Induction of Cytotoxic T Lymphocytes by CEA Peptide-pulsed γδ T-Cells Isolated from Patients with Advanced Cancer

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Abstract. *Cytotoxic γδ T-cells recognize antigens directly without the need for antigen processing and presentation. Recently, it was reported that they can also present antigens and proliferate in vitro. In this study, we examined whether γδ T-cells isolated from patients with advanced cancer can be used for immunotherapy. Twenty-two inoperable patients with multiple cancer metastases were enrolled in the study. There was no significant difference in the ratio of γδ T-cells within the peripheral blood mononuclear cell population isolated from healthy volunteers and cancer patients. γδ T-Cells isolated from cancer patients were expanded 2- to 5-fold using zoledronic acid or 2-methyl-3butenyl-1-pyrophosphate and IL-2. Autologous CD8$^+$ T-cells co-cultured with expanded CEA peptide-pulsed γδ T-cells from cancer patients with HLA-A24 killed more CEA-positive HLA-A24-matched gastric cancer cells and secreted higher levels of interferon-γ. These results suggest that γδ T-cells from cancer patients may be ideal candidates for adoptive immunotherapy.*

γδ T-Cells are essential constituents of innate antimicrobial and antitumor defense, but their role in adaptive immunity is less clear (1-3). γδ T-Cells account for 2-10% of CD3$^+$ peripheral blood T-cells, but constitute a dominant fraction of T-cells at other anatomical sites, such as the intestinal epithelia (4-5). γδ T-Cells differ from conventional αβ T-cells in several aspects (5). Firstly, most γδ T-cells lack CD4 and CD8 antigens, so they display a double-negative phenotype. This leads to a lack of major histocompatibility complex (MHC) restriction during antigen recognition (1). It is clear that γδ T-cells recognize antigens directly, without the need for antigen processing and presentation (6); however, it is unclear what the majority of γδ T-cell ligands are and how they are recognized. Secondly, the germline encoded T-cell receptor (TCR) repertoire of γδ T-cells is strikingly small compared with the large TCR repertoire of αβ T-cells. This means that only six expressed Vγ genes, and a similarly small number of Vδ genes, are expressed in humans. The majority of γδ T-cells (typically >80%) express Vγ9Vδ2 TCRs (7). Thirdly, the functional capacity of γδ T-cells includes cytokine production, and potent cytotoxic effector and killer cell abilities. They also express perforin and granzymes, and use this pathway to kill macrophages infected by mycobacteria (8, 9).

Recently, it was reported that they can also act as professional antigen-presenting cells (4, 10). γδ T-Cells from several mammalian species can, surprisingly, present antigens to CD4$^+$ αβ T-cells on MHC class II (4, 11-12). They can also efficiently cross-present soluble proteins, and have similar abilities to monocyte-derived dendritic cells (Mo-DCs) in terms of inducing CD4$^+$ T-cell responses or presenting peptides to CD8$^+$ T-cells (10, 13). Moreover, human peripheral blood γδ T-cells can phagocytose synthetic beads (1 μm diameter) and *Escherichia coli via* CD16-mediated uptake. This process is functionally linked to antigen processing and presentation on MHC class II (14).

As mentioned above, γδ T-cells have both cytotoxic and antigen-presentation abilities. Recently, it was reported that γδ T-cells were expanded in *in vitro* systems using zoledronic acid or pyrophosphate (15–17). Therefore, γδ T-cells may be strong candidates for use in immunotherapy. Indeed, a pilot study looking at adoptive immunotherapy using γδ T-cells (activated and expanded *in vitro* with 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP)) as effectors against advanced renal cell carcinoma was recently undertaken (18); however, the antigen-presenting ability of γδ T-cells isolated from patients with advanced cancer remains unclear. In this study, we

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Table I. *Profiles of patients included in this study.*

Patient	Age, years (gender)	Origin	Stage	Metastatic site
1	86 (M)	Prostate	IV	Bone, lung
2	65 (F)	Breast	IV	Bone, liver
3	51 (F)	Breast	IV	Pleura
4	52 (F)	Breast	IV	Pleura, lymph node
5	75 (F)	Ovary	IV	Lymph node, skin
6	70 (F)	Breast	IV	Lymph node, lung
7	60 (M)	Breast	IV	Liver
8	44 (M)	Pancreas	IV	Liver, lymph node
9	67 (M)	Pancreas	IV	Peritoneum
10	76 (F)	Stomach	IV	Liver
11	72 (F)	Esophagus	IV	Liver
12	63 (F)	Uterus	IV	Lymph node
13	78 (M)	Lung	IV	Bone
14	73 (F)	Uterus	III	Lymph node
15	54 (F)	Ovary	IV	Peritoneum
16	74 (F)	Colon	IV	Liver
17	47 (F)	Colon	IV	Lung, pleura
18	72 (M)	Stomach	IV	Liver, peritoneum
19	56 (M)	Pancreas	IV	Liver
20	48 (F)	Stomach	IV	Peritoneum
21	82 (M)	Pancreas	IV	Lymph node
22	67 (M)	Colon	IV	Lymph node

Figure 1. *The proportion of $\gamma\delta$ T-cells in PBMCs from healthy volunteers (n =10) and advanced cancer patients (n=22) was analyzed by FACS. Data represent the mean±SD.*

isolated $\gamma\delta$ T-cells from patients with advanced cancer and examined their antigen-presenting capacity. The results suggest that $\gamma\delta$ T cells isolated from cancer patients can be used for immunotherapy.

Patients and Methods

Patients. Twenty-two inoperable patients with multiple metastases of cancer (1 patient with stage III and 21 patients with stage IV) in the Fukuoka Cancer General Clinic (Fukuoka, Japan) were enrolled in the study. The patient profiles are summarized in Table I. Staging was performed in accordance with the criteria drawn up by the American Joint Committee on Cancer. Ten healthy volunteers, whose gender and age were matched with those of the patient group, were enrolled as controls. Informed consent was obtained from all individuals.

Generation of $\gamma\delta$ T cells. Human peripheral blood mononuclear cells (PBMCs) were maintained in RPMI-1640 basal medium (Sanko Pure Chemicals, Tokyo, Japan) supplemented with 1% human albumin, 100 µg/ml penicillin (Meijiseika, Tokyo, Japan) and 100 µg/ml streptomycin (Meijiseika) (hereafter referred to as RPMI medium). $\gamma\delta$ T-Cells were generated from the non-adherent fraction of PBMCs. The non-adherent fraction of PBMCs was cultured for 14 days in human AB serum in the presence of 1 µg/ml of zoledronic acid (Novartis, Basel, Switzerland) or 2M3B1PP (final concentration, 200 µM/ml) and 100 U/ml IL-2 (Nipro, Tokyo, Japan). 2M3B1PP was kindly provided by Dr. Y. Tanaka (Kyoto University, Kyoto, Japan). Zoledronic acid or 2M3B1PP were added to the culture medium on day 1, and IL-2 was added on days 2, 4 and 7 (as the cells expanded).
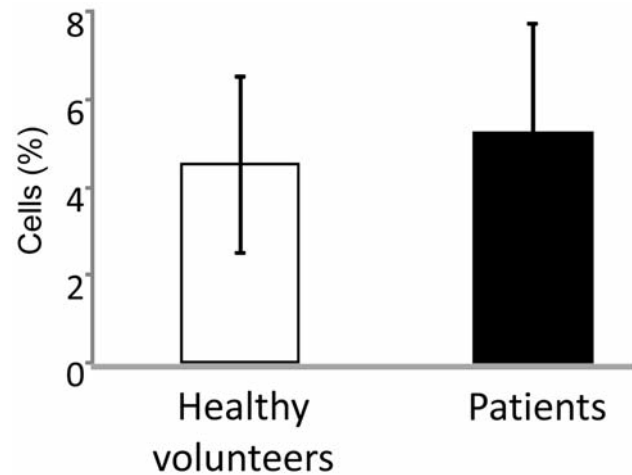
Generation of Mo-DCs. PBMCs were allowed to adhere to 6-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). After an overnight incubation at 37˚C, non-adherent cells were removed and the adherent cells were harvested and cultured in RPMI medium. Granulocyte macrophage colony-stimulating factor (GM-CSF) (500 ng/ml, Novartis Pharma Co., Switzerland) and IL-4 (500 U/ml, Ono, Tokyo, Japan) were added on day 1. On day 7, the non-adherent fraction (immature Mo-DCs) was collected and examined immunobiologically.

FACS analysis. To analyze the expression of $\gamma\delta$ TCRs and antigen-presenting molecules, such as CD80, CD86 and HLA-DR, on $\gamma\delta$ T-cells, the cells were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-CD80 or anti-pan TCR $\gamma\delta$, or phycoerythrin (PE)-conjugated anti-HLA DR or anti-CD86 (BD Pharmingen, San Diego, CA, USA). In some experiments, Kato III gastric cancer cells were stained with a PE-conjugated anti-CEA monoclonal antibody (mAb) (Affinity Bioreagents, Rockford, IL, USA). Mouse IgG1 was used as an isotype control (BD Pharmingen). For staining, cells were washed twice with PBS and incubated in PBS containing 3% BSA (Sigma, St. Louis, MO, USA) and 0.1% NaN3 (FACS buffer; Sigma) and the appropriate concentration of labeled mAb for 1 h at 4˚C. After washing with FACS buffer, the fluorescence intensity of the gated lymphocyte populations was measured using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed with CELLQuest software (BD Biosciences).

Generation of cytotoxic T cells (CTLs). $\gamma\delta$ T-Cells, pulsed with 30 µg/ml of carcinoembryonic antigen (CEA) peptide (CAP-1; Operon Biotechnologies, Tokyo, Japan) on days 1 and 7 were co-cultured with naïve autologous CD8+ T-cells purified using FlowComp® human CD8 Dynabeads (Invitrogen, Tokyo, Japan) for 14 days. After 14 days of co-culture, the CD8+ T-cells were selectively harvested using FlowComp® human CD8 Dynabeads and used as cytotoxic T-cells (CTLs).
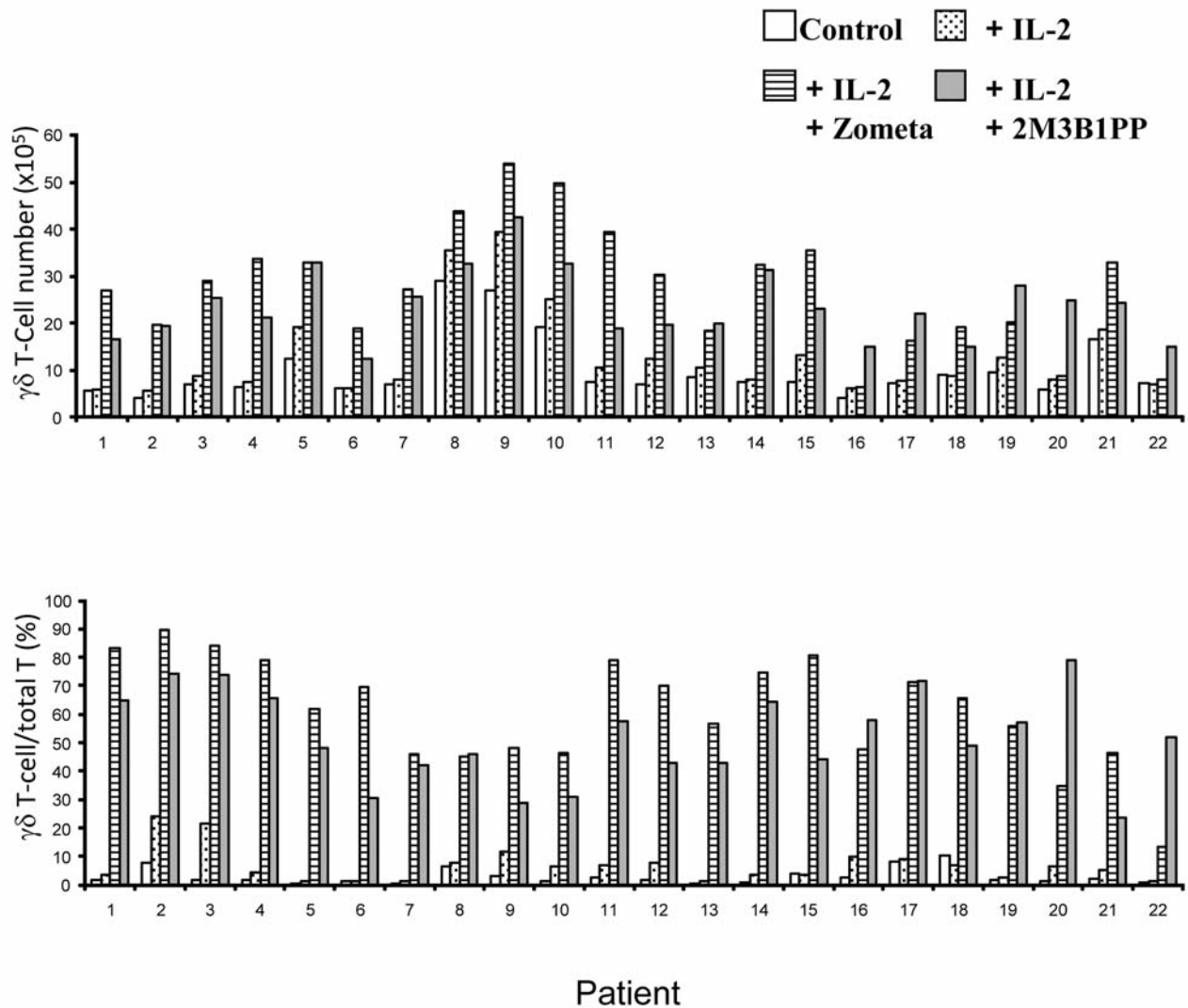
Figure 2. *PBMCs from 22 advanced cancer patients were co-cultured with IL-2 plus zoledronic acid (zometa) or 2-methyl-3butenyl-1-pyrophosphate (2M3B1PP), or IL-2 alone, for 14 days. The number of γδ T-cells (upper panel) and the ratio of γδ T-cell to total T-cells (lower panel) were estimated by FACS.*

[51]Cr release assay. Target cells (Kato III cells) were labeled with 100 μCi of [51]Cr (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at 37˚C. The labeled cells were washed twice in RPMI medium, resuspended in RPMI medium, and viable cell counts performed. Cells were co-incubated at effector to target (E:T) ratios of 40, 20, and 10 for the CTL assays. Co-cultures were set up in triplicate in 96-well flat-bottom plates (Nalge Nunk International, Chiba, Japan) and incubated for 4 h at 37˚C. Controls included targets incubated in medium alone for spontaneous release, and targets incubated with 5% (v/v) Triton X-100 (Sigma) in PBS for maximal release. Radioactivity was measured using a Wallac Wizard 1470 Automatic Gamma Counter (PerkinElmer, Tokyo, Japan). The percentage cytotoxic activity was calculated using the following formula: % specific lysis=(sample cpm–spontaneous cpm)/(maximal cpm–spontaneous cpm) ×100%.

ELISA. The supernatant from each well of the ^{51}Cr release assay was collected and the concentration of IFN-γ measured using an ELISA kit according to the manufacturer's instructions (Biosource, Carlsbad, CA, USA). The detection limit of the assay was 15.6 pg/ml.

Statistical analysis. An unpaired two-tailed Student's *t*-test was used for statistical analysis. A *p*-value of <0.05 was considered significant.

Results

There was no significant difference in the percentage of γδ T-cells in PBMCs from cancer patients compared to those from healthy volunteers. Firstly, we looked at whether the ratio of γδ T-cells within the PBMC population isolated from patients
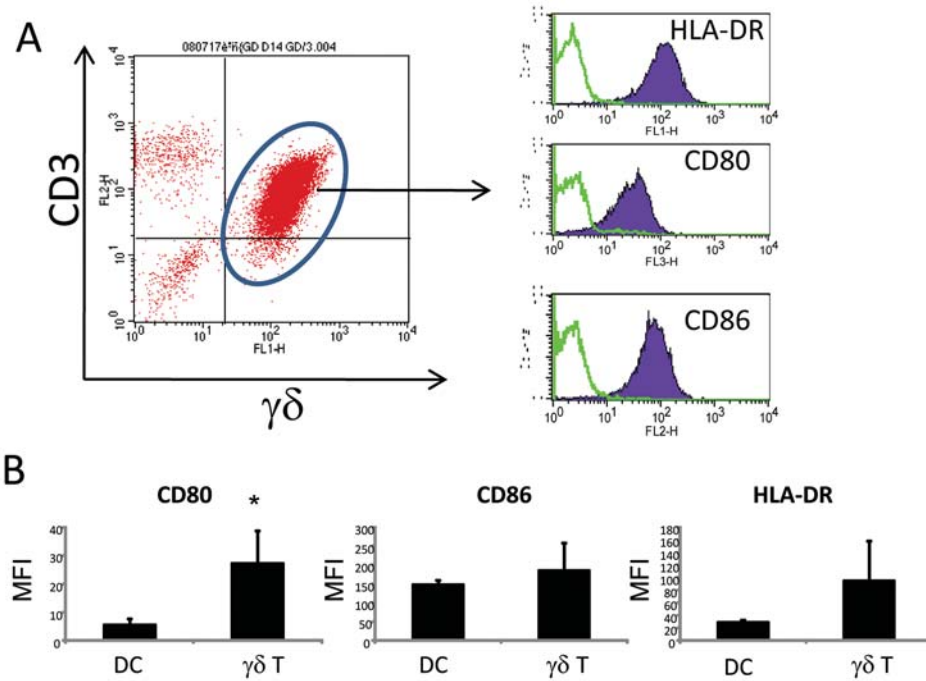
Figure 3. *A: After PBMCs were co-cultured with IL-2 plus zoledronic acid for 14 days, the expression of HLA-DR, CD80 and CD86 (filled histogram) was analyzed by FACS. The open histogram shows isotype control. B: Monocyte-derived dendritic cells (Mo-DCs) were generated using 500 U/ml of IL-4 and 500 ng/ml of GM-CSF and the expression levels of HLA-DR, CD80 and CD86 on immature Mo-DCs were compared with those on γδ T-cells. Data represent the mean±SD. *p<0.05.*
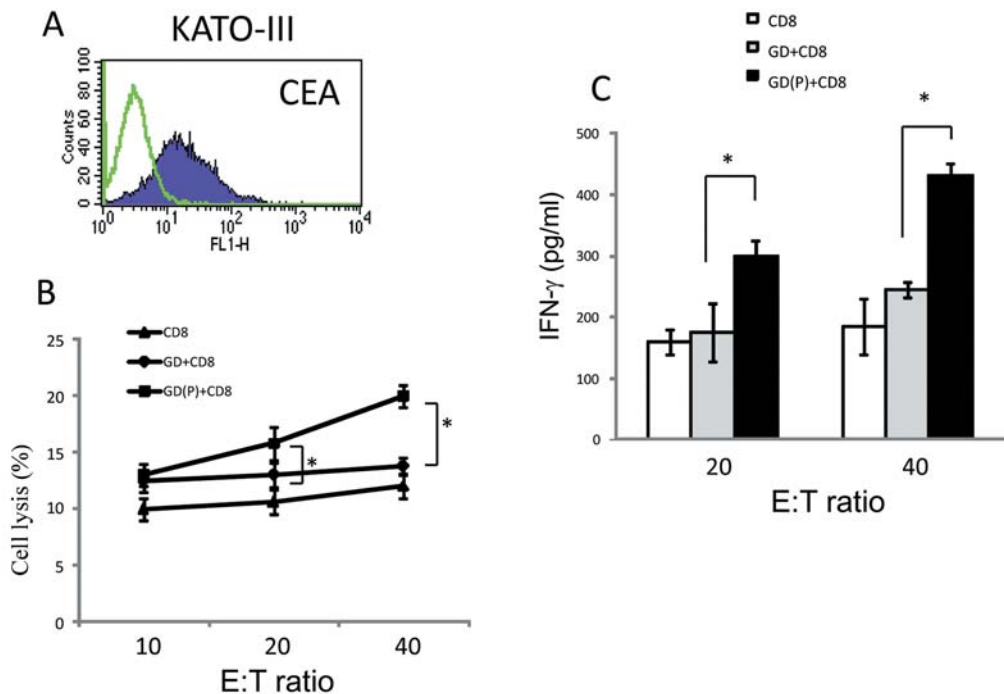

Figure 4. *A: CEA expression by KATO III cells was estimated by FACS. B and C: γδ T-Cells were expanded from PBMCs isolated from a patient with HLA-A24 using zoledronic acid and100 U/ml of IL-2. The γδ T-cells were then cultured with or without CEA peptide for 5 days. Autologous CD8+ T-cells were then co-cultured with the CEA peptide-pulsed γδ T-cells (GD(P)), or non-pulsed γδ T-cells (GD). After 14 days of culture, the CD8+ T-cells were purified using CD8 Dynabeads. The purified CD8+ T-cells were co-cultured with KATO III cells labeled with 100 μCi of ^{51}Cr at the indicated effector-target ratios for 16 h and then the radioactivity (B) and IFN-γ secretion (C) in the supernatants were measured using an automatic gamma counter or by ELISA, respectively. Data represent the mean±SD. *p<0.05.*

with advanced cancer was different from that in healthy volunteers. The ratios of γδ T-cells within the PBMCs from patients with advanced cancer and those from healthy volunteers were 5.3±2.5% and 4.6±1.8%, respectively, with there being no significant difference in the percentage (Figure 1). These results suggest that PBMCs from patients with advanced cancer are a good source of γδ T-cells.

γδ T-Cells from cancer patients can be expanded in vitro. Next, to examine whether γδ T-cells from patients with advanced cancer could be used for immunotherapy, we attempted to expand them *in vitro*. Recently, it was reported that γδ T-cells were expanded *in vitro* using zoledronic acid or pyrophosphate (15-17). Therefore, we used IL-2 plus zoledronic acid or 2M3B1PP to expand the cells. We found that γδ T-cells could be expanded from the PBMCs derived from all 22 patients with advanced cancer, using zoledronic acid, and 2M3B1PP (Figure 2). This suggests that γδ T-cells from cancer patients can be expanded to provide sufficient quantities for use in immunotherapy.

γδ T-Cells expressed levels of the molecules required for antigen-presentation equivalent to those on Mo-DCs. First, we estimated the expression molecules related to antigen-presentation, including HLA-DR (MHC class II) and the co-stimulatory molecules CD80 and CD86, on expanded-γδ T-cells. The results showed that HLA-DR, CD80 and CD86 were highly expressed on γδ T-cells (Figure 3A). Next, to evaluate whether γδ T-cells from cancer patients could be used instead of 'professional' antigen-presenting cells, Mo-DCs, we compared the expression of HLA-DR, CD80 and CD86 on γδ T-cells and Mo-DCs from the same patients. Surprisingly, the expression of HLA-DR, CD80 and CD86 on γδ T-cells was equivalent to that on Mo-DCs (Figure 3B). These results suggest that γδ T-cells from patients with advanced cancer have almost the same antigen-presenting capacity as Mo-DCs, *i.e.* sufficient for immunotherapy.

CTLs can be generated using expanded γδ T-cells from cancer patients. Finally, we generated CTLs using CEA peptide-pulsed γδ T-cells, as described in the Patients and Methods section. Kato III cells were used as target cells because they express CEA (Figure 4A). CD8^{+} T-cells stimulated by CEA peptide-pulsed γδ T-cells lysed target cancer cells to a greater extent than did CD8^{+} T-cells stimulated by non peptide-pulsed γδ T-cells (Figure 4B). We next measured the concentration of IFN-γ in the supernatants from the ^{51}Cr release assay as an index of T-cell activation. Consistent with the results of the ^{51}Cr release assay, CD8^{+} T-cells stimulated by CEA peptide-pulsed γδ T-cells secreted significantly higher levels of IFN-γ than did CD8^{+} T-cells stimulated by non-peptide-pulsed γδ T-cells (Figure 4C). These results suggest that γδ T-cells from patients with advanced cancer have the capacity to induce CTLs.

Discussion

Because *in vitro* γδ T-cells have cytotoxic, antigen-presenting and proliferative capabilities, they are thought to be ideal candidates for immunotherapy. However, the antigen-presenting ability of γδ T-cells in cancer patients remains unclear. In this study, we examined the antigen-presenting capacity of γδ T-cells isolated from patients with various types of advanced malignancy.

Although cancer patients are immunocompromised, the results of our study showed that the percentage of γδ T-cells within PBMCs isolated from cancer patients was similar to that in healthy volunteers (Figure 1). This is in contrast to a report suggesting that the percentage of γδ T-cells in patients with advanced renal cell carcinoma was significantly lower than that in healthy individuals (18). We also showed that γδ T-cells were successfully expanded from PBMCs derived from 22 patients with advanced cancer using either zoledronic acid or 2M3B1PP (Figure 2). These results suggest that sufficient quantities of γδ T-cells can be obtained from PBMC of cancer patient for use in immunotherapy.

Many studies report that the antigen-presenting capacity (expression of molecules related to antigen-presentation and the ability to stimulate naïve T-cells) of GM-CSF/IL-4-expanded cells, such as Mo-DCs, is very high (19-22). Therefore, Mo-DCs are often used as a source of DCs in clinical trials (23). However, we have shown that Mo-DCs from cancer patients are dysfunctional, short-lived, and difficult to generate (24). In the present study, we showed that the expression of molecules related to antigen-presentation, such as HLA-DR, CD80 and CD86, by γδ T-cells isolated from cancer patients, is equivalent to that of Mo-DCs (Figure 3B). These findings suggest that γδ T-cells from cancer patients could be used instead of Mo-DCs in immunotherapy.

Recently, antigen-presenting function in γδ T-cells was reported (4, 10). However, most data are derived from healthy volunteers, not patients with advanced cancer. In the present study, we also showed that it was possible to generate antigen-specific CTLs using γδ T-cells from cancer patients (Figure 4). Collectively, our data suggest that γδ T-cells from cancer patients are strong candidates for use in immunotherapy because of their ability to present antigens and proliferate *in vitro*. Further analysis of the mechanisms involved in γδ T-cell recognition of antigens and antigen presentation to MHC class I or MHC class II may provide new strategies for developing cancer immunotherapy.

Acknowledgements

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