The chromosomal inversion does not always occur in the same location, and multiple EML4-ALK variants have been identified (19). At least 11 variants have been reported. The most common variants are E13;A20 (variant 1) and E6a/b;A20 (variant 3a/b), which have been detected in 33% and 29% of NSCLC patients, respectively (14).

PF-02341066 (crizotinib) is an ALK inhibitor currently under clinical development. Kwak *et al* conducted an open-label, multi-center, two-part phase I trial and found a remarkable 57% overall response rate and a 72% 6-month progression-free survival rate (20).

In spite of the marked antitumor activity of crizotinib, ALK-positive cancers invariably gain resistance to crizotinib. In the case of ALK-positive cancers, as well as EGFR-mutant lung cancer, resistance develops on average within the first 2 years of therapy (21). The main resistance mutations are L1196M, a gatekeeper mutation and C1156M. In addition to ALK mutations, other known mechanisms for acquired resistance include ALK amplification (21,22) and EGFR activation (23,24). To overcome resistance, new ALK inhibitors are currently in early phase studies (25). Novel combinatorial strategies to overcome crizotinib resistance and further improve the clinical outcome are needed.

We focused on this new fusion array as a novel target of immunotherapy. There are several methods to detect EML4-ALK NSCLC, including polymerase chain reaction (PCR), immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) (19). These methods detect high-level EML4-ALK fusion gene expression. Passoni *et al* identified two HLA-A*02:01-restricted ALK-derived peptides that induce peptide-specific CTL lines (26).

We focused on the EML4 array as a novel epitope of immunotherapy. We identified a candidate 9- or 10-amino acid array of novel epitopes using the Bioinformatics and Molecular Analysis Section (BIMAS) software and analyzed its potential as a new immunotherapy epitope, with respect to its ability to induce anticancer activity. We then induced and generated a peptide-specific CTL clone from peripheral blood lymphocytes of HLA-A*02:01-positive healthy donors. We report here that an EML4-ALK-derived peptide-specific human CTL clone recognized peptide-pulsed T2 cells and HLA-A*02:01-positive and EML4-ALK-positive tumor cells pretreated with IFN-γ. Furthermore, we showed that immunotherapy with this novel epitope peptide has potential for treatment of EML4-ALK-positive NSCLC.

Materials and methods

Peptides. Human EML4-ALK-derived peptides carrying binding motifs for HLA-A*02:01-/HLA-A*24:02-encoded molecules were identified by HLA-peptide binding predictions using the BIMAS program (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html). We purchased a total of seven EML4-ALK-derived peptides carrying HLA-A*02:01 binding motifs and two peptides carrying HLA-A*24:02 binding motifs from Geneworld (Tokyo, Japan).

Cell lines. The H2228 human lung adenocarcinoma cell line and EML4-ALK fusion protein variant 3 (E6;A20) were kindly provided by Professor S. Yano (Kanazawa University).

T2 is a lymphoblastoid cell line that lacks TAP function and has HLA-A*02:01 molecules that can easily be loaded with exogenous peptides. T2A24 is the same cell line but with HLA-A*24:02 instead. T2 and T2A24 cells were cultured in RPMI medium supplemented with 10% heat-inactivated FBS.

*HLA-A*02:01/HLA-A*24:02 binding assay*. In order to determine the binding ability of the predicted peptides to HLA-A*02:01/HLA-A*24:02 molecules, an *in vitro* cellular binding assay was performed as reported previously (27).

Briefly, after incubation of the T2/T2A24 cells in culture medium at 26˚C for 18 h, cells were washed with PBS and suspended in 1 ml Opti-MEM (Invitrogen, Carlsbad, CA, USA) with or without 100 μg peptide and then incubated at 26˚C for 3 h and at 37˚C for 3 h. After washing with PBS, HLA-A*02:01/HLA-A*24:02 expression was measured by flow cytometry using a FITC-conjugated and HLA-A*02:01-/HLA-A*24:02-specific monoclonal antibody (mAb) and the mean fluorescence intensity was recorded.

Generation of dendritic cells. $CD14^+$ cells were isolated from human peripheral blood mononuclear cells (PBMCs) using human CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Immature dendritic cells (DCs) were generated from $CD14^+$ cells using interleukin (IL)-4 (10 ng/ml; PeproTech Inc., Rocky Hill, NJ, USA) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml; PeproTech) in RPMI-1640 medium supplemented with 10% FBS. Maturation of DCs was induced by prostaglandin E2 (PGE2; 1 μg/ml; Sigma, St. Louis, MO, USA) and tumor necrosis factor (TNF-)-α (10 ng/ml; PeproTech).

Induction of EML4-ALK-derived peptide-specific CTLs from PBMCs. $CD8^+$ cells were isolated from PBMCs using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ cells ($2x10^6$) were stimulated by peptide-pulsed irradiated autologous mature DCs ($1x10^5$). Autologous DCs were prepared from a limited supply; artificial antigen presenting cells (aAPCs) (K562/A2 or A24/CD80/CD83) were alternatively used for further examination. After 1 week, these cells were stimulated twice per week by peptide-pulsed irradiated artificial APC-A2 or artificial APC-A24 cells ($1x10^5$). Supplementation with 10 IU/ml IL-2 (Proleukin; Novartis Pharmaceuticals, Basel, Switzerland) and 10 ng/ml IL-15 (PeproTech) was performed every 3 to 4 days between stimulations (28).

IFN-γ ELISPOT assay. Specific secretion of IFN-γ from human CTLs in response to stimulator cells was assayed using the IFN-γ ELISPOT kit (BD Biosciences), according to the manufacturer's instructions. Stimulator cells were pulsed with peptide for 2 h at room temperature and then washed. Responder cells were incubated with stimulator cells for 20 h. The resulting spots were counted using an ELIPHOTO counter (Minerva Tech, Tokyo, Japan). HIV-gag (77-85) (SLYNTYATL) was used as an irrelevant peptide in the CTL assay.

Generation of CTL clones. Cultured cells were incubated with peptide-pulsed T2/T2A24 cells at a ratio of 2:1 for 3.5 h

Table I. HLA-A2 peptide binding predictions of the BIMAS program.

Peptide name	Peptide sequence	Binding score[a]
A	RLSALESRV	69.552
B	AISEDHVASV	90.183
C	TVLKAALADV	51.79
D	KLIPKVTKT	59.989
E	YLLPTGEIV	237.82
F	MLIWSKTTV	118.238
G	VMLIWSKTTV	315.95

[a]Binding scores were estimated using BIMAS software (http://www-bimas.cit.nih.gov/mobio/hla_bind/).

Table II. HLA-A24 peptide binding predictions of the BIMAS program.

Peptide name	Peptide sequence	Binding score[a]
H	NYDDIRTEL	369.6
I	VYFIASVVVL	200

[a]Binding scores were estimated using BIMAS software (http://www-bimas.cit.nih.gov/mobio/hla_bind/).

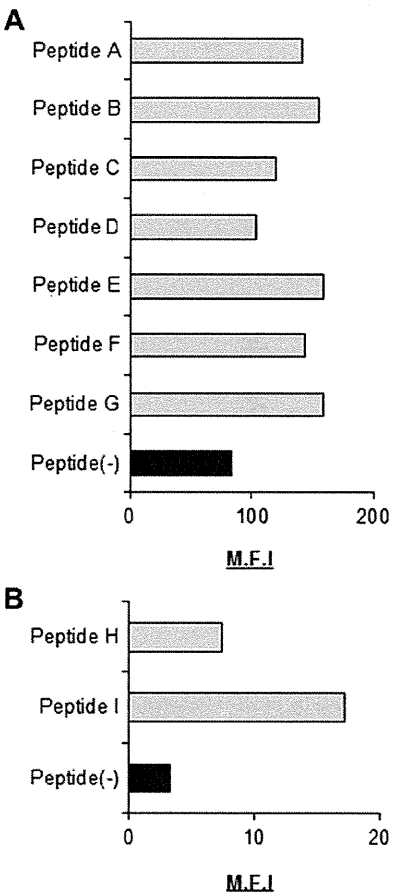

Figure 1. EML4-ALK-derived peptides bound to HLA-A2 or HLA-A24 molecules. *In vitro* cellular peptide binding assays for HLA-A*02:01 (A) or HLA-A*24:02 (B) were performed using a FACS system.

at 37°C. CD107a-specific antibodies (BioLegend, San Diego, CA, USA) were included in the mixture during the incubation period. $CD8^+CD107a^+$ cells were sorted using a FACSAria II cell sorter (BD Biosciences). Sorted CTLs were stimulated and the CTL clones were established as described previously (29).

Flow cytometry. H2228 cells with or without pretreatment with 100 U/ml IFN-γ (PeproTech) for 48 h were harvested and stained with anti-HLA-A2 Ab-FITC (MBL, Japan) and analyzed using a FACSCanto II flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software.

Cytotoxicity assay. The cytotoxic capacity was analyzed using the Terascan VPC system (Minerva Tech, Tokyo). The CTL clone was used as the effector cell type. Target cells treated with 100 U/ml IFN-γ (PeproTech) 42 h previously were labeled through incubation in calcein-AM solution for 30 min at 37°C. The labeled cells ($1x10^4$) were then co-cultured with the effector cells for 4-6 h. Fluorescence intensity was measured before and after the culture period, and specific cytotoxic activity was calculated as described previously (29).

HLA-A*02:01 blocking of T-cell activity was tested by pre-incubating the target cells with anti-HLA-A, -B, -C mAb (W6/32) or an isotype control mAb (mIgG2a,κ; BioLegend San Diego, CA, USA).

Results

*Identification of HLA-A*02:01-/HLA-A*24:02-restricted EML4-ALK-derived peptides.* As candidate EML4-ALK-derived and HLA-A*02:01-/HLA-A*24:02-restricted CTL epitopes, we selected nine peptides with highly predicted scores for HLA-A*02:01/HLA-A*24:02 binding calculated using BIMAS software (Tables I and II) and evaluated their ability to bind to HLA-A*02:01/HLA-A*24:02 molecules. All nine peptides were able to bind HLA-A*02:01/HLA-A*24:02 molecules (Fig. 1).

Generation of an EML4-ALK-derived peptide-specific CTL clone from human PBMCs. We next assessed the capacity of EML4-ALK-derived peptides to generate peptide-specific CTLs *in vitro* from human PBMCs of HLA-A*02:01/HLA-A*24:02 healthy donors. CTLs were induced by three stimulations with DCs or artificial APCs loaded with the EML4-ALK-derived peptides. CTLs were tested for specificity for each peptide using the IFN-γ ELISPOT assay. Peptides A, B and C could induce peptide-specific CTLs that were able to specifically recognize T2 cells pulsed with each peptide, but not T2 cells without peptides (Fig. 2). Peptides B and C were able to induce CTLs from only one donor (healthy donor 3 for peptide B and healthy donor 4 for

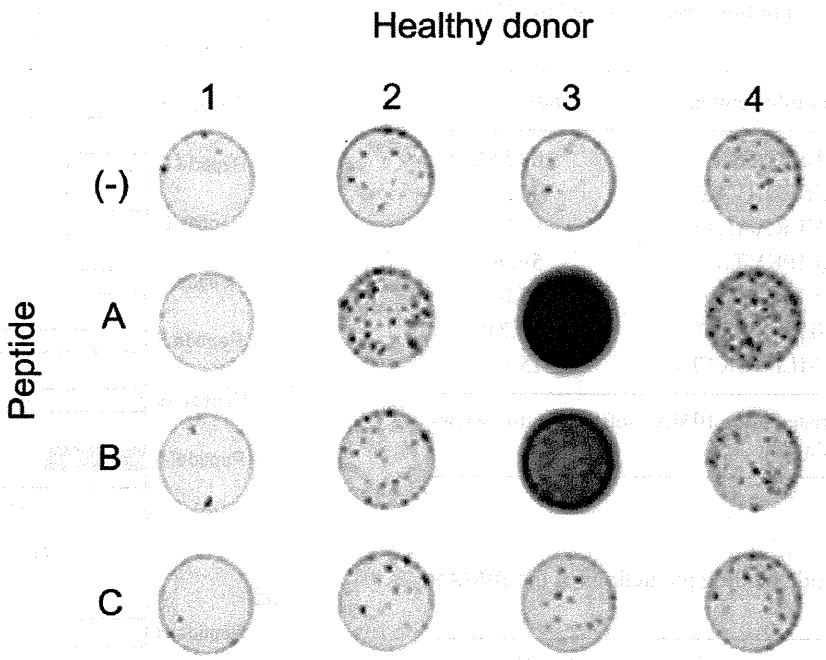

Figure 2. IFN-γ release by *in vitro*-induced anti-EML4-ALK CTLs. $CD8^+$ T cells from four healthy donors were stimulated with EML4-ALK-derived peptide-pulsed autologous DCs and aAPCs. CTLs induced by EML4-ALK-derived peptides ($1x10^5$) were stimulated with T2 cells pulsed with or without 1 μM EML4-ALK-derived peptides. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT assay. DCs, dendritic cells; aAPCs, artificial antigen presenting cells; CTLs, cytotoxic T cells.

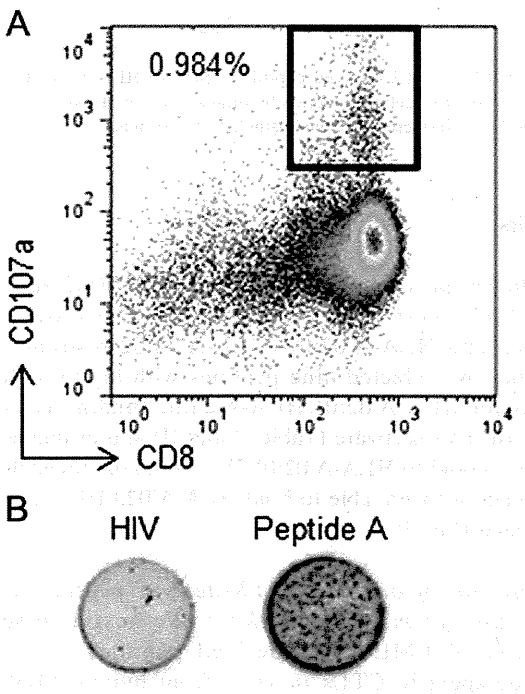

Figure 3. Peptide A-specific CTL clone established from anti-EML4-ALK CTL. (A) Peptide A-specific CTL clones established using CD107a single cell sorting. Peptide A-specific CTLs ($1x10^5$) were incubated with peptide-pulsed T2 cells ($5x10^4$) with CD107a-specific antibodies for 3.5 h at 37°C. $CD8^+CD107a^+$ cells were sorted using a FACSAria II cell sorter. Square, $CD8^+CD107a^+$ cells that are peptide A-specific CTL clones. (B) Recognition of peptide-pulsed T2 cells by peptide A-specific CTL clones. A peptide A-specific CTL clone ($1x10^4$ cells) was incubated with stimulator cells that had been pulsed with 1 μM peptide A or HIV-gag peptide. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT assay. CTLs, cytotoxic T cells.

peptide C), but peptide A was able to induce CTLs in three of four donors (healthy donors 2, 3 and 4). Based on this result, we used peptide A for further examinations.

Next, we obtained one CTL clone from peptide A-specific CTLs that was able to specifically recognize T2 cells pulsed with peptide A, but not T2 cells pulsed with an irrelevant HIV-gag peptide, using single cell sorting with a CD107a antibody. The population of $CD8^+CD107a^+$ cells represented 0.984% of all stimulated cells (Fig. 3A). These cells were sorted as single cells in each well of a 96-well plate. Twenty-one days after cell sorting, peptide specificity was assessed using the IFN-γ ELISPOT assay (Fig. 3B). The established clone reacted to the T2 cells pulsed with peptide A, but not to T2 cells pulsed with the irrelevant HIV-gag peptide. These results indicate that a peptide A-specific CTL clone was successfully established from PBMCs from a healthy donor.

*The EML4-ALK-specific CTL clone recognizes HLA-A*02:01$^+$ lung carcinoma cells with the EML4-ALK variant 3a/b incubated with IFN-γ.* We next evaluated the ability of the EML4-ALK-specific CTL clone to recognize the cancer cell line H2228, which expresses HLA-A*02:01 and EML4-ALK, using the IFN-γ ELISPOT assay. Even though the EML4-ALK-specific CTL clone failed to recognize H2228 cells, it did recognize those pretreated with 100 U/ml IFN-γ 48 h prior to examination (Fig. 4A). We examined the effect of IFN-γ on H2228 cells. Incubating target cells with IFN-γ for 48 h increased the expression of MHC class I molecules on the cell surface (Fig. 4B). This result indicates that the peptide A-specific CTL clone was able to recognize H2228 cells because of increased expression of MHC-class I on the H2228 cell surface.

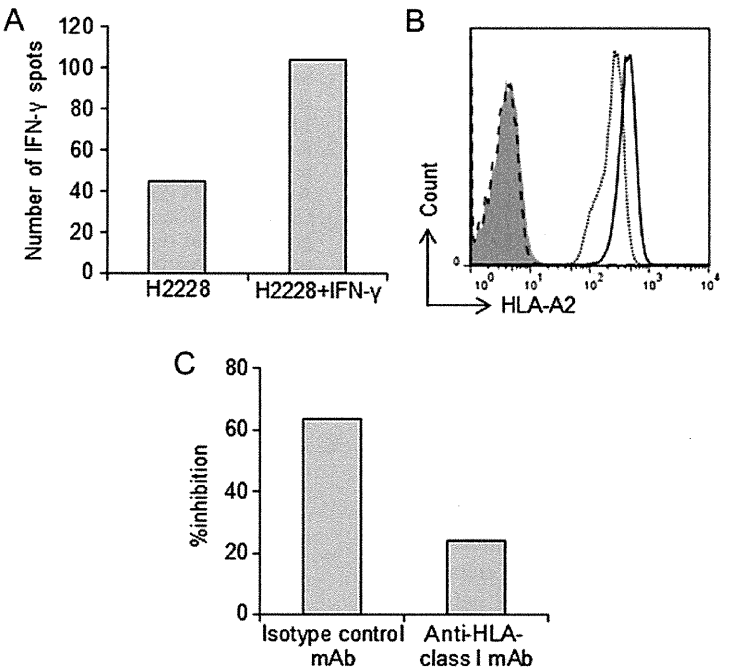

Figure 4. Recognition of lung carcinoma cells expressing HLA-A*02:01 and the EML4-ALK fusion gene by the peptide A-specific CTL clone. The peptide A-specific CTL clone recognized H2228 cells pretreated with IFN-γ 48 h prior to the assay. (A) The peptide A-specific CTL clone ($1x10^4$ cells) was incubated with H2228 cells with or without IFN-γ. IFN-γ production was detected by IFN-γ ELISPOT assay. (B) IFN-γ increased expression of HLA-A2 presented on H2228 cells. Incubation of H2228 cells with 100 U/ml IFN-γ for 48 h increased HLA-A2 presentation on the cells. Dotted line, HLA-A2 on H2228 cells without IFN-γ. Black line, HLA-A2 on H2228 cells incubated with IFN-γ (higher than on H2228 cells without IFN-γ). Dashed line and shaded region: no staining of H2228 cells with/without IFN-γ. (C) Inhibition of IFN-γ production by an anti-HLA-class I mAb. Blocking experiments were performed using an HLA-A, -B, -C-specific mAb (W6/32) or an isotype control mAb (mIgG2a,κ). The peptide A-specific CTL clone was incubated with H2228 cells (HLA-A*02:01+/EML4-ALK+) pretreated with IFN-γ 48 h prior to examination. IFN-γ-producing CTL clones were detected by IFN-γ ELISPOT assay. The bar graph shows the percentage of inhibition. CTL, cytotoxic T cell.

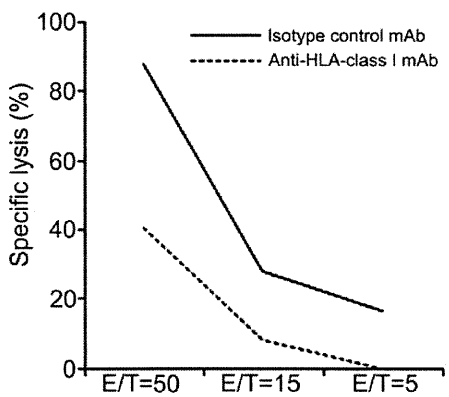

Figure 5. Cytotoxic activity of the peptide A-specific CTL clone against H2228 cells. The peptide A-specific CTL clone was incubated with H2228 cells pretreated with IFN-γ 48 h prior to the assay at various E/T ratios, and specific lysis was assessed. Blocking experiments were performed using the HLA-A, -B, -C-specific mAb (W6/32) or the isotype control mAb (mIgG2a,κ). CTL, cytotoxic T cell.

Specific IFN-γ production by the peptide A-specific CTL clone was detectable in H2228 cells treated with IFN-γ. The specificity was abolished by an anti-HLA-class I mAb, but not by an isotype control, suggesting that the observed production was HLA-A2 restricted (Fig. 4C).

A cytotoxicity assay was also performed. The peptide A-specific CTL clone was able to specifically lyse H2228 cells pretreated with IFN-γ 48 h prior to examination. This specific lysis was blocked by the anti-HLA-class I mAb, but not by the isotype control. These results indicate that the peptide A-specific CTL clone showed cytotoxicity and the ability to produce IFN-γ against HLA-A*02:01+ EML4-ALK+ NSCLC cell lines (Fig. 5).

Discussion

In the present study, we identified a new tumor-associated CTL epitope (peptide A) derived from EML4-ALK, which binds to HLA-A*02:01 molecules, and we were able to establish a peptide-specific CTL clone from human PBMCs that specifically recognized cognate peptide-pulsed T2 cells and HLA-A*02:01 tumor cells expressing EML4-ALK that had been pretreated with IFN-γ.

EML4-ALK-positive lung cancers are highly sensitive to ALK inhibition. However, as with trastuzumab or gefitinib (30,31), patients typically gain resistance within 1 to 2 years of starting therapy (23). We aimed to overcome these difficulties with immunotherapy.

We identified a glypican-3 (GPC3)-derived peptide and showed that GPC3-specific CTL frequency after vaccination correlated with OS. OS was significantly longer in patients with high GPC3-specific CTL frequencies than in those

with low frequencies (32). This indicates that the ability to induce a peptide-specific CTL clone is important for effective immunotherapy. We also revealed that GPC3 is an ideal target for anticancer immunotherapy since it is specifically overexpressed in hepatocellular carcinoma (HCC) (33-35).

In the present study, we chose a peptide array from EML4-ALK, from which we were able to induce a peptide-specific CTL clone. EML4-ALK is a strong oncogene overexpressed in cancer cells of NSCLC, breast cancer, kidney cancer and colon cancer (17). We performed RT-PCR and assayed the EML4 DNA levels of certain lung cancer cell lines. H2228 cells express EML4 moderately but at higher levels than other lung cancer cell lines. EML4 expression has been reported as highly expressed in $CD8^+$ T cells. RT-PCR showed that EML4 DNA levels were high in PBMCs and $CD8^+$ T cells. Because of a lack of suitable antibodies, we could not perform western blotting. However, our success at inducing a peptide A-specific CTL clone from $CD8^+$ T cells indicated that the CTL clone had no cytotoxicity against $CD8^+$ T cells.

This CTL clone could not recognize cancer cell lines without the ability to increase the amount of HLA class I presented on cell surfaces. Further examination is needed to achieve higher tumor reactivity. Combination chemotherapy or radiation therapy plus immunotherapy was recently reported to have a synergistic effect (36). Moreover, some mechanisms of synergy between radiation therapy, chemotherapy and immunotherapy have been revealed (37). In one of the mechanisms, these therapies upregulated tumor antigens and MHC moieties. These results suggest that combination therapy could be used to make tumor cell lines more susceptible to this peptide A-specific CTL clone-mediated cytolysis (38-41).

In addition, this treatment may be able to overcome resistance to ALK inhibition. Some resistance mechanisms for targeting drugs have been examined. The most commonly identified causes of resistance are point mutations such as L1196M (42-44), G1269A (22) and S1206Y (21). These point mutations occur in the tyrosine kinase domain, which plays an important role in oncogenesis. Our peptide array was selected from EML4, which has no correlation with these point mutations. It is possible that this treatment is effective for tumor cells resistant to ALK inhibitors.

In this study, we identified a new epitope peptide derived from the EML4-ALK fusion gene. We successfully induced an HLA-A*02:01-restricted peptide-specific CTL clone that demonstrated cytotoxicity for EML4-ALK-positive tumor cells. This is a new epitope-based vaccine therapy design for EML4-ALK-positive cancer cells. In order to obtain a stronger effect, further analysis is needed.

Acknowledgements

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REVIEW

Critical analysis of the potential of targeting GPC3 in hepatocellular carcinoma

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Keigo Saito
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Abstract: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide. The treatment options for patients with advanced HCC are limited, and novel treatment strategies are required urgently. Glypican-3 (GPC3), a member of the glypican family of heparan sulfate proteoglycans, is overexpressed in 72%–81% of HCC cases, and is correlated with a poor prognosis. GPC3 regulates both stimulatory and inhibitory signals, and plays a key role in regulating cancer cell growth. GPC3 is released into the serum, and so might be a useful diagnostic marker for HCC. GPC3 is also used as an immunotherapeutic target in HCC. A Phase I study of a humanized anti-GPC3 monoclonal antibody, GC33, revealed a good safety profile and potential antitumor activity, and a Phase II trial is currently ongoing. In addition, the authors' investigator-initiated Phase I study of a GPC3-derived peptide vaccine showed good safety and tolerability, and demonstrated that the GPC3 peptide-specific cytotoxic T-lymphocyte frequency in peripheral blood correlated with overall survival in HCC patients. A sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway. GPC3 is currently recognized as a promising therapeutic target and diagnostic marker for HCC. This review introduces the recent progress in GPC3 research, from biology to clinical impact.

Keywords: GPC3, hepatocellular carcinoma, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide.[1] HCC patients are often diagnosed at an advanced stage, and so the prognosis is often poor. Currently, surgery or locally ablative treatments such as percutaneous ethanol injection or radiofrequency ablation are the standard treatments for early-stage HCC. However, these treatments are no longer available and options are limited for most patients with advanced HCC.[2] Generally, transarterial chemoembolization or systemic chemotherapy is used. However, these therapeutic approaches are not curative in most patients. Sorafenib, a multi-targeted tyrosine kinase inhibitor, is the only drug that has significantly prolonged the survival of patients with advanced HCC;[3,4] therefore, it has become the standard agent for first-line systemic treatment. However, the incidence of adverse effects is high, and there are no effective second-line treatments for patients who do not respond to sorafenib. Therefore, new treatment strategies for patients with advanced HCC should be established.

To date, several immunotherapeutic clinical trials in patients with advanced HCC have been performed. These studies have shown feasibility and safety, but no dramatic clinical responses.[5,6] Nevertheless, some randomized controlled trials have shown the

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potential to reduce the risk of cancer recurrence in adjuvant settings.[6] Therefore, an immunotherapeutic approach is potentially an attractive treatment option for HCC.

Various tumor antigens for HCC have been identified and investigated as immunotherapeutic targets.[7] GPC3 is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface via glycosylphosphatidylinositol (GPI) anchors.[8] Mutations in *GPC3* cause Simpson–Golabi–Behmel syndrome,[9] which is an X-linked disorder characterized by pre- and postnatal overgrowth with visceral and skeletal anomalies. *GPC3*-deficient mice exhibited similar characteristics as Simpson–Golabi–Behmel syndrome patients.[10] GPC3 is overexpressed in 72%–81% of patients with HCC.[11–15] Therefore, GPC3 has been recognized as a potential immunotherapeutic target or diagnostic marker for HCC. This paper reviews the biology of GPC3 and discusses recent advances in GPC3-targeted HCC immunotherapy.

Tumor-associated antigens (TAAs) in HCC

TAA-specific immunotherapy is an attractive strategy because it is associated with fewer adverse events. Therefore, identifying appropriate TAAs is important for the development of TAA-specific cancer immunotherapies. Boon et al initially reported that MAGE-A was a human TAA in a melanoma patient, and that the human immune system could recognize TAA expressing-cancer cells as foreign bodies and exclude them.[16] Subsequently, a novel approach termed serological analysis of recombinant complementary DNA expression libraries (SEREX) was developed to identify TAAs.[17,18] Complementary DNA microarray technology is also useful for identifying novel cancer-associated genes and for classifying human cancers at the molecular level.[19,20] In HCC, some TAAs, such as AFP, MAGE-A, NY-ESO-1, SSX2, and telomerase reverse transcriptase, have been identified.[7] Although GPC3 is overexpressed in HCC,[11–15] it is not expressed in most normal adult tissues. Furthermore, GPC3-expression was correlated with poor prognosis in patients with HCC: GPC3-positive HCC patients had a significantly lower 5-year survival rate than GPC3-negative individuals (54.5% versus 87.7%; P=0.031).[15] These results suggest that GPC3 might be a promising target for cancer immunotherapy.

Biological aspects of GPC3

General considerations

Glypicans are a family of heparan sulfate proteoglycans. To date, six glypicans have been identified (GPC1 to GPC6) in mammals, and two orthologs of the mammalian genes were identified in *Drosophila melanogaster* (Dally- and Dally-like).[8,21] Glypicans of all species are classified into two subfamilies according to their sequence homology.[21] In general, the function of glypicans is to regulate morphogenesis during embryonic development,[22] and mutations cause the overgrowth genetic disease Simpson–Golabi–Behmel syndrome.[23] Several recent studies have revealed that GPC3 is overexpressed in many cancers.

Structure and function of GPC3

GPC3 is a 580-amino acid protein (~60 kDa) that is encoded by nine exons on chromosome X (Xq26). Alternative splicing results in four variants that were isolated from the HepG2 cell line. Fourteen cysteine residues located in the core region are well conserved among glypicans, and contribute to the formation of a unique ternary structure via disulfide bonds. The amino-terminus contains a signal peptide sequence (residues 1–24), which is required for targeting to the cell surface. The carboxyl-terminus contains a hydrophobic region that is associated with the lipid bilayer of the Golgi apparatus. During the transport of GPC3 to the cell surface, the hydrophobic region is truncated by transamidase, and then covalently attached to a GPI anchor via the C-terminus of serine 560.[24] Therefore, the attachment of a GPI anchor is a key post-translational modification that regulates the cellular localization of GPC3.

GPC3 regulates both stimulatory and inhibitory signals through the binding of heparan sulfate chains to signaling molecules such as Wnt, Hedgehog, fibroblast growth factors, bone morphogenetic proteins.[25–31] The core protein also plays an important role for regulating the activity in Wnt and Hedgehog signaling.[27,28,32] Structural information regarding GPC3 is needed to understand these signaling mechanisms, but the three-dimensional structure of GPC3 is yet to be elucidated. Nevertheless, the crystal structure of *Drosophila* Dlp, an ortholog of the mammalian gene, is available.[33] Structural analysis of the Dlp core region revealed an elongated conformation with α-helix packing: this is a unique structure when compared with other proteins. Further structural studies of glypicans are necessary to understand their complex and multifunctional signaling pathways and their regulation of cancer cell growth.

GPC3 biology and disease

GPC3 is expressed in many embryonic tissues in addition to fetal liver and placenta.[34] The overexpression of GPC3 is observed in liver cancer, ovarian cancer, lung cancer, malig-

nant melanoma, and embryonal cancers such as neuroblastoma medulloblastoma and Wilms' tumor.[35–41] Capurro et al demonstrated that the binding of GPC3 to Wnt and Hedgehog activates signaling pathways that promote the growth of HCC cells.[27,28] Moreover, the knockdown of GPC3 using small interfering RNA and subsequent gene expression analysis revealed that suppressing GPC3 inhibited the transforming growth factor-β (TGF-β) receptor pathway and the subsequent growth of HCC cell lines.[42] These suggest that GPC3 is an important target for cancer therapy.[43,44]

It is noteworthy that GPC is a novel serological cancer marker.[12,45,46] Secreted circulating GPC3 is detected in the blood of cancer patients with HCC[11,45] and melanoma,[37,47]and the presence of soluble GPC3 correlates with cancer progression. However, because GPC3 is initially membrane-bound via a GPI anchor, it is currently unknown how GPC3 is secreted into the circulation. It was reported that GPC3 can be cleaved by Notum (α/β-hydrolase enzyme) and furin-like convertase,[48,49] releasing the N-terminal domain and full-length GPC3 from the cell surface.[50,51] Secreted GPC3 might be useful for cancer diagnosis.

GPC3 as a diagnostic marker for HCC

GPC3 expression in HCC at the messenger RNA or protein level

Several studies have suggested that GPC3 is a potential therapeutic target in liver cancer because it is overexpressed in HCC, but is not expressed or is expressed at only low levels in normal adult tissue.[52–54] Hsu et al performed pioneering work to identify GPC3 as a potential biomarker for HCC.[55] When GPC3 was compared with AFP, another established HCC marker, data revealed higher *GPC3* messenger RNA expression compared with serum α-fetoprotein (AFP), levels (71.7% versus 51.3%) based on the analysis of 113 patients with unicentric primary HCC. The authors also reported previously that *GPC3* is specifically overexpressed in HCC by analyzing complementary DNA microarrays containing 23,040 genes. The expression profiles of 20 HCC samples, corresponding noncancerous liver tissues, and various normal human tissues revealed that GPC3 was overexpressed specifically in HCC.[11]

Capurro et al confirmed increased GPC3 expression in HCC patients using a mouse monoclonal antibody (1G12) against a GPC3 C-terminal peptide.[12] Immunohistochemistry revealed that GPC3 was overexpressed in 72% of HCC samples. Therefore, GPC3 might also be useful as an ancillary tool during histopathological diagnostic processes to distinguish HCC from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules.[56]

GPC3 as a serum marker for HCC

Several studies have been performed to validate the diagnostic potential of GPC3 as a serum marker by developing methodologies such as enzyme-linked immunosorbent assays and radioimmunoassays.[45,57] Several antibody-based immunoassays have been developed to assess potential serum biomarkers. Using multiple serum markers, including AFP and protein induced by vitamin K absence or antagonists-II (PIVKA-II), might increase diagnostic accuracy. Although GPC3 is a cell-surface marker, it can be released into the serum by the lipase Notum, which cleaves the GPI anchor.[49] Specifically, Hippo et al reported that GPC3 is cleaved between Arg358 and Ser359, and that the N-terminal fragment of GPC3 is also released into circulation. They reported the usefulness of the N-terminal fragment of GPC3 for diagnosing early-stage HCC.[51] Therefore, GPC3 also exhibits diagnostic value as a serum marker.[57,58] Qiao et al compared the serum levels of three markers (GPC3, human cervical cancer oncogene [HCCR], and AFP) for diagnosing HCC in 189 patients (101 HCC, 40 cirrhosis, and 18 hepatitis cases and 30 healthy control donors). They reported that GPC3 was the most accurate diagnostic marker: using a cutoff of 26.8 ng/mL for the diagnosis of HCC, GPC3 had a sensitivity of 51.5% and a specificity of 92.8%. In addition, the simultaneous detection of three markers increased the sensitivity significantly to 80.2% higher than AFP alone.[58] In a meta-analysis comparing AFP and GPC3 as serum markers for HCC, the pooled sensitivities for AFP and GPC3 were 51.9% and 59.2%, and the pooled specificities were 94% and 84.8%, respectively.[59] This suggests that GPC3 and AFP are comparable serum markers. Serum GPC3 might be a useful tumor marker in patients with HCC. However, the biochemistry of serum GPC3 is yet to be elucidated, and so further studies are needed.

GPC3 as an immunotherapeutic target in HCC

Identification of human leukocyte antigen (HLA)-A2- or A24-restricted GPC3-derived epitope peptides

Identifying TAA-derived epitope peptides is the first step in the development of peptide vaccines. *HLA-A24* is the most common HLA class I allele in the Japanese population (60%).[60,61] Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H-2K^d are similar,[62,63] and the amino acid sequences of human and mouse GPC3 have 95% homology. These studies identified the mouse GPC3-derived

and K^d- restricted cytotoxic T-lymphocyte (CTL) epitope peptide $GPC3_{298-306}$ (EYILSLEEL) in BALB/c mice. This peptide-specific CTL showed specific cytotoxicity against GPC3-expressing or peptide-pulsed cancer cell lines, suggesting that GPC3 was highly immunogenic and could elicit effective antitumor immunity in mice. Importantly, there was no evidence of autoimmune reactions in the treated mice.[64] Because of the similarities in the peptide binding motifs between H-$2K^d$ and HLA-A24, this peptide was applicable for immunotherapy in HLA-A24-positive patients.

HLA-A2 is also expressed in 40% of Japanese individuals, as well as other ethnic populations.[60,65] An HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide was also identified using *HLA-A2.1* transgenic mice.[58] A binding assay was performed, and it was reported that the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide could bind to HLA-A*02:06 and HLA-A*02:07. This suggests that HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) might be effective in HLA-A*02:06 and HLA-A*02:07 patients.

These GPC3-derived peptide-specific CTLs could be induced from the peripheral blood mononuclear cells of HCC patients by in vitro stimulation with peptide. The adoptive transfer of these GPC3-derived peptide-specific CTLs reduced the mass of human HCC tumors implanted into non-obese diabetic/severe combined immunodeficiency mice.[66]

GPC3-targeted vaccine therapy

The authors recently completed an investigator-initiated Phase I clinical trial of GPC3-derived peptide vaccines to evaluate their safety, tolerability, and efficacy in patients with advanced HCC.[67] Thirty-three advanced HCC patients were enrolled and received escalating doses of GPC3-derived peptide vaccine (0.3, 1.0, 3.0, 10, and 30 mg/patient). On days 1, 15, and 29, peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant by intradermal injection. $GPC3_{298-306}$ (EYILSLEEL) peptide was used in 17 HLA-A24-positive patients, and $GPC3_{144-152}$ (FVGEFFTDV) peptide was used in 16 HLA-A2-positive patients.

Dose-limiting toxicity and dose-specific adverse events were not seen, and GPC3-derived peptide vaccine treatment was well tolerated. One of the thirty-three patients was judged to have a partial response, whereas 19 patients exhibited stable disease after 2 months according to Response Evaluation Criteria In Solid Tumors (RECIST).[68] The disease control rate (partial response plus stable disease) was 60.6% after 2 months. The median time to tumor progression was 3.4 months (95% confidence interval [CI] 2.1–4.6), and the median overall survival was 9.0 months (95% CI 8.0–10.0).

Immunologically, the frequency of GPC3-peptide-specific CTL in the peripheral blood correlated with the overall survival of HCC patients. In the multivariate analysis, GPC3 peptide-specific CTL frequency was a predictive factor for overall survival. The median overall survival of all 33 patients was 12.2 months (95% CI 6.5–18.0) in patients with a high frequency of GPC3-specific CTLs compared with 8.5 months (95% CI 3.7–13.1) in individuals with a low frequency (P=0.033). Moreover, the infiltration of cluster of differentiation (CD)8-positive T-cells into HCC cells was confirmed.

Based on this Phase I study, a Phase II study of the GPC3-derived peptide vaccine is ongoing in an adjuvant setting (UMIN-CTR: 000002614). Forty-four patients with HCC who had undergone surgery or radiofrequency ablation were enrolled. The primary end points of this study were the 1- and 2-year recurrence rates, and the secondary end point was the immunological response. Patient enrollment has been completed, and the study is ongoing. An additional sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway.

Anti-GPC3 antibody therapy

GPC3 has been suggested as a potential target for antibody-based therapy in liver cancer because of its high-level expression in HCC. The murine monoclonal antibody GC33, which binds specifically to the C-terminal region of GPC3 with a high affinity, caused significant antibody-dependent cellular cytotoxicity against HCC cells, and exhibited potent antitumor activity in xenograft models.[69–72] For the clinical application of GC33, a humanized GC33 was generated using complementarity-determining region grafting with the aid of both the hybrid variable region and two-step design methods. To improve the stability of the humanized GC33, it was further optimized by replacing the amino acid residues that might affect the structure of the variable region of its heavy chain.[73]

Because of these preclinical data highlighting the relevance of GPC3 as a potential therapeutic target in HCC, a first-in-man Phase I clinical trial to assess the safety, tolerability, and pharmacokinetics of GC33 in patients with advanced HCC was performed.[74] A total of 20 patients were enrolled, and were assigned to receive GC33 at one of four sequentially increasing dose levels (2.5, 5, 10, and 20 mg/kg) weekly by intravenous infusion. The tumor expression of GPC3 was examined in biopsied specimens using immunohistochemical staining. A total of 56% of the patients had a high total GPC3-staining score. This study provided the

initial clinical data regarding the safety profile and pharmacokinetic features of GC33, and revealed potential antitumor activity that might be associated with the expression of GPC3 in tumors. Stable disease was seen in four patients, all of whom exhibited high GPC3 expression. The median time to progression was significantly longer in patients with tumors expressing high levels of GPC3 than in patients with low GPC3 expression.

GC33 is now being assessed in Phase II clinical trials in second-line HCC patients who have progressed after one line of systemic therapy and whose tumors exhibit positive GPC3 immunohistochemical staining (NCT01507168). Additional antibodies that target GPC3 for HCC treatment, human (MDX-1414 and HN3) and humanized mouse (YP7) antibodies, are at different stages of preclinical development.[75] These trials will define the potential of GPC3 as a novel antibody therapy.

Potential of GPC3 for other cancers

GPC3 is also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian clear-cell carcinoma (CCC), and lung squamous cell carcinoma.[37-41] However, Kim et al reported that GPC3 is downregulated in lung cancer. Thus, the overexpression of GPC3 in lung cancer is controversial.[76] GPC3 has been investigated in some of these tumors as a potential immunotherapeutic target or diagnostic marker.

Melanoma

GPC3 messenger RNA and protein was identified in >80% of melanoma and melanocytic nevus patients.[39] In the authors' previous study, GPC3 protein was detected in the sera of 39.6% melanoma patients, but not in healthy donors. The positive detection of serum GPC3 was significantly higher than that of 5-S-cysteinyldopa and melanoma-inhibitory activity, both of which are well-known tumor markers for melanoma. Surprisingly, GPC3 could be detected even in patients with stage 0 in situ melanoma.[37] The combination of secreted protein acidic and rich in cysteine (SPARC) and GPC3 was also a useful tumor marker for melanoma: 66.2% of melanoma patients at stages 0–II exhibited positive SPARC or GPC3 expression.[47] This suggests that GPC3 is a novel tumor marker that is useful for the diagnosis of melanoma, particularly during the early stages.

Ovarian carcinoma

Ovarian CCC is the second most common epithelial ovarian carcinoma subtype in Japan. Ovarian CCC is associated with a poor prognosis and increased chemoresistance compared with other epithelial ovarian carcinoma subtypes.[77,78] GPC3 was expressed in ~40% of CCC patients, and there was a tendency toward poor progression-free survival in GPC3-positive patients at stage I.[79] GPC3 expression was responsible for CTL recognition, and subtoxic dose chemotherapy made tumor cells more susceptible to the cytotoxic effects of CTL.[80] A Phase II trial of a GPC3-derived peptide vaccine in ovarian CCC patients is ongoing (UMIN-CTR: 000003696), and some chemotherapy-refractory ovarian CCC patients have achieved a significant clinical response.[81]

Pediatric tumors

A Phase I trial using a GPC3-derived peptide vaccine for pediatric patients with hepatoblastoma, nephroblastoma, or yolk sac tumors is ongoing (UMIN-CTR: 000006357). The safety and optimal dose of GPC3 peptide vaccines for pediatric cancer patients has not yet been reported.

Conclusion

Although immunotherapy is a potentially attractive treatment modality, its antitumor effects in advanced HCC are not dramatic. GPC3 is overexpressed in HCC but its expression in most adult normal tissues is low. GPC3 expression is correlated with poor prognosis in HCC, suggesting it to be an ideal tumor antigen. GPC3 is thought to play a role in regulating cancer cell growth, although our structural and biological knowledge of GPC3 remain limited. Recent studies have shown the utility of GPC3 as a serum and immunohistochemical marker for the diagnosis of HCC. In addition, although studies assessing GPC3-targeted immunotherapies against HCC (such as vaccine and antibody therapies) have shown good safety and tolerability, sufficient clinical effects have not yet been observed. Further analysis and knowledge of GPC3 biology and its potential as an immunotherapeutic target are needed to allow the development of more effective GPC3-targeted cancer therapies. Although current GPC3-targeted immunotherapies for HCC are in the preclinical and clinical trial phases of development, they are expected to yield clinical success in the near future.

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Disclosure

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Large-scale expansion of γδ T cells and peptide-specific cytotoxic T cells using zoledronate for adoptive immunotherapy

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Abstract. Specific cellular immunotherapy for cancer requires efficient generation and expansion of cytotoxic T lymphocytes (CTLs) that recognize tumor-associated antigens. However, it is difficult to isolate and expand functionally active T-cells *ex vivo*. In this study, we investigated the efficacy of a new method to induce expansion of antigen-specific CTLs for adoptive immunotherapy. We used tumor-associated antigen glypican-3 (GPC3)-derived peptide and cytomegalovirus (CMV)-derived peptide as antigens. Treatment of human peripheral blood mononuclear cells (PBMCs) with zoledronate is a method that enables large-scale γδ T-cell expansion. To induce expansion of γδ T cells and antigen-specific CTLs, the PBMCs of healthy volunteers or patients vaccinated with GPC3 peptide were cultured with both peptide and zoledronate for 14 days. The expansion of γδ T cells and peptide-specific CTLs from a few PBMCs using zoledronate yields cell numbers sufficient for adoptive transfer. The rate of increase of GPC3-specific CTLs was approximately 24- to 170,000-fold. These $CD8^+$ cells, including CTLs, showed GPC3-specific cytotoxicity against SK-Hep-1/hGPC3 and T2 pulsed with GPC3 peptide, but not against SK-Hep-1/vec and T2 pulsed with human immunodeficiency virus peptide. On the other hand, $CD8^-$ cells, including γδ T cells, showed cytotoxicity against SK-Hep-1/hGPC3 and SK-Hep-1/vec, but did not show GPC3 specificity. Furthermore, adoptive cell transfer of $CD8^+$ cells, $CD8^-$ cells, and total cells after expansion significantly inhibited tumor growth in an NOD/SCID mouse model. This study indicates that simultaneous expansion of γδ T cells and peptide-specific CTLs using zoledronate is useful for adoptive immunotherapy.

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Introduction

Current therapeutic options for cancer treatment, including surgery, radiotherapy and chemotherapy, have made advancements in recent years and the survival rate of patients with cancer has gradually improved. However, these therapies remain far from satisfactory in most cancers (1,2). Therefore, the development of novel treatment modalities, including antigen-specific cancer immunotherapies with peptide vaccines, dendritic cell vaccines and adoptive cell transfer therapies, is critical for the further advancement of effective cancer treatments (3-5).

We found that glypican-3 (GPC3), which is an oncofetal antigen that is overexpressed in human hepatocellular carcinoma (HCC), was shown to be a useful target antigen for immunotherapy in several studies (6-10). Based on results obtained from preclinical studies, we conducted a phase I clinical trial using a GPC3-derived peptide vaccine in 33 patients with advanced HCC. In almost all vaccinated patients, the frequency of GPC3 peptide-specific CTLs increased after vaccination. Furthermore, this was the first study to show that the frequency of peptide-specific CTLs was correlated with overall survival in patients with HCC receiving peptide vaccines (11,12). Although the peptide vaccine is a potentially attractive treatment modality, the antitumor effects of the peptide vaccine alone are not dramatic in patients with advanced HCC. Therefore, the establishment of an innovative strategy to enhance the power of antigen-specific cancer immunotherapy is urgently required.

Cellular immunotherapy of solid and hematopoietic malignancies is regarded as a promising approach to treat relapse after or resistance to conventional treatments. The adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) results in objective cancer regression in 49 to 72% of patients with metastatic melanoma (13). However, due to the scarcity of TILs, this therapy is only possible for a limited number of patients. It is difficult to isolate and expand functionally active T cells. Development of a new method of CTL expansion may be useful in addressing this problem.

It was recently reported that γδ T cells are attractive mediators of cancer immunotherapy (14). Several clinical studies that included manipulation of γδ T cells by aminobisphosphonate administration or adoptive transfer of γδ

T cells were performed (15-17). γδ T cells recognize their targets independently of major histocompatibility complex (MHC)-mediated antigen presentation (18-21). Human γδ T cells kill a vast repertoire of tumor cell lines and primary samples *in vitro*, including leukemia, lymphoma, melanoma, neuroblastoma and multiple types of carcinomas (22-25). In addition, human γδ T cells mediate antibody-dependent cellular cytotoxicity (26,27). On the other hand, activated human γδ T cells produce large amounts of interferon-γ (28,29), a central cytokine in antitumor immune responses. Moreover, it has been reported that zoledronate stimulates proliferation of γδ T cells, which then stimulate CTLs as antigen-presenting cells (APCs) (30-33). Therefore, we have attempted to use γδ T cells as both effector cells and APCs.

We report on the development of a more effective adoptive immunotherapy. We investigated a new method to induce expansion of γδ T cells and peptide-specific CTLs using zoledronate.

Materials and methods

Patient samples. Patient blood samples were obtained during the performance of clinical trials at National Cancer Center Hospital East. We carried out two clinical trials involving GPC3-derived peptide vaccine. The phase I trial was carried out among 33 patients with advanced or metastatic HCC from February, 2007 to November, 2009 (11,12). The trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR no. 000001395). We subsequently conducted a phase II trial involving the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC. Forty patients with initial HCC who had undergone surgery or radiofrequency ablation were enrolled in this phase II trial (UMIN-CTR no. 000002614). These patients were enrolled after providing a written informed consent. Patients were intradermally injected with HLA-A24-restricted $GPC3_{298\text{-}306}$ (EYILSLEEL) or HLA-A2-restricted $GPC3_{144\text{-}152}$ (FVGEFFTDV) peptide vaccine emulsified with incomplete Freund's adjuvant (IFA, Montanide ISA-51VG; SEPPIC, Paris, France). This study was approved by the Ethics Committee of the National Cancer Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

PBMCs. Peripheral blood (30 ml) was obtained from healthy volunteers or patients at the times designated in the protocol (before the first vaccination and 2 weeks after each vaccination). Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll density gradient centrifugation from buffy coats. In this study, we used the remaining PBMCs after immunological monitoring in the clinical trials.

Cell lines. The human liver cancer cell lines SK-Hep-1 ($GPC3^-$, HLA-A*02:01/A*24:02) and SK-Hep-1/hGPC3 ($GPC3^+$, HLA-A*02:01/A*24:02) were used as target cells. SK-Hep-1/hGPC3 is an established stable GPC3-expressing cell line transfected with a human GPC3 gene, and SK-Hep-1/vec is an established counterpart cell line in which an empty vector was transfected. T2 (HLA-A*02:01, TAP^-) and T2A24 (HLA-A*02:01/A*24:02, TAP^-) cells were pulsed with GPC3 peptide or human immunodeficiency (HIV) peptide at room temperature for 1 h. They were conserved in our laboratory. Cells were cultured at 37°C in RPMI-1640 or DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2.

Synthetic peptides. The peptides used in this study were as follows: HLA-A*02:01-restricted $GPC3_{144\text{-}152}$ (FVGEFFTDV) peptide (American Peptide Company, Sunnyvale, CA), HLA-A*24:02-restricted $GPC3_{298\text{-}306}$ (EYILSLEEL) peptide (American Peptide Company), HLA-A*02:01-restricted cytomegalovirus $(CMV)_{495\text{-}503}$ (NLVPMVATV) peptide (ProImmune, Rhinebeck, NY, USA), HLA-A*24:02-restricted $CMV_{341\text{-}349}$ (QYDPVAALF) peptide (ProImmune), and HLA-A*02:01-restricted $HIV_{77\text{-}85}$ (SLYNTYATL) peptide (ProImmune). The peptides were dissolved and diluted in 7% $NaHCO_3$ or dimethyl sulfoxide.

Large-scale expansion using zoledronate. PBMCs were cultured ($2x10^6$ cells/well) with zoledronate (5 μM) (Novartis Pharma, Basel, Switzerland) and CMV or GPC3 peptide (10 μM) in AIM-V medium (Gibco) supplemented with 10% human AB serum (Sigma) and recombinant human interleukin (IL)-2 (1,000 IU/ml) (Novartis Pharma) for 14 days. The stimulation procedure was performed at 37°C and 5% CO_2. Scale-up of cells was performed in accordance with their growth.

Expansion of peptide-specific CTLs in the absence of zoledronate. To obtain zoledronate-activated γδ T cells, PBMCs were stimulated with zoledronate and IL-2 for 7 days. On day 7, zoledronate-activated γδ T cells were sorted using FACSAria II. $CD8^+$ cells and γδ T cells without zoledronate activation were sorted from non-cultured PBMCs using microbeads and FACSAria II, respectively. Dendritic cells (DCs) were induced from $CD14^+$ cells using GM-CSF and IL-4. On day 5, DCs were stimulated with TNF-α for 2 days. We used γδ T cells with or without zoledronate activation and TNF-α-stimulated DCs as stimulator cells. Stimulator cells were pulsed with CMV peptide (10 μM) for 1 h at room temperature. After washing out the peptide, stimulator cells were co-cultured for 2 weeks with responder $CD8^+$ cells and the addition of IL-2 in the absence of zoledronate. We compared the percentages of CMV peptide-specific CTLs in responder $CD8^+$ cells using dextramer assays.

In vitro stimulation of GPC3 peptide-specific CTL clones. GPC3 peptide-specific CTL clones were previously generated by single cell sorting using a GPC3-dextramer or CD107a antibody. CTL clones were stimulated as described previously (34).

Dextramer staining and flow cytometry analysis. The PBMCs were stained with CMV, GPC3 or HIV Dextramer-RPE (Immudex, Copenhagen, Denmark) for 10 min at room temperature and with anti-CD8-FITC (ProImmune) or anti-CD8-APC (BioLegend, San Diego, CA), anti-CD45RA-FITC (BD Biosciences, San Jose, CA, USA), and anti-CCR7-PerCP/Cy5.5 (BioLegend) for 20 min at 4°C. To detect γδ T cells, PBMCs were stained with anti-TCR-Vγ9-FITC (Beckman Coulter, Erembodegem,

Table I. Rate of increase in the number of cells in 16 patients with HCC.

		Total			γδ			GPC3 specific CTLs		
Sample	HLA-A	Day 0	Day 14	The rate of increase	Day 0	Day 14	The rate of increase	Day 0[a]	Day 14[b]	The rate of increase
1	02:01	$2.0x10^6$	$1.7x10^8$	85	$1.2x10^4$	$2.8x10^7$	$2.3x10^3$	$2.0x10^3$	$6.1x10^7$	$3.1x10^4$
2	02:01	$2.0x10^6$	$5.4x10^8$	$2.7x10^2$	$2.8x10^4$	$3.1x10^8$	$1.1x10^4$	$1.6x10^2$	$2.7x10^7$	$1.7x10^5$
3	02:01	$2.0x10^6$	$1.7x10^8$	85	$8.8x10^3$	$7.8x10^7$	$8.9x10^3$	92	$1.0x10^6$	$1.1x10^4$
4	02:01	$2.0x10^6$	$1.7x10^8$	85	$2.4x10^4$	$9.5x10^7$	$4.0x10^3$	$1.3x10^3$	$1.9x10^7$	$1.5x10^4$
5	02:01	$2.0x10^6$	$7.4x10^7$	37	$9.4x10^3$	$5.2x10^7$	$5.5x10^3$	92	$1.8x10^5$	$2.0x10^3$
6	02:01	$2.0x10^6$	$2.5x10^7$	13	$2.0x10^4$	$1.2x10^7$	$6.0x10^2$	$1.0x10^3$	$1.9x10^6$	$1.9x10^3$
7	02:01	$2.0x10^6$	$1.6x10^8$	80	$1.1x10^4$	$7.4x10^7$	$6.7x10^3$	$2.1x10^2$	$4.4x10^6$	$2.1x10^4$
8	02:01	$2.0x10^6$	$4.0x10^8$	$2.0x10^2$	$1.2x10^4$	$1.3x10^8$	$1.1x10^4$	$4.0x10^2$	$2.6x10^7$	$6.5x10^4$
9	02:01	$2.0x10^6$	$4.0x10^7$	20	$8.0x10^3$	$4.4x10^6$	$5.5x10^2$	$8.4x10^2$	$5.9x10^6$	$7.0x10^3$
10	02:01	$2.0x10^6$	$2.2x10^6$	1.1	$2.0x10^3$	$4.5x10^4$	23	92	$2.2x10^3$	24
11	02:01	$2.0x10^6$	$1.5x10^8$	75	$6.0x10^3$	$4.8x10^7$	$8.0x10^3$	$3.0x10^2$	$2.0x10^7$	$6.7x10^4$
12	02:01	$2.0x10^6$	$1.1x10^8$	55	$6.0x10^3$	$1.2x10^7$	$2.0x10^3$	$1.2x10^3$	$3.5x10^7$	$2.9x10^4$
13	24:02	$2.0x10^6$	$5.8x10^6$	2.9	$1.8x10^4$	$3.8x10^6$	$2.1x10^2$	$1.3x10^2$	$6.3x10^4$	$4.9x10^2$
14	24:02	$2.0x10^6$	$4.0x10^6$	2	$2.2x10^4$	$2.6x10^6$	$1.2x10^2$	$1.0x10^2$	$3.1x10^4$	$3.1x10^2$
15	24:02	$2.0x10^6$	$9.9x10^7$	50	$3.8x10^3$	$3.8x10^7$	$1.0x10^4$	$1.8x10^2$	$1.2x10^6$	$6.7x10^3$
16	24:02	$2.0x10^6$	$4.0x10^7$	20	$9.8x10^3$	$5.6x10^6$	$5.7x10^2$	$1.4x10^2$	$1.4x10^5$	$1.0x10^3$

[a]Frequency of GPC3-specific CTLs of $2x10^6$ PBMCs was measured by *ex vivo* IFN-γ ELISPOT assay. [b]GPC3-specific CTLs after cell culture were measured by flow cytometry.

Belgium) and anti-CD3-PC5 (BioLegend) for 20 min at 4°C. γδ T cells, with or without zoledronate activation, and TNF-DCs were stained with anti-HLA-class I-FITC, anti-CD80-FITC, anti-CD83-FITC and anti-CD86-PE (BD Biosciences) antibodies for 20 min at 4°C. Flow cytometry analysis was carried out using FACSCanto II (BD Biosciences).

Cytotoxicity assay. Cytotoxic activity against target cells was analyzed using the Terascan VPC system (Minerva Tech, Tokyo, Japan) as described previously (34). Target cells were labeled with calcein AM (Dojindo, Kumamoto, Japan) solution for 30 min at 37°C. The labeled cells were then incubated with effector cells for 4 to 6 h. As effector cells, $CD8^+$ and $CD8^-$ T cells were isolated using human CD8 microbeads (BD Bioscience) from PBMCs stimulated for 14 days. Assays were conducted in duplicate.

Transfer of effector cells to NOD/SCID mice implanted with the GPC3$^+$ or GPC3$^-$ cell line. Female NOD/SCID (6-8 weeks old) were purchased from Japan Charles River Laboratories (Yokohama, Japan). All animal procedures were performed according to the guidelines for the Animal Research Committee of the National Cancer Center, Japan. We inoculated SK-Hep-1/hGPC3 or SK-Hep-1/vec cells subcutaneously into the right flank of NOD/SCID mice. We intravenously injected the $CD8^+$ cells, $CD8^-$ cells, or both, as effector cells. We injected PBS as a negative control. Before adoptive transfer, we examined the percentage of $CD8^+$ cells in expanded cells using flow cytometry. The percentage of $CD8^+$ cells after expansion was ~25% of all cells. We injected immune cells at this ratio. We injected $5x10^6$ cells per mouse for the $CD8^+$-cell-treatment group. We injected $1.5x10^7$ cells per mouse for the $CD8^-$ cell treatment group and $2.0x10^7$ cells per mouse for the all cells treatment group. We performed adoptive cell transfer of expanded cells using five mice per group. The tumor volume was monitored and calculated using the following formula: tumor volume (mm^3) = a x b^2 x 0.5, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid.

Statistical analysis. The correlation between the number of GPC3-specific CTLs and γδ T cells at days 0 and 14 was analyzed using the Spearman's rank correlation coefficient. Comparisons of tumor volume at the last time point were performed using the Mann-Whitney U test. Differences were considered significant at $P<0.05$.

Results

Zoledronate induces expansion of γδ T cells and peptide-specific CTLs from PBMCs. To assess whether this new culture method can induce the expansion of γδ T cells and peptide-specific CTLs, PBMCs were stimulated once with zoledronate and an antigen-derived peptide. Fig. 1A shows the representative data using PBMCs from a healthy volunteer. The number of total cells increased $3.2x10^2$-fold after 14 days (from $2.0x10^6$ to $6.4x10^8$). In flow cytometry analysis, γδ T cells increased $8.0x10^3$-fold after 14 days [from $5.6x10^4$ (2.8%) to $4.5x10^8$ (70%)]. Simultaneously with γδ T cells, CMV

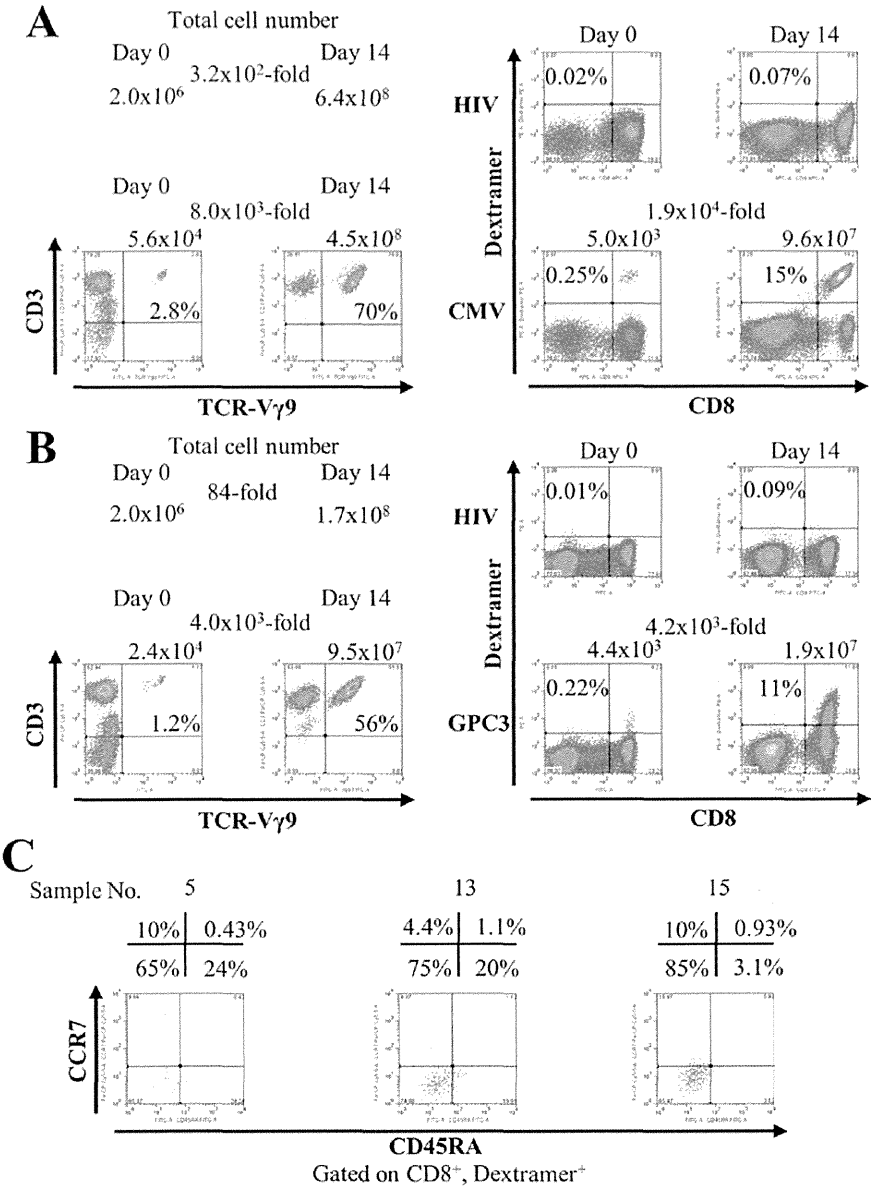

Figure 1. Zoledronate induces expansion of γδ T cells and peptide-specific CTLs. We performed flow cytometry analysis before (day 0) and after (day 14) cell culture. Representative data are shown. (A) PBMCs from a healthy volunteer were stimulated with CMV-derived peptide and zoledronate. (B) PBMCs from a patient vaccinated with GPC3 peptide were stimulated with GPC3-derived peptide and zoledronate. The number indicates the number of cells. The presence of TCR-Vγ9$^+$, CD3$^+$ cells indicated γδ T cells. The presence of CD8$^+$, dextramer$^+$ cells indicated antigen-specific CTLs. HIV-dextramer was used as a negative control. (C) Analysis of the phenotype of CD8$^+$, GPC3-dextramer$^+$ cells at day 14. The CD45RA$^-$, CCR7$^-$ phenotype indicated the effector memory phenotype.

peptide-specific CTLs increased 1.9x10^4-fold after 14 days [from 5.0x10^3 (0.25%) to 9.6x10^7 (15%)]. Similar results were obtained from three healthy subjects (data not shown).

Next, we investigated the capacity of this culture method to induce expansion of CTLs specific for peptides derived from the weakly immunogenic tumor-associated self-antigen GPC3. PBMCs from vaccinated patients were stimulated once with zoledronate and a GPC3-derived peptide. In Fig. 1B, the number of total cells increased 84-fold after 14 days (from 2.0x10^6 to 1.7x10^8). γδ T cells increased 4.0x10^3-fold after 14 days [from 2.4x10^4 (1.2%) to 9.5x10^7 (56%)]. GPC3 peptide-specific CTLs increased 4.2x10^3-fold after 14 days [from 4.4x10^3 (0.22%) to 1.9x10^7 (11%)]. In addition, during expansion, GPC3 peptide-specific CTLs acquired mainly an effector memory phenotype (CD45RA$^-$, CCR7$^-$) (Fig. 1C). One of the features of this culture method is the rate of increase in the number of cells. Table I shows the rate of increase in the number of cells in 16 patients with HCC. We found that the total cell number increased (range, 1.1-270-fold), γδ T cells increased (range, 23-1.1x10^4-fold), and GPC3 peptide-specific CTLs increased (range, 24-1.7x10^5-fold) after 14 days. These results suggest that peptide-specific CTLs were successfully expanded with the proliferation of γδ T cells from PBMCs by this new culture method.

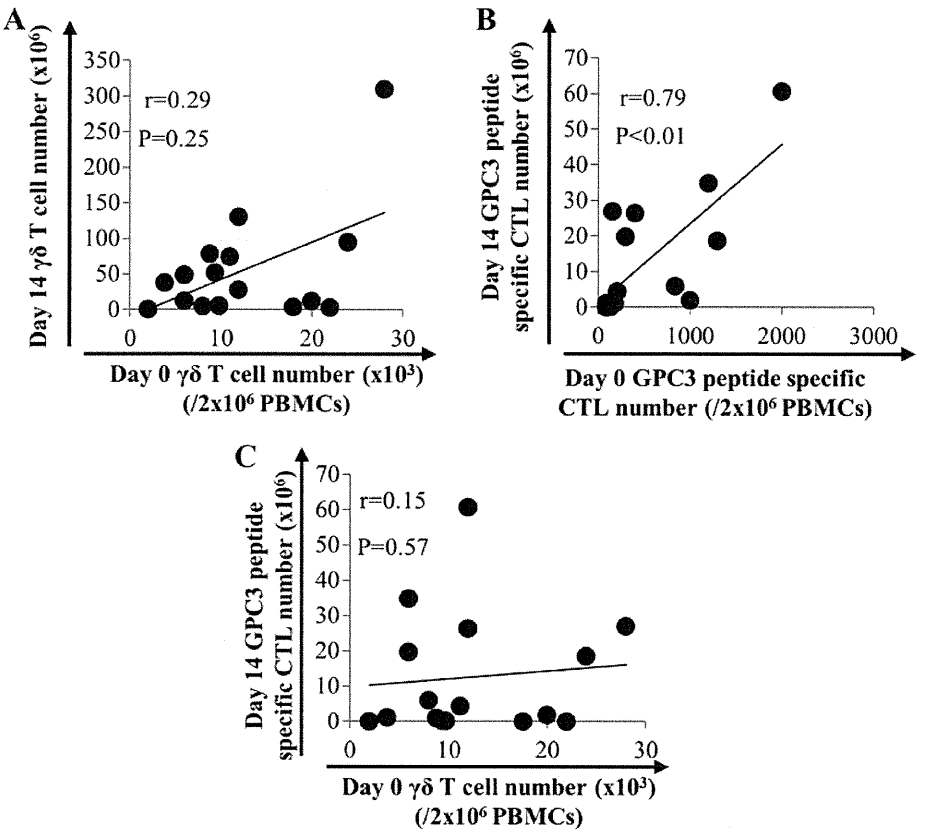

Figure 2. Efficacy of the method to induce expansion of GPC3 peptide-specific CTLs. (A) The correlation between the number of γδ T cells before and after expansion (n=16). (B) The correlation between the number of GPC3 peptide-specific CTLs before and after expansion. The number of GPC3 peptide-specific CTLs after expansion was correlated with that before expansion (n=16). (C) The correlation between the number of γδ T cells and the number of GPC3 peptide-specific CTLs after expansion (n=16).

Efficiency of the culture method to induce expansion of GPC3 peptide-specific CTLs. One of the problems of cell transfer therapy is that it cannot predict cell growth prior to cell culture. Therefore, to identify predicting factors, we investigated the ability of this culture method to induce expansion of γδ T cells and GPC3 peptide-specific CTLs in 16 patients with HCC. As shown in Fig. 2, the number of γδ T cells after expansion did not correlate with that before expansion (Fig. 2A). On the other hand, the number of GPC3 peptide-specific CTLs after expansion correlated with that before expansion ($P<0.01$, $r=0.79$) (Fig. 2B). This result indicates that the number of GPC3 peptide-specific CTLs before expansion is a predicting factor. We expected a positive correlation between the number of γδ T cells and the number of GPC3 peptide-specific CTLs after expansion. However, no such correlation was observed (Fig. 2C).

Activated γδ T cells function as antigen-presenting cells. To examine whether the expansion of peptide-specific CTLs is enhanced by simultaneous activation/expansion of γδ T cells, we expanded peptide-specific CTLs in the absence of zoledronate. The purity of sorted $CD8^+$ cells and γδ T cells with or without zoledronate activation was greater than 99% (Fig. 3A). The expansion of peptide-specific CTLs stimulated by γδ T cells with zoledronate activation (70.8%) was higher than by γδ T cells without zoledronate activation (43.6%). Moreover, the CTL-expanding ability of zoledronate-activated γδ T cells was comparable to that of TNF-DCs (62.0%), which are known professional antigen-presenting cells. These results indicate that zoledronate-activated γδ T cells function as antigen-presenting cells in co-cultures in the absence of zoledronate (Fig. 3B). We compared cell surface expression of antigen-presenting molecules and co-stimulatory molecules on γδ T cells (with or without zoledronate activation) and TNF-DCs. All cells expressed HLA-class I; however, γδ T cells without zoledronate activation did not express co-stimulatory molecules. Furthermore, CD86 expression in zoledronate-activated γδ T cells was comparable with that of TNF-DCs (Fig. 3C). These results indicate that γδ T cells activated by zoledronate acquire antigen-presenting properties accompanied by CD86 expression.

Cytotoxic activity of expanded cells. We performed a cytotoxicity assay to assess the peptide specificity and cytotoxic activity of expanded cells against cancer cells. We used $CD8^+$ and $CD8^-$ cells that were isolated from cultured cells using CD8 microbeads at day 14 as effector cells. The purity of $CD8^+$ cells was 99.4%. We performed further immunophenotyping of $CD8^-$ cells. $CD3^+$ $Vg9^+$ cells were 80.0% of $CD8^-$ cells. $CD8^-$ cells also included $CD3^+$ $CD4^+$ cells (4.1%), $CD3^+$ $CD8^+$ cells (9.4%), and $CD3^-$ $CD56^+$ cells (NK cells; 3.6%). $CD14^+$ cells (monocytes; 0.1%) and

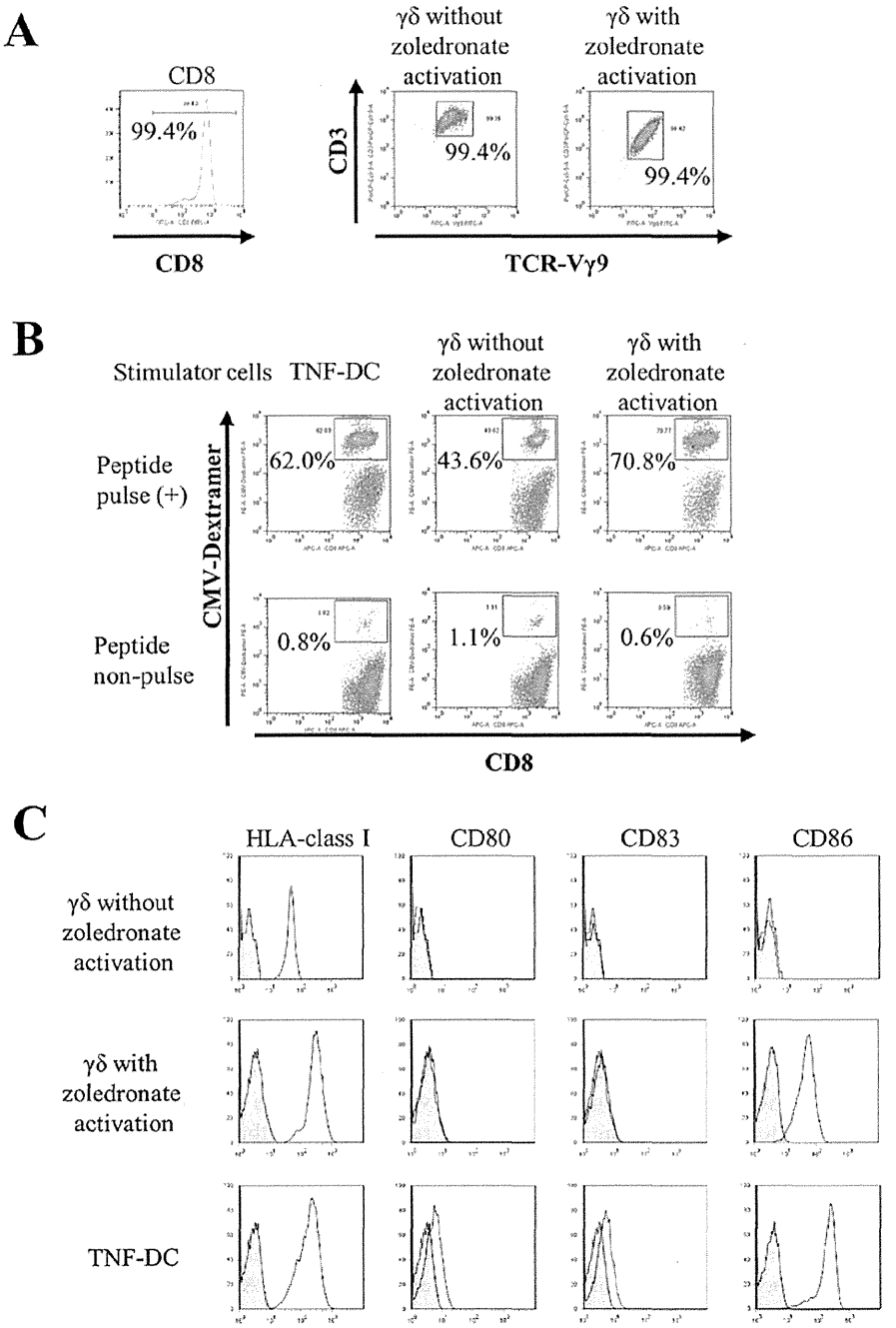

Figure 3. Activated γδ T cells function as antigen-presenting cells. (A) The percentages of sorted cells were analyzed using flow cytometry. The purity of sorted $CD8^+$ cells, γδ T cells without zoledronate activation and γδ T cells with zoledronate activation were greater than 99%. (B) The responder $CD8^+$ cells were co-cultured with stimulator cells pulsed with CMV peptide in the absence of zoledronate. After 2 weeks, flow cytometry analyses were performed using CMV-Dextramer. Non-pulsed stimulator cells were co-cultured with responder $CD8^+$ as negative controls. Representative data are shown. Similar results were obtained from three healthy subjects. (C) Cell surface expression of antigen-presenting molecules (HLA-class I) and co-stimulatory molecules (CD80, CD83 and CD86) on γδ T cells (with or without zoledronate activation) and TNF-DCs using flow cytometry. Black line shows a specific antibody. Gray-filled area shows negative control. Representative data are shown. Similar results were obtained from three healthy subjects.

$CD19^+$ cells (B cells; 0.1%) were not observed in $CD8^-$ cells. These results indicate that $CD8^-$ cells were predominantly γδ T cells (Fig. 4A). Similar results were obtained from four patients. $CD8^+$ cells showed cytotoxicity against T2 cells pulsed with GPC3 peptide, whereas $CD8^-$ cells did not show cytotoxicity against T2 cells pulsed with both GPC3 and HIV peptide (Fig. 4B). Moreover, we used SK-Hep-1/hGPC3 cells as target cells; they were transfected with the GPC3 gene and endogenously presented GPC3 peptide. $CD8^+$ cells showed GPC3-specific cytotoxicity, whereas $CD8^-$ cells showed cytotoxicity against SK-Hep-1 cells but did not show GPC3 specificity (Fig. 4C). We performed cytotox-