Antitumor Activity and Some Immunological Properties of γδ T-Cells from Patients with Gastrointestinal Carcinomas

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Abstract. *Objectives: Human γδ T-cells expressing Vγ2Jγ1.2Vδ2-TCR recognize microbial pyrophosphomonoesters in an MHC-independent manner and exert cytotoxic activity on a wide variety of tumor cells. In the present study, the immunological properties of γδ T-cells derived from patients with gastrointestinal carcinomas were examined and compared with those from healthy adult individuals, aiming to develop a novel cancer immunotherapy using γδ T-cells stimulated with one of the nonpeptide antigens, 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP). Materials and Methods: Peripheral blood mononuclear cells (PBMs) and tumor-associated lymphocytes (TAL) were obtained from patients with gastrointestinal carcinomas. The mononuclear cells were stimulated with 2M3B1PP for 2 weeks and the expanded γδ T cells were examined for cytokine production upon T-cell receptor (TCR) engagement and cytotoxic activity against allogeneic tumors and autologous tumor cells. For comparison, PBMCs derived from healthy adult volunteers were similarly stimulated with 2M3B1PP and the resulting γδ T-cells were analyzed for effector functions. Results: All the peripheral blood- and tumor-associated γδ T-cell preparations from patients with gastrointestinal carcinomas proliferated vigorously in response to 2M3B1PP to comparable levels to those from healthy donors. When challenged with CD3 monoclonal antibodies, the carcinoma patient-derived γδ T-cells secreted a large amount of inflammatory cytokine, IFN-γ, and exhibited a potent cytotoxic activity against allogeneic tumor cell lines as well as autologous tumor cells. Conclusion: Both peripheral blood- and tumor-associated γδ T-cells derived from patients with gastrointestinal carcinomas were as immunologically active as those from healthy individuals and could be utilized for a novel cancer immunotherapy for gastrointestinal malignancies.*

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Human γδ T-cells expressing Vγ2, Jγ1.2 and Vδ2 germline genes recognize nonpeptide antigens derived from microbial pathogens such as mycobacteria and malaria parasites, and malignant cells in a major histocompatibility complex (MHC)-independent manner, strongly suggesting that γδ T-cells play important roles in infection immunity as well as tumor immunity (1-5). The major microbial antigen is (E)-4-hydroxy-3-methyl-2- butenyl pyrophosphate (HMBPP), which is the direct precursor of isopentenyl pyrophosphate (IPP), a basic donor unit of isoprenoid metabolites (6-8). The entity of tumor antigens recognized by γδ T-cells remains enigmatic.

Recently, nitrogen-containing *bis*phosphonates (N-BP) were found to elicit expansion of peripheral blood γδ T-cells in patients infused with the drugs (9-10). Biochemical and structural analyses revealed that the compounds directly bound and inhibited farnesysl pyrophosphate synthase (FPPS), leading to the accumulation of IPP (11). It is thus currently believed that γδ T-cells recognize IPP or IPP derivatives in antigen-presenting cells such as monocyte-lineage cells primed with N-BP (12). It is worth noting that up-regulation of the mevalonate pathway is observed in some tumor cells, indicating a possibility for IPP serving as an endogenous antigen. We previously reported that human tumor cells pretreated with N-BP could be efficiently recognized and lyzed by γδ T-cells in a γδ T-cell receptor (TCR) dependent manner by using a Jurkat gene transfer system (13-15). When certain tumor cell lines including Daudi (Burkitt lymphoma) and RPMI8226 multiple myeloma, were challenged with γδ T-cells, γδ T-cells similarly recognize these cells in a TCR-dependent manner (16). In contrast, most other tumor cell lines are not detected

by the γδ-TCR in the absence of N-BP (17). Taken together, these data suggest γδ T-cells appear to have two distinct mechanisms for tumor recognition: CD8 killer cell-like TCR-dependent cytotoxicity and natural killer-like TCR-independent pathway.

On the basis of the potent tumoricidal activity of γδ T-cells, protocols for phase I/II clinical trials have been designed and extensive efforts have been made to harness γδ T-cells for the treatment of patients with B-cell malignancies, renal cell carcinoma and prostate cancer (18-22). In the present study, we attempted to examine the relevance of γδ T-cell immunotherapy for treatment of patients with gastrointestinal carcinomas. Since immune systems are sometimes impaired in patients with advanced cancer, it is essential to study the immunological properties of γδ T-cells in patients and, in particular, to determine the cytotoxic activity of γδ T-cells against autologous tumor cells. Therefore, we compared the immunological features of peripheral blood γδ T-cells derived from healthy donors with those of peripheral blood- and tumor-associated γδ T-cells from patients with gastrointestinal carcinomas.

Materials and Methods

Nonpeptide antigens. A pyrophosphomonoster antigen, 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP) (Figure 1a), was synthesized as described elsewhere (23). Pamidronate (disodium 3-amino-1-hydroxypropylidene-1, 1-bisphosphonate pentahydrate) (Figure 1b) was purchased from Novartis Pharma K. K. (Minato-Ku, Tokyo, Japan). For enzymatic treatment, 2M3B1PP was treated with shrimp alkaline phosphatase (10 U/400 μl, Takara Bio Inc., Otsu, Shiga, Japan) at a final concentration of 2 mM at 37˚C for 16 h. The reaction mixture was ultrafiltrated through a 3,000 MWCO membrane filter (Gelman Sciences, Ann Arbor, MI, USA) at 10,000 ×g for 2 h and sterilized by filtration through a 0.22 μm PVDF membrane filter (Millipore, Carrigtwohill Co., Cork, Ireland).

Proliferation assay. Assays were performed in triplicate using 1×10^5 12G12 γδ T-cells per round-bottom well of a 96-well plate (Corning Inc., Corning, NY, USA). The γδ T -cells were incubated in the presence of 2M3B1PP, pamidronate or shrimp alkaline phosphatase (AP)-pretreated 2M3B1PP at a final concentration of 0, 50, 3,125 and 1,2500 nM for 36 h at 37˚C, 5% CO_2, pulsed with 2 μCi of [^{3}H]-thymidine, harvested at 48 h, and examined for tritium incorporation into DNA. The means and SE are depicted as histograms.

Expansion of peripheral blood γδ T- cells. Healthy volunteers were free from malignancies and microbial infections. Informed consent was obtained from healthy volunteers and patients with gastrointestinal carcinomas. Peripheral mononuclear cells (PBMCs) were isolated from heparinized blood samples derived from the donors by using Ficoll-Conray density gradient centrifugation for 30 min at 1,500 rpm at room temperature. The purified PBMCs, 2×10^6, were then incubated with 200 μM of 2M3B1PP in 1.5 ml of modified Yssel's medium containing 0.25% human serum albumin at 37˚C, 5% CO_2 in a flat-bottom well of 24-well plates (BD Biosciences, Franklin Lakes, NJ, USA) (24). On day 2, 1.4 ml of the culture supernatant was carefully removed. To the well was added 1.4 ml of modified Yssel's medium supplemented with 10% pooled human AB serum (Cat.No.12181301. Cosmo Bio Co., Ltd., Koto-Ku, Tokyo, Japan) and 100 U/ml of recombinant human interleukin-2 (rIL-2). When the cultures became confluent, the cells were transferred into wells of 6-well plates (Corning, NY, USA), then into 75 cm^2 flasks (BD Biosciences, Franklin Lakes, NJ, USA) containing an appropriate volume of the medium plus rIL-2. On day 14, the cells were harvested and examined for cell surface markers on a flow cytometer.

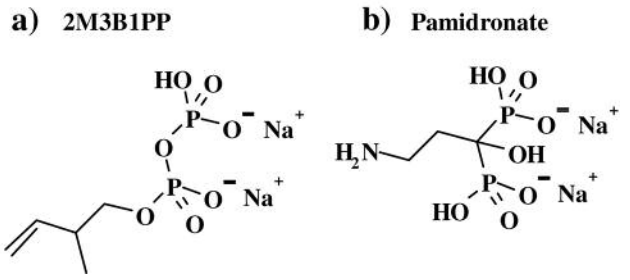

Figure 1. *Structures of nonpeptide antigens for human γδ T-cells. a) 2-Methyl-3-butenyl-1-pyrophosphate (2M3B1PP) and b) pamidronate.*

Preparation of mononuclear cells and tumor cells from patients with gastro-intestinal malignancies. Heparinized malignant ascites of patients with gastrointestinal malignancies were centrifuged at 1,500 rpm for 5 min and resuspended in phosphate-buffered saline (PBS). The cell suspensions were loaded on Ficoll-Conray for centrifugal separation. The fluffy layer at the interface of Ficoll-Conray and PBS was collected, washed once with PBS, and then subjected to Percoll density gradient (density 1.055 and 1.068) centrifugation at 2,600 rpm for 20 min at room temperature. The large cells obtained at the interface between 1.055 and 1.068 were morphologically identified as tumor cells. The resulting cells were maintained in 30 ml of complete RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) in a 75-cm^2 flask as target cells until used. Mononuclear cells at the bottom of the centrifuge tube, representing tumor-associated lymphocytes (TALs), were stimulated with 2M3B1PP as described above for PBMCs.

Flow cytometric analysis. For detection of surface antigens on cultured cells, harvested cells were stained using monoclonal antibodies (mAbs): phycoerythrin (PE)- conjugated-anti-CD3 (SK7; Becton Dickinson Immunocytometory Systems, San Jose, CA, USA), anti-CD4 (SK3; Becton Dickinson), anti-CD8 (SK1; Becton Dickinson), anti-CD56(IM2073U; Immunotech, Marseille, France), fluorescein isothiocyanate (FITC)-conjugated-anti-pan-TCR γδ (Immu515; Immunotech), anti-Vγ2 chain (Immu360; Immunotech), anti-Vδ2 chain (15D; Serotec Ltd, Kidlington, Oxford, UK), anti-CD16 (41116015; Beckman Coulter Inc., Fullerton, CA, USA), anti-HLA-DR (TU36; Becton Dickinson), anti-αβ (555547; Becton Dickinson) and anti-CD45RO (313044; Becton Dickinson). The stained cells were analyzed on an EPICS XL flow cytometer (Coulter Electrics, Hialeah, FL, USA), as described elsewhere (25). Controls with isotype-matched antibodies established the quadrants so that greater than 99% of the cells were in the double-negative region.

Cytokine assay. PBMCs and TALs expanded with 2M3B1PP were subjected to magnetic bead separation to remove $CD4^+$ and $CD8^+$ cells. After the negative sorting, more than 95% of cells were $CD4^-$ $CD8^-$ γδ T-cells based on flow cytometric analysis. The cells were incubated in 1.0 ml of complete RPMI 1640 medium in a flat-bottom well of a 24-well plate coated with anti-CD3 mAb. Culture supernatants were harvested at 1, 3, 6, 12 and 24 h and frozen at –20˚C until used. The supernatants were thawed and examined for the amounts of INF-γ and IL-4 by using a standard enzyme-linked immunosorbent assay (High Sensivity (h) INFγ and IL-4 ELISA System. Code No. RPN2783. GE Healthcare UK Ltd., Amersham Place, Little Chalfront, Bucks, UK).

Cytotoxicity assay. Tumor cell lines, Daudi (Burkitt lymphoma), T24 (bladder carcinoma), VMRC-RCW (renal cell carcinoma) and K562 (chronic myelogenous leukemia) were purchased from Health Science Research Resources Bank (Sennan, Osaka, Japan). CW-2 (colon carcinoma), KATO III (gastric carcinoma) and autologous tumor cells were maintained in our laboratories. The tumor cells, 1×10^6, were incubated with 100 mCi of [^{51}Cr]-sodium chromate for 1 h, washed three times with complete RPMI-1640 medium, and resuspended in 10 ml of the medium. Tumor cells, 1×10^4, were challenged in triplicate by the γδT-cells in 200 ml of the medium at an effector to target (E/T) ratio of 0.625:1, 1.25:1, 2.5:1, 5:1, 10:1, 20:1 and 40:1 for 4 h at 37˚C, 5% CO_2. The supernatants were harvested and the tumoricidal activity was examined using a γ-counter. Specific lysis (%) of tumor cell lines by γδ T-cells was calculated based on the formula, [(test release-spontaneous release)/(complete release-spontaneous release)] ×100 as described elsewhere (26). Relative spontaneous release never exceeded 10% of the maximum release.

Results

Recognition of alkaline phosphatase-sensitive nonpeptide antigens by primed Vγ2Jγ1.2Vδ2-bearing T-cells. In cancer immunotherapy, repetitive stimulation of T-cells is pivotal for a relatively long series of treatments. It is thus essential to examine the reactivity of primed γδ T-cells to nonpeptide antigens. As shown in the left and center panels of Figure 2, a primed γδ T-cell clone, 12G12, proliferated in response to 2M3B1PP, but not to pamidronate, consistent with our previous observations (28-29). This clearly demonstrates that pyrophosphomonoester compounds are superior to N-BP drugs in terms of repetitive stimulation of γδ T-cells in clinical settings. However, pyrophosphomonoester antigens are alkaline phosphatase sensitive as indicated in the right panel of Figure 2, compelling us to use serum-free medium for the first 2 days in the cell culture. Based on the above finding, we attempted to examine the possibility of γδ T-cell immunotherapy in patients with gastrointestinal carcinomas in this study.

Expansion of peripheral blood- and tumor-associated γδ T-cells by using 2M3B1PP. In γδ T-cell immunotherapy, it is crucial to establish a reproducible strategy for efficient expansion of γδ T-cells. We first compared the expansion levels of PBMCs obtained from 9 healthy donors and from

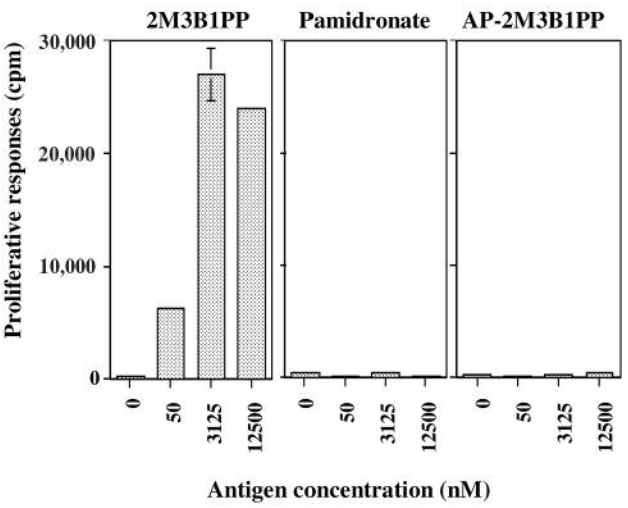

Figure 2. *Proliferative responses of primed γδ T-cells to nonpeptide antigens. An established γδ T-cell line, 12G12, was challenged with 2M3B1PP, pamidronate, or alkaline phosphatase-pretreated 2M3B1PP (AP-2M3B1PP), at concentrations of 0, 50, 3125 and 12500 nM, and proliferative responses were determined by standard [^{3}H]-thymidine incorporation assay. The means and SE of triplicate cultures are indicated.*

5 patients with colon carcinomas, and TALs from a patient with colon carcinoma, when challenged with 2M3B1PP. In PBMCs derived from healthy adult donors, the percentage of Vγ2-bearing γδ T-cells was a mean of 4.7±3.0% and the total number $6.0\times10^4\pm3.0\times10^4$ (Table I upper panel) (n=9), consistent with previous results (30-31). After stimulation with 2M3B1PP for 2 weeks, γδ T-cells increased to 84.7±10.9% and the total number to $3.8\times10^7\pm4.1\times10^7$. This confirms that 2M3B1PP can be utilized for effective expansion of γδ T-cells from healthy adult donors. In five patients with colon carcinomas, the percentage and total number of γδ T-cells increased from a mean of 5.6±6.4% on day 0 to 94.3±2.6% on day 14 and $11.2\times10^4\pm12.7\times10^4$ on day 0 to $3.2\times10^7\pm1.8\times10^7$ on day 14 (Table I center panel). Peripheral blood γδ T-cells of patients with colon carcinomas can generally be expanded using 2M3B1PP *in vitro* to comparable levels to those found with healthy donors (*t*-test; p=0.7301). Moreover, tumor-associated γδ T-cells derived from ascites of a patient with colon carcinoma were also elevated after stimulation with 2M3B1PP from 3.1% and 3.1×10^4 on day 0 to 88.0% and 6.7×10^7 on day 14 as shown in Table I lower panel. When it comes to expansion rates, both peripheral blood- and tumor-associated γδ T-cells increased effectively in response to 2M3B1PP, comparable to peripheral blood γδ T-cells from healthy donors, with repeated measure ANOVA being p=0.478 and 0.760, respectively. Figure 3 shows a representative flow cytometric profile of γδ T-cells derived from a healthy donor.

Table I. *Expansion of peripheral blood γδ T-cells and tumor-associated γδ T-cells in response to 2M3B1PP. Peripheral blood mononuclear cells and tumor-associated cells were purified and stimulated with 2M3B1PP. After 2 weeks, the cellularity of Vγ2-bearing γδ T-cells were determined.*

	Freshly isolated PBMCs (Day 0)			After stimulation (Day 14)		
Donors	Whole cells	Vγ2 T-cells (%)	Vγ2 T-cells	Whole cells	Vγ2 T-cells (%)	Vγ2 T-cells
Healthy donor 1	2×10^6	4.6	9.2×10^4	1.3×10^8	92.8	1.3×10^8
Healthy donor 2	2×10^6	3.8	7.6×10^4	7.9×10^6	78.4	1.0×10^7
Healthy donor 3	2×10^6	3.7	7.3×10^4	3.7×10^6	96.5	3.9×10^6
Healthy donor 4	2×10^6	3.7	7.5×10^4	2.9×10^7	82.4	2.2×10^7
Healthy donor 5	2×10^6	12.0	2.4×10^4	5.7×10^7	89.2	3.3×10^7
Healthy donor 6	2×10^6	1.1	2.3×10^4	6.5×10^7	59.4	9.7×10^7
Healthy donor 7	2×10^6	5.4	1.1×10^4	1.1×10^7	90.0	7.2×10^7
Healthy donor 8	2×10^6	4.6	9.2×10^4	1.9×10^7	86.3	1.3×10^7
Healthy donor 9	2×10^6	3.5	7.8×10^4	1.7×10^7	87.2	2.0×10^7
Mean		4.7±3.0	$6.0\pm3.0\times10^4$	$4.5\pm4.5\times10^7$	84.7±10.9	$3.8\pm4.1\times10^7$
Patient 1	2×10^6	16.8	3.6×10^5	2.5×10^7	96.8	2.4×10^7
Patient 2	2×10^6	3.9	7.9×10^4	2.0×10^7	93.0	1.8×10^7
Patient 3	2×10^6	3.9	7.8×10^4	3.8×10^7	91.1	3.4×10^7
Patient 4	2×10^6	1.9	3.8×10^4	6.3×10^7	97.3	6.2×10^7
Patient 5	2×10^6	1.4	2.8×10^4	2.2×10^7	93.5	2.0×10^7
Mean		5.6±6.4	$11.2\pm12.7\times10^4$	$3.4\pm1.8\times10^7$	94.3±2.6	$3.2\pm1.8\times10^7$
	Freshly isolated TALs (Day 0)			After stimulation (Day 14)		
Patient 3 TAL	1×10^6	3.1	3.1×10^4	7.6×10^7	88.0	6.7×10^7

Phenotypic analysis of γδ T-cells stimulated with 2M3B1PP. PBMCs expanded with 2M3B1PP for 14 days were analyzed for cell surface markers by using a flow cytometer. Table II summarizes cell surface antigens expressed on the stimulated peripheral blood cells derived from representative individuals and two healthy donors. Most of the cultured cells, 97.6%, were $CD3^+$ and the majority of them seemed to consist of Vγ2Vδ2-bearing T-cells and some expressed natural killer cell markers (Table II a). In addition, ~90% of the Vγ2-bearing cells were $CD4^-CD8^-$, judging from the two-color flow cytometric analysis (Table II). Since more than 90% of the γδ T-cells expressed CD45RO and HLA-DR, these cells were highly activated. Regarding other cell types, only marginal proportions were αβ T-cells after stimulation with 2M3B1PP. Essentially the same results were observed in TAL expanded with the nonpeptide antigen (data not shown).

Cytokine production by γδ T-cells stimulated with 2M3B1PP. To further characterize the γδ T-cells expanded by the use of 2M3B1PP, PBMCs derived from 9 healthy individuals and 6 patients with colon carcinomas, and TAL from 2 patients with colon carcinomas were stimulated with the nonpeptide antigen, from which γδ T-cells were then purified. The resulting γδ T-cell fractions were challenged with anti-CD3 mAb and the amounts of IFN-γ and IL-4 in the culture supernatants were determined by the standard ELISA assay. As shown in Table III, most of the γδ T-cell preparations secreted much greater amounts of IFN-γ, compared to IL-4. Figure 4 depicts representative time courses of the cytokine production by γδ T-cells after TCR stimulation. The amounts and production kinetics of IFN-γ and IL-4 were very similar among the three independent samples of peripheral blood γδ T-cells derived from a healthy donor, peripheral blood γδ T-cells from a colon carcinoma patient and tumor associated γδ T-cells from a colon carcinoma patient. This demonstrates that peripheral blood- and tumor-associated γδ T-cells in patients with colon carcinomas seem to retain potent cytokine productivity, comparable to the levels observed in peripheral blood γδ T-cells from healthy individuals upon TCR engagement.

Tumoricidal activity exhibited by 2M3B1PP-stimulated peripheral blood γδ T-cells from healthy individuals. In order to develop a novel γδ T-cell cancer immunotherapy, it is essential to examine the target specificity of γδ T-cells. As shown in Figure 5, peripheral blood γδ T-cells derived from healthy donors exerted potent cytotoxicity to different degrees on Daudi, T24, K562, CW-2, VMRC-RCW and KATO III cells consistent with previous observations (27). To examine the tumorigenicity of peripheral blood γδ T-cells from patients with colon carcinomas, we next determined the

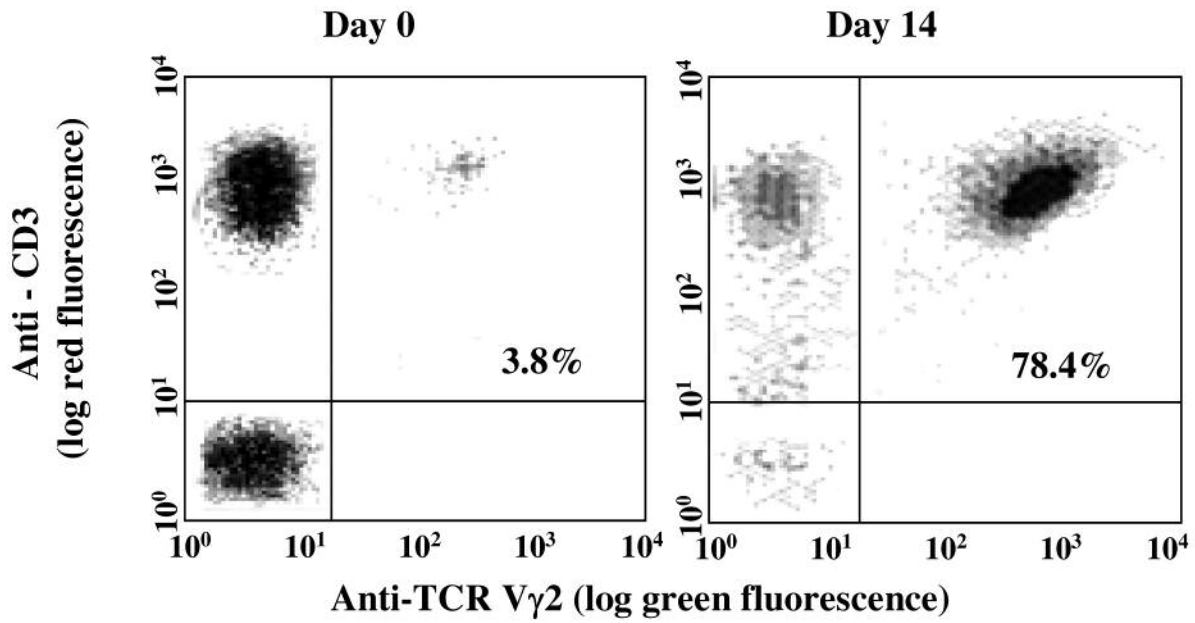

Figure 3. *Expansion of peripheral blood γδ T-cells with 2M3B1PP. PBMCs were purified from a healthy donor and stimulated with one of the pyrophosphomonoester antigens, 2M3B1PP. The proportion of γδ T-cells before and after incubation with the antigen was analyzed using an EPICS XL flow cytometer.*

Table II. *Phenotypic analysis of peripheral blood cells expanded with 2M3B1PP. Peripheral blood mononuclear cells derived from healthy donors were stimulated with 2M3B1PP and their surface markers were analyzed using an EPICS XL flow cytometer.*

a) Surface markers of the expanded cells (%)

	γδ	γ2	δ2	CD3	CD4	CD8	CD16	CD56
Healty donor 1	93.4	90.5	86.9	97.6	11.5	20.2	10.1	16.5
Healty donor 2	77.4	76.1	79.2	97.6	19.4	12.2	11.9	25.1

b) Surface markers on Vγ2 T cells (%)

	CD4	CD8	CD16	CD56
Healty donor 1	3.3	11.3	5.1	11.5
Healty donor 2	5.0	8.2	1.9	25.3

c) Characterization of the expanded cells (%)

	γδ/3 CD	γ2/ CD3	δ2/ CD3	γ2/ CD4	γ2/ CD8	γδ/ CD45 RO	γδ/ HLA-DR	αβ/ CD3
Healty donor 1	94.9	92.1	95.1	2.9	4.9	99.8	91.0	4.3
Healty donor 2	88.3	87.2	88.9	4.8	3.7	99.3	96.6	3.0

Table III. *Cytokine production from 2M3B1PP-stimulated γδ T-cells in response to anti-CD3 mAb. Peripheral blood mononuclear cells and tumor-associated lymphocytes were stimulated with 2M3B1PP. The expanded γδ T-cells were isolated and analyzed for cytokine production induced by anti-CD3 mAb.*

Donors	IL-4 (pg/ml)	IFN-γ (ng/ml)
Healthy donor 1	235.0	21.7
Healthy donor 2	195.0	11.8
Healthy donor 3	n.d.	n.d.
Healthy donor 4	n.d.	n.d.
Healthy donor 5	34.2	1.1
Healthy donor 6	291.6	10.1
Healthy donor 7	239.2	9.8
Healthy donor 8	587.0	35.1
Healthy donor 9	225.1	27.8
Patient 1	125.0	21.0
Patient 2	n.d.	n.d.
Patient 3	59.1	17.0
Patient 3 TAL	227.0	27.5
Patient 4	290.3	18.3
Patient 5	151.7	28.7
Patient 6	105.5	26.8
Patient 6 TAL	333.1	18.7

cytotoxic activity of 2M3B1PP-stimulated γδ T-cells prepared from PBMCs of colon carcinoma patients. Figure 6 clearly demonstrates that the allogeneic tumor cell lines Daudi and CW-2 were efficiently lyzed by peripheral blood γδ T-cells prepared from the patients. Furthermore, tumor-associated γδ T-cells stimulated with 2M3B1PP were also able to recognize and kill the allogeneic tumor cell lines, even at a E/T ratio of 2.5:1 (Figure 7).

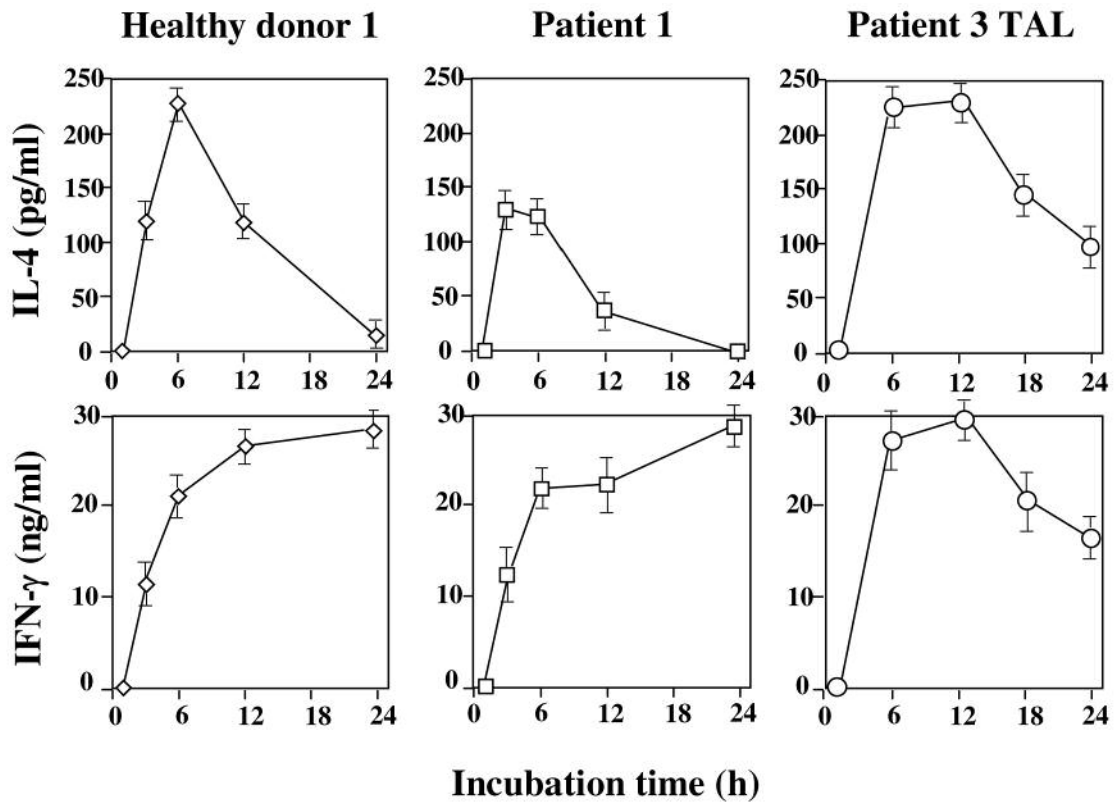

Figure 4. *Time course of cytokine production by peripheral blood- and tumor-associated γδ T-cells expanded by 2M3B1PP after treatment with anti-CD3 mAb. PBMCs derived from a healthy donor and a colon carcinoma patient were stimulated with 2M3B1PP, and γδ T-cell-rich fractions were isolated using anti-CD4 and anti-CD8 mAb-coated magnetic beads. The purified γδ T-cells were challenged with anti-CD3 mAb and the production of IFN-γ and IL-4 was measured by the use of standard ELISA assay. Similarly, TAL derived from another colon carcinoma patient were expanded by 2M3B1PP, and the magnetic bead-purified γδ T-cell fractions were examined for cytokine production in response to anti-CD3 mAb.*

Potent cytotoxic activity of γδ T-cells against autologous tumor cells. Since γδ T-cells recognize tumor cells in an MHC-independent manner, a variety of allogeneic tumor cell lines can be lyzed by 2M3B1PP-stimulated γδ T-cells. It is, however, pivotal to study the cytotoxicity of γδ T-cells on autologous tumor cells. As shown in Figure 8, both peripheral blood- and tumor-associated γδ T-cells stimulated with 2M3B1PP exerted a potent cytolytic activity on autologous tumor cells, comparable to the levels of γδ T-cells from healthy indivisuals *versus* allogeneic tumors.

Discussion

For the development of a novel cancer immunotherapy using γδ T-cells, it is cardinal to stimulate γδ T-cells with antigenic substances regardless of treatment strategies, infusion or adoptive transfer. Monoethyl phosphate was originally identified as a simple, synthetic phosphomonoester compound which could stimulate human Vγ2Jγ1.2Vδ2- bearing T-cells (28). Based on this finding, phosphorylated metabolites in mycobacterial extracts were systematically screened and purified, leading to a discovery of isopentenyl pyrophosphate (IPP) as one of the antigenic entities for γδ T-cells (29). A microbial precursor of IPP, (*E*)-4-hydroxy-3-methyl-2-butenyl pyrophosphate, was then found to have an even more potent biological activity in stimulating γδ T-cells (30). In addition, some alkyl amine compounds and N-BPs also turned out to induce expansion of peripheral blood γδ T-cells (31). It is, thus, vital to select compounds for clinical studies utilizing γδ T-cells. Regarding the nonpeptide antigen recognition mechanism by γδ T-cells, the precise mode of action has not yet fully been elucidated. Pyrophospho-monoester antigens seem to bind weakly and transiently to the surface on certain cells, including γδ T-cells, in an as yet unidentified manner and the nonpeptide antigen-presenting cells might be recognized by γδ T-cells through their γδ TCR (32). In contrast, N-BPs and alkyl amine compounds appear to be internalized into monocyte-lineage cells or tumor cells, where farnesyl pyrophosphate synthase (FPPS) could be inhibited, resulting in the accumulation of IPP (33-38). IPP itself or its derivative then seem to stimulate γδ T-cells. The most important features of the three classes of nonpeptide antigens manifest themselves

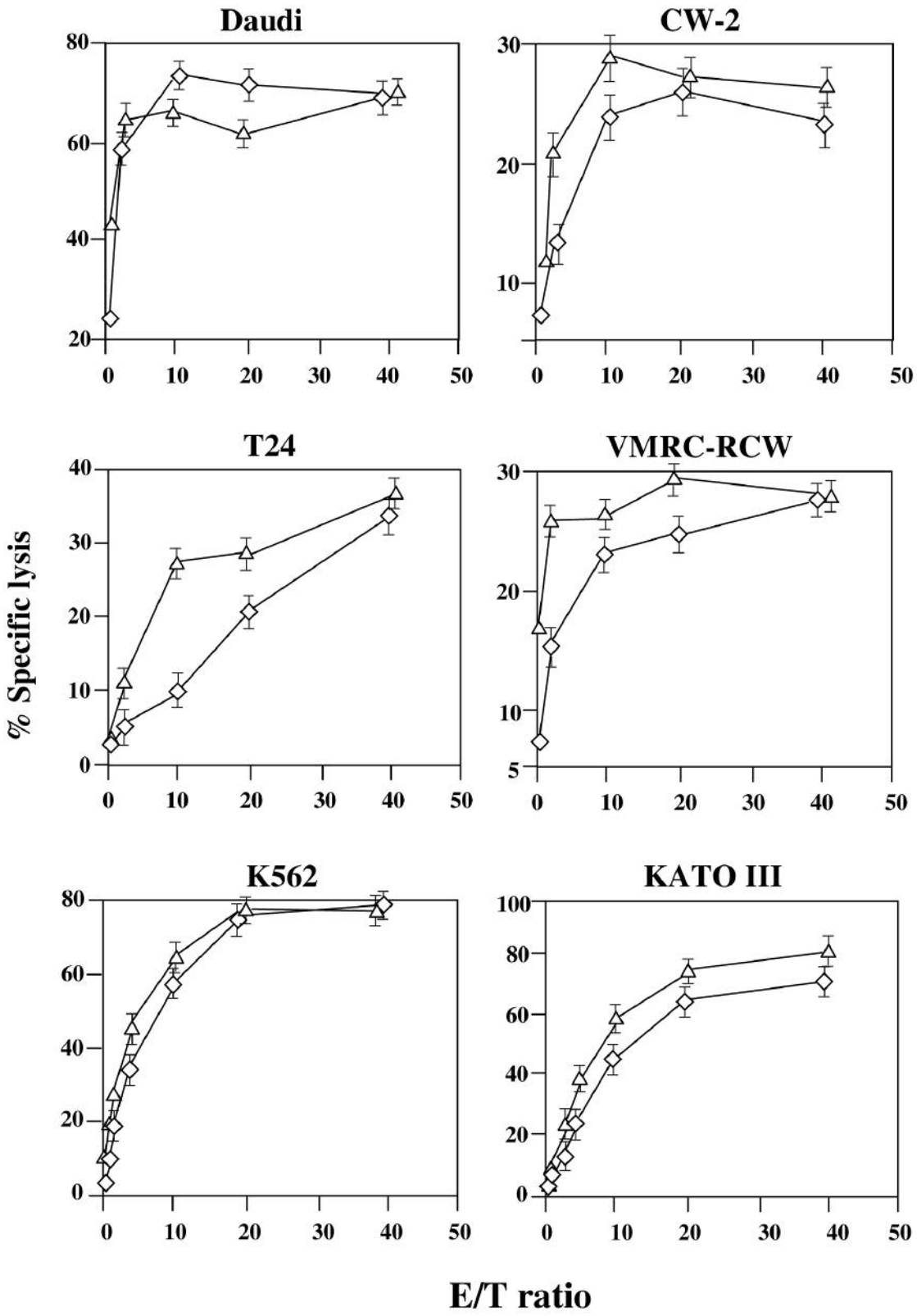

Figure 5. *Induction of tumoricidal activity by 2M3B1PP in γδ T-cells derived from healthy donors. PBMCs from two healthy donors were stimulated with 2M3B1PP and allogeneic tumor cell lines were challenged by the resulting γδ T-cells. The cytotoxic activity was examined by standard [^{51}Cr]-sodium chromate assay. The symbols, open diamond and open triangle, represent PBMCs from healthy donor 1 and healthy donor 2, respectively.*

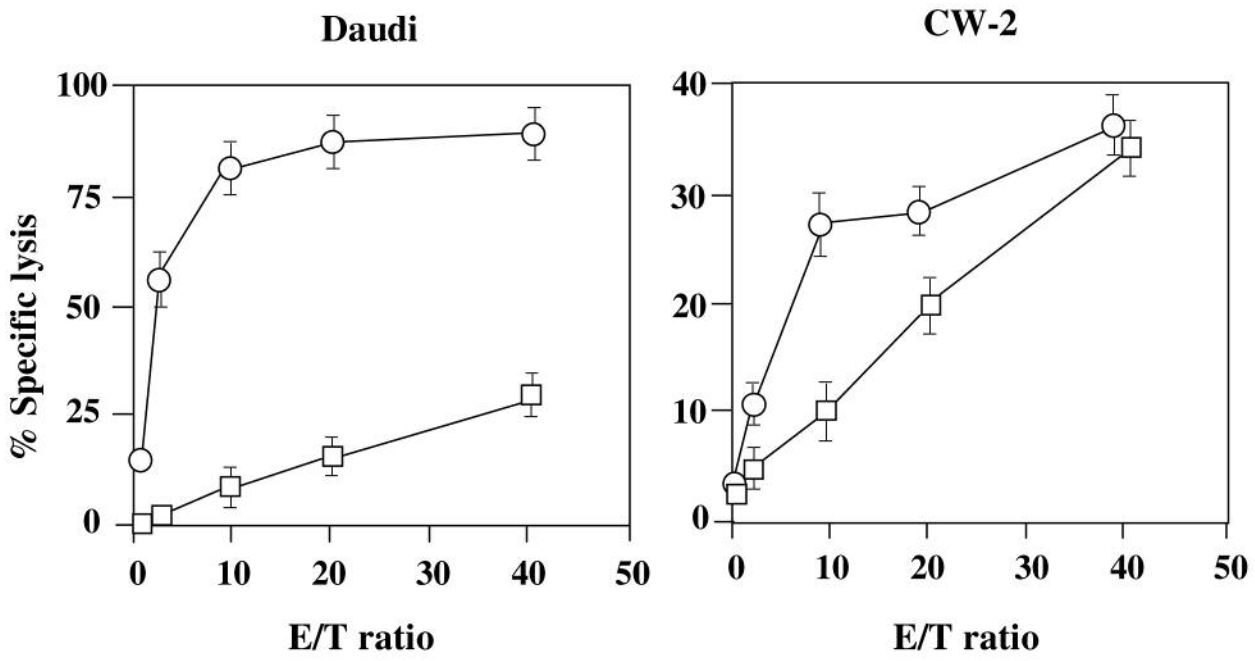

Figure 6. *Cytotoxic activity exhibited by 2M3B1PP-stimulated γδ T-cells derived from patients with colon carcinomas. PBMCs were isolated from colon carcinoma patients, and co-incubated with 2M3B1PP for 2 weeks. Allogeneic tumor cell lines, Burkitt lymphoma, Daudi, and colon carcinoma CW-2 cells were then challenged by the expanded γδ T-cells. Specific [^{51}Cr]-sodium chromate release was determined by γ-counter. Open square and open circle indicate PBMCs from colon carcinoma patients 1 and 2, respectively.*

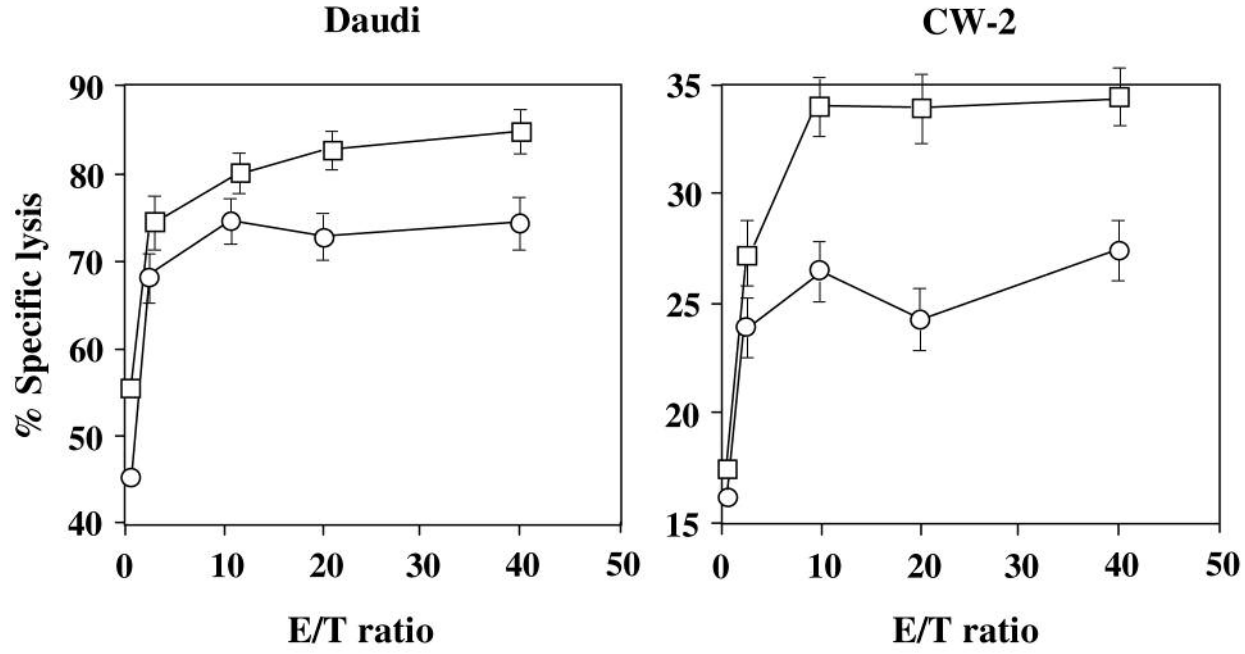

Figure 7. *Tumoricidal activity of peripheral blood- and tumor-associated γδ T-cells stimulated by 2M3B1PP. PBMCs and TALs were prepared from a colon carcinoma patient and incubated in the presence of 2M3B1PP. Allogeneic Burkitt lymphoma cells, Daudi, and colon carcinoma CW-2 cells were challenged by the 2M3B1PP-expanded γδ T-cells at an effector/target (E/T) ratio of 40:1, 20:1, 10:1, 1.25:1 and 0.625:1. Open square and open circle symbols represent peripheral blood γδ T-cells and tumor-associated γδ T-cells, respectively.*

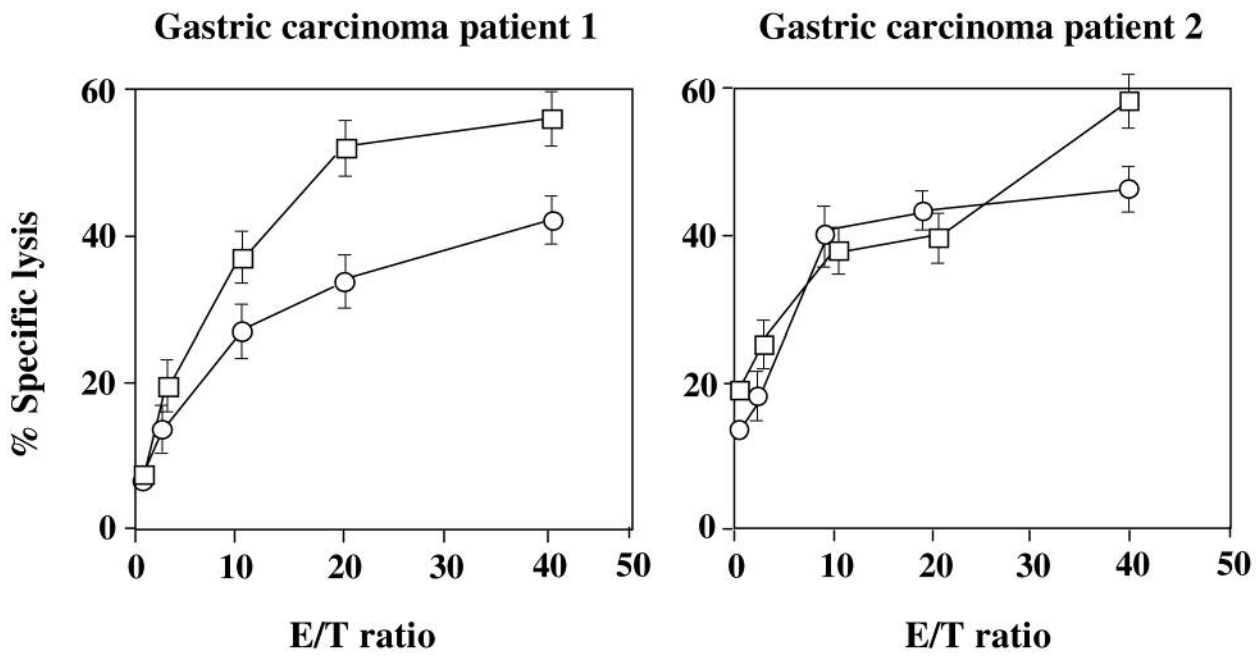

Figure 8. *Cytotoxic activity of peripheral blood- and tumor-associated γδ T-cells derived from patients with gastric carcinomas. PBMCs and TALs were prepared from two gastric carcinoma patients and stimulated with 2M3B1PP. After 2 weeks, autogenic tumor cells were incubated with the γδ T-cells at an E/T ratio of 40:1, 20:1, 10:1, 1.25:1 and 0.625:1. Open square and open circle symbols indicate peripheral blood γδ T-cells and tumor-associated γδ T-cells, respectively.*

in secondary responses. While all three nonpeptide antigens can successfully elicit vigorous expansion in freshly prepared peripheral blood γδ T-cells, only pyrophosphomonoester antigens can induce proliferative responses in primed γδ T-cells. This unequivocally demonstrates that alkyl amines and N-BPs cannot be utilized for repetitive stimulation of γδ T-cells for cancer immuno-therapy, though the precise mechanism for unresponsiveness of primed γδ T-cells to nitrogen-containing nonpeptide antigens remains unknown.

Pyrophosphomonoester compounds are essentially alkaline phosphatase sensitive and are thus swiftly hydrolyzed by the enzyme in cell culture media containing serum. Generally, only small amounts of antigens are required for γδ T-cell expansion when stimulated with highly bioactive species, but even a trace of alkaline phosphatase would suffice to completely inactivate that amount of antigen. In contrast, weakly active compounds require high levels of blood concentrations, possibly causing adverse effects. Consequently, moderately active, synthetic substances are ideal for *in vitro* expansion of γδ T-cells. We previously synthesized a moderately active compound, 2M3B1PP, with the concentration required for half maximal proliferative responses being 400 nM. Although this compound is inevitably sensitive to alkaline phosphatase, the use of this chemical at 200 μM efficiently expands both naive and primed γδ T-cells, as shown in this study. This demonstrates that 2M3B1PP is a good synthetic compound for *in vitro* expansion of γδ T-cells aimed at cancer immunotherapy.

It is known that the immune system can be down-regulated in patients with malignancies. It is, therefore, essential to examine the responsiveness of γδ T-cells derived from patients with carcinomas. Since we aim at developing γδ T-cell immunotherapy in patients with gastrointestinal carcinomas, we purified PBMCs and TALs from the patients and compared the reactivity of γδ T-cells with that from healthy individuals. As reported in this study, both peripheral blood- and tumor-associated γδ T-cells efficiently recognize 2M3B1PP and proliferated to a level comparable to that for γδ T-cells from healthy donors. Moreover, there was no significant difference in effector functions of γδ T-cells of patients with carcinoma and healthy individuals. Based on these findings, it seems promising to treat gastrointestinal carcinoma patients with 2M3B1PP-activated γδ T-cells.

To date, several pilot studies and phase I/II clinical trials have been carried out in patients with B-cell malignancies, renal cell carcinoma and prostate cancer. According to the reports, the γδ T-cell immunotherapy using nonpeptide antigens was well tolerated and major adverse events, fever, chills and fatigue, appeared to be attributable to concomitantly administered IL-2 (20). Early studies on γδ T-cell immuno-therapy utilized N-BP intravenously; patients with B-cell malignancies and multiple myelomas were infused with N-BP periodically (18). The principle is that N-BP is internalized by monocyte-lineage cells, creating antigenic structures on the surface of the cells, which are then recognized by γδ T-cells.

Finally, the activated γδ T-cells recognize B-cell lymphomas and myelomas in a TCR-dependent manner. More recently, adoptive transfer of γδ T-cells was performed in patients with renal cell carcinomas. In these pilot studies, peripheral blood γδ T-cells prepared from patients with renal cell carcinomas were expanded by using pyrophosphomonoester antigens, and the patients were infused with the effector cells together with a low dose of IL-2. Since renal cell carcinomas are known to be recognized by γδ T-cells in a TCR-independent manner, certain natural killer receptors may account for the effector functions of γδ T-cells. More recently, a phase I clinical trial of γδ T-cell immunotherapy using an N-BP, zoledronate, plus rIL-2 was carried out in patients with hormone-refractory prostate cancer. This report further extended the possible tumor targets for γδ T-cell immunotherapy (22).

Our target malignant cells, gastrointestinal tumors, are also known to be recognized by γδ T-cells in a TCR-independent manner. Thus, tumor targeting is indispensable for achieving more efficient immunotherapy. One possibility is to employ intravenous administration of N-BP right before adoptive transfer of γδ T-cells. In this protocol, two different nonpeptide antigens are required: pyrophosphomonoesters for *in vitro* expansion of γδ T-cells and N-BP for tumor targeting. A phase I/II clinical trial is currently ongoing. It is noteworthy that N-BP administration without IL-2 might lead to functional unresponsiveness of γδ T-cells, consistent with the previous observation that some patients with Paget's disease suffered from low-grade fever and chills after the first infusion of N-BP, but no significant adverse events were observed after the third or fourth administration (39, 40). In fact, peripheral blood γδ T cells derived from carcinoma patients previously infused with N-BP aimed at controlling bone metastasis failed to respond to nonpeptide antigens (our unpublished data). Taken together, it is clear that pyrophosphomonoesters such as 2M3B1PP are essential and ideal for expanding γδ T-cells in possible γδ T-cell immunotherapy (41, 42), though great attention should be paid to the use of N-BP because of ramifications such as γδ T-cell anergy or exhaustion.

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References

1 Tanaka Y, Brenner MB, Bloom BR and Morita CT: Recognition of nonpeptide antigens by T-cells. J Mol Med *74*: 223-231, 1996.

2 Morita CT, Lee HK, Leslie DS, Tanaka Y, Bukowski JF and Märker-Hermann E: Recognition of nonpeptide prenyl pyrophosphate antigens by human γδ T-cells. Microbes Infect *1*: 175-186, 1999.

3 Hayday AC: γδ Cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol *18*: 975-1026, 2000.

4 Kabelitz D: Effector functions and control of human γδ T-cell activation. Microbes Infect *1*: 255-261, 1999.

5 Chien YH, Jores R and Crowley MP: Recognition by γδ T-cells. Annu Rev Immunol 14: 511-532, 1996.

6 Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind RM, Kollas AK, Beck E, Wiesner J, Eberl M and Jomma H: Identification of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human γδ T-cells in *Escherichia coli*. FEBS Lett *509*: 317-322, 2001.

7 Rohdich F, Hecht S, Gärtner K, Adam P, Krieger C, Amslinger S, Arigoni D, Bacher A and Eisenreich W: Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein. Proc Natl Acad Sci USA *99*: 1158-1163, 2002.

8 Cunningham FX Jr, Lafond TP and Gantt E: Evidence of a role for LytB in the nonmevalonate pathway of isoprenoid biosynthesis. J Bacteriol *182*: 5841-5848, 2000.

9 Kunzmann V, Bauer E and Wilhelm M: γδ T-cell stimulation by pamidronate. N Engl J Med *340*: 737-738, 1999.

10 Kunzmann V, Bauer E, Feurle J, Weißinger F, Tony HP and Wihelm M: Stimulation of γδ T-cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood *96*: 384-392, 2000.

11 Gober HJ, Kistowska M, Angman L, Jenö P, Mori L and Libero GD: Human T-cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med *197*: 163-168, 2003.

12 Morita CT, Jin C, Sarikonda G and Wang H: Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T-cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev *215*: 59-76, 2007.

13 Miyagawa F, Tanaka Y, Yamashita S and Minato N: Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human γδ T-cells by aminobisphosphonate antigen. J Immunol *166*: 5508-5514, 2001.

14 Miyagawa F, Tanaka Y, Yamashita S, Mikami B, Danno K, Uehara M and Minato N: Essential contribution of germline-encoded lysine residues in Jγ1.2 segment to the recognition of nonpeptide antigens by human γδ T-cells. J Immunol *167*: 6773-6779, 2001.

15 Das H, Wang L, Kamath A and Bukowski JF: Vγ2Vδ2 T-cell receptor-mediated recognition of aminobisphosphonates. Blood *98*: 1616-1618, 2001.

16 Bukowski JF, Morita CT, Tanaka Y, Bloom BR, Brenner MB and Band H:Vγ2Vδ2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. J Immunol *154*: 998-1006, 1995.

17 Tanaka Y: Human γδ T-cells and tumor immunotherapy. J Clin Exp Hematopathol *46*: 11-23, 2006.

18 Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T and Tony HP: γδ T-cells for immune therapy of patients with lymphoid malignancies. Blood *102*: 200-206, 2003.

19 Dieli F, Gebbia N, Poccia F, Caccamo N, Montesano C, Filfaro F, Arcara C, Valerio MR, Meraviglia S, Sano CD, Sireci G and Salemo A: Induction of γδ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients *in vivo*. Blood *102*: 2310-2311, 2003.

20 Kobayashi H, Tanaka Y, Yagi J, Toma H and Uchiyama T: γδ T cells provide innate immunity against renal cell carcinoma. Cancer Immunol Immunother *50*: 115-24, 2001.

21 Burjanadze M, Condomines M, Reme T, Quittet P, Latry P, Lugagne C, Romagne F, Morel Y, Rossi JF, Klein B and Lu ZY: *In vitro* expansion of γδ T-cells with anti-myeloma cell activity by Phosphostim and IL-2 in patients with multiple myeloma. Br J Haematol *139*: 206-216, 2007.
22 Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N, Salerno A, Eberl M and Hayday AC: Targeting human γδ T-cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res *67*: 7450-7457, 2007.
23 Yamashita S, Tanaka Y, Harazaki M, Mikami B and Minato N: Recognition mechanism of non-peptide antigens by human γδ T-cells. Int Immunol *15*: 1301-1307, 2003.
24 Yssel H, De Vries JE, Koken M, Bitterswijk WV and Spits H: Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T-cell clones. J Immunol Methods *72*: 219-227, 1984.
25 Takahashi N, Imanishi K, Nishida H and Uchiyama T: Evidence for immunologic immaturity of cord blood T-cells: cord blood T-cells are susceptible to tolerance induction to *in vitro* stimulation with a superantigen. J Immunol *351*: 1614-1619, 1995.
26 Migita K, Eguchi K, Tezuka H, Otsubo T, Kawakami A, Nagao H, Ueki Y, Shimomura C, Matsunaga M, Ishikawa N, Ito K and Nagataki S: Cytotoxic activity of interleukin-2 (IL-2) activated killer cells toward thyroid epithelial cells. Clin exp Immunol *77*: 196-201, 1989.
27 Morita CT, Lee HK, Wang H, Li H, Mariuzza RA and Tanaka Y: Structural features of nonpeptide prenyl pyrophosphates that determine their antigenicity for human γδ T-cells. J Immunol *167*: 36-41, 2001.
28 Kato Y, Tanaka Y, Tanaka H, Yamashita S and Minato N: Requirement of species-specific interactions for the activation of human γδ T-cells by pamidronate. J Immunol *170*: 3608-3613, 2003.
29 Kato Y, Tanaka Y, Miyagawa F, Yamashita S and Minato N: Targeting of tumor cells for human γδ T cells by nonpeptide antigens. J Immunol *167*: 5092-5098, 2001.
30 Morita CT, Tanaka Y, Bloom BR and Brenner MB: Direct presentation of non-peptide prenyl pyrophosphate antigens to human γδ T-cells. Res Immunol *147*: 347-353, 1996.
31 Morita CT, Mariuzza RA, and Brenner MB: Antigen recognition by human γδ T cells: pattern recognition by the adaptive immune system. Springer Semin Immunopathol *22*: 191-217, 2000.
32 Tanaka Y, Sano S, Nieves E, De Libero G, Rosa D, Modlin RL, Brenner MB, Bloom BR and Morita CT: Nonpeptide ligands for human γδ T-cells. Proc Natl Acad Sci USA *91*: 8175-8179, 1994.
33 Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB and Blom BR: Natural and synthetic non-peptide antigens recognized by human γδ T-cells. Nature *375*: 155-158, 1995.
34 Allison TJ and Garboczi DN: Structure of γδ T-cell receptors and their recognition of non-peptide antigens. Mol Immunol *38*: 1051-1061, 2002.
35 Bukowski JF, Morita CT and Brenner MB: Human γδ T-cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. Immunity *11*: 57-65, 1999.
36 Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, Golan DE and Brenner MB: Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T-cells. Immunity *3*: 495-507, 1995.
37 Thompson K, Dunford JE, Ebetino FH and Rogers MJ: Identification of a bisphosphonate that inhibits isopentenyl diphosphate isomerase and farnesyl diphosphate synthase. Biochem Biophys Res Commun *290*: 869-873, 2002.
38 Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH and Rogers MJ: Structure-activity relationships for inhibition of farnesyl diphosphate synthase *in vitro* and inhibition of bone resorption *in vivo* by nitrogen-containing bisphosphonates. J Pharmacol Exp Ther *296*: 235-242, 2001.
39 van Beek E, Pieterman E, Cohen L, Löwik C and Papapoulos S: Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem Biophys Res Commun *264*: 108-111, 1999.
40 van Beek E, Pieterman E, Cohen L, Löwik C and Papapoulos S: Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their antiresorptive potencies *in vitro* and *in vivo*. Biochem Biophys Res Commun *255*: 491-494, 1999.
41 Thompson K and Rogers MJ: Statins prevent bisphosphonate-induced γδ-T-cell proliferation and activation *in vitro*. J Bone Miner Res *19*: 278-288, 2004.
42 Martin MB, Arnold W, Heath HT 3rd Urbina JA and Oldfield E: Nitrogen-containing bisphosphonates as carbocation transition state analogs for isoprenoid biosynthesis. Biochem Biophys Res Commun *263*: 754-758, 1999.
43 Buckler HM, Mercer SJ, Davison CE, Hollis S, Richardson PC and Anderson DC: Evaluation of adverse experiences related to pamidronate infusion in Paget's disease of bone. Ann Rheum Dis *57*: 572, 1998.
44 Adami S, Bhalla AK, Dorizzi R, Montesanti F, Rosini S, Salvagno G and Lo Cascio V: The acute-phase response after bisphosphonate administration. Calcif Tissue Int *41*: 326-331, 1987.
45 Poupot M and Fournie JJ: Non-peptide antigens activating human Vγ9/Vδ2 T lymphocytes. Immunol Lett *95*: 129-138, 2004.
46 Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, Bonneville M and Fournié JJ: Stimulation of human γδ T-cells by nonpeptidic mycobacterial ligands. Science *264*: 267-270, 1994.

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