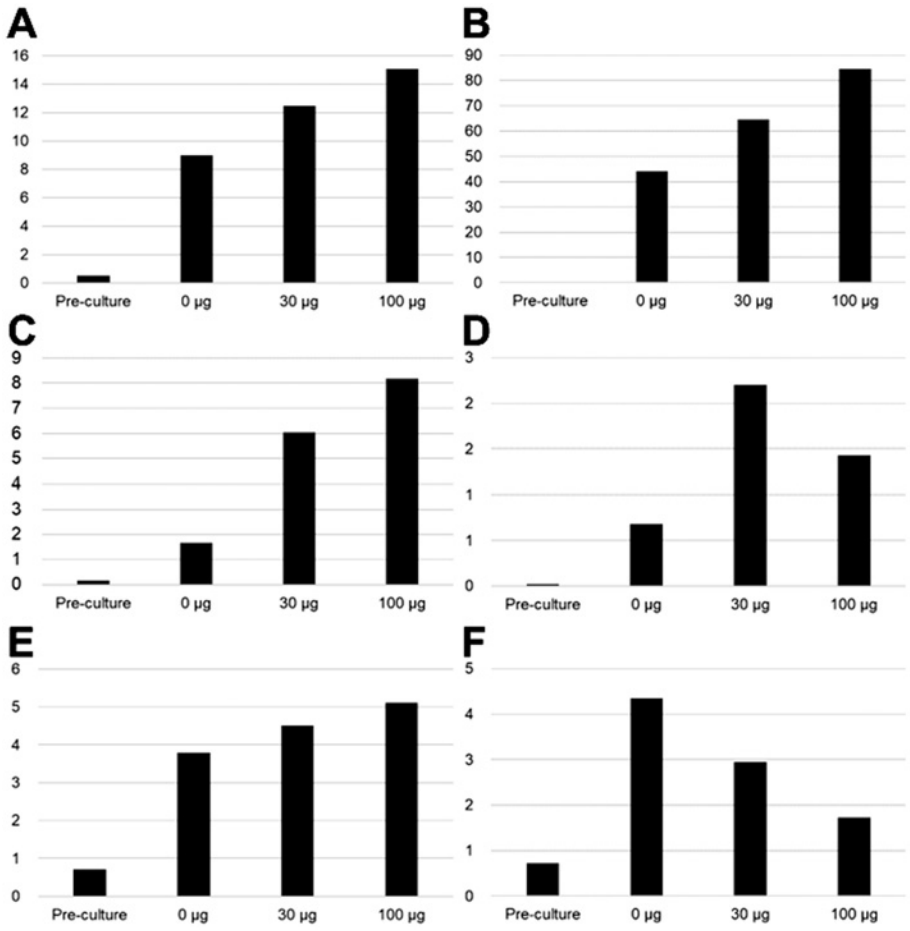

Figure 2. Proliferation effect of β-TCP stimulation on Vδ2-expressing cells. **A**; Control 1-1 (C1-1), **B**; Control 1-2 (C1-2), **C**; Control 2 (C2), **D**; Control 3 (C3), **E**; Control 4 (C4), and **F**; Control 5 (C5). The *y*-axis indicates the cell count ($\times 10^5$).

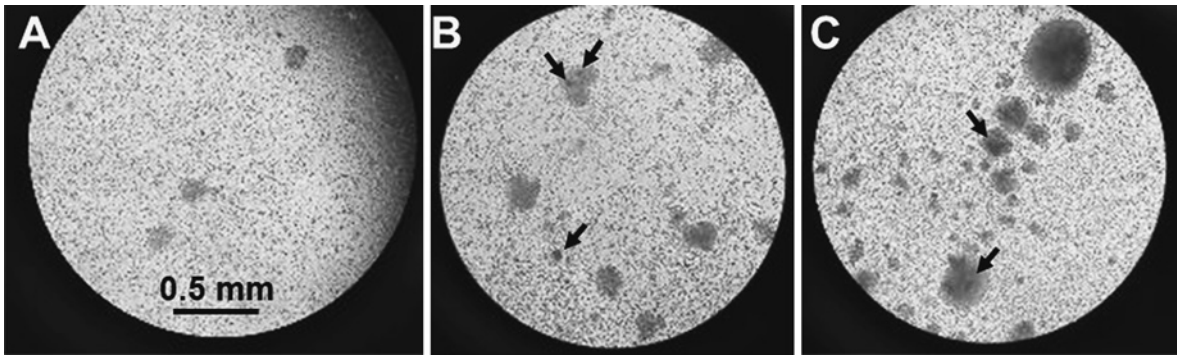

Figure 3. Appearance of the cultured cells in plates visualized by light microscopy. The γδ T cells in culture expanded after the addition of 0 (**A**), 30 (**B**), and 100 μg (**C**) of β-TCP. The arrows indicate β-TCP particles.

ration and dendritic cell activation. In addition, dendritic cells stimulated by β-TCP released various types of cytokines and chemokines, which induced splenocyte migration. Murayama et al.[17] further confirmed the ability of β-TCP to stimulate the immune system via an *in vitro* investigation of mouse dendritic cells and macrophages. Activated dendritic cells and macrophages increase the production of IL-1 in a caspase-1-dependent manner, and caspase-1 transforms pro-IL-18 into activated IL-18. Tsuda et al.[18] reported that IL-18 strongly affects the pro-

liferation of γδ T cells. Based on these findings, we speculate that β-TCP might be useful for expanding γδ T cells for γδ T cell-based adoptive immunotherapy applications.

Originally, 2M3B1PP was added to the medium and used to proliferate γδ T cells. This study confirmed that β-TCP addition to the culture medium could further enhance γδ T cell proliferation. However, some subjects did not show the expected level of increase. In this study, human γδ T cells derived from the peripheral blood of healthy subjects were used. Ou et al.[19] examined 98 blood samples from healthy individuals to measure the γδ T cell expansion stimulated by zoledronic acid. They reported that the expansion ability is independent of gender, age, and HLA type, and is dependent on the proportion of γδ T cells in peripheral blood mononuclear cells (PBMCs) before starting the culture. Proliferation was significantly lower in PBMCs with less than 0.82% γδ T cells than that in PBMCs with > 0.82% γδ T cells. In our study, one healthy subject (C5), whose γδ T cell percentage was 0.98% of CD3+ cells in PBMC, did not respond to the β-TCP additive. In good responders (C1, C2, and C4), the γδ T cell percentage varied from 1.37% to 5.58% of CD3 + cells in PBMC. Our results are similar to those reported by Ou et al.[19] One healthy individual (C3), whose γδ T cell percentage was 0.16% of CD3+ cells in PBMC, responded to 30 μg of β-TCP additive but not to 100 μg. Macrophages stimulated by β-TCP produce IL-18, and γδ T cells may proliferate in response to IL-18.[17] The promotion of the expansion capability of γδ T cells by β-TCP additives might depend not only on the percentage of γδ T cells in PBMC, but also on the ability of macrophages to produce IL-18. Therefore, the ability of β-TCP to increase cultured γδ T cells may depend on the different genetic backgrounds of individuals. Further research is necessary to confirm these findings.

β-TCP comprises particles of various sizes. Larger particles may act as a scaffold, as shown in the light microscopic image of larger colonies with β-TCP particles. Conversely, small particles are phagocytosed by macrophages and dendritic cells, resulting in the production of various cytokines and chemokines, including IL-18, which facilitates γδ T cell proliferation. We believe that β-TCP plays a dual role in γδ T cell proliferation. In conclusion, this is the first report to suggest β-TCP as a possible adjuvant for γδ T-cell-based immune therapy for human disorders.

Sources of Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of Interest: The authors declare that there are no conflicts of interest regarding the publication of this article.

Author Contributions: KT designed the study, collected and analyzed the data, conducted the literature review, and wrote the original draft of the manuscript. MI helped prepare the manuscript and reviewed the draft. YS and TN helped design the study, supported the writing of the draft, and reviewed the original manuscript. All the authors have read and approved the final manuscript.

Acknowledgments: We express our gratitude to the volunteers for their cooperation. We would like to thank Editage (www.editage.com) for English language editing.

Ethical Approval: Tokyo Women's Medical University, approval number: 2436.

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