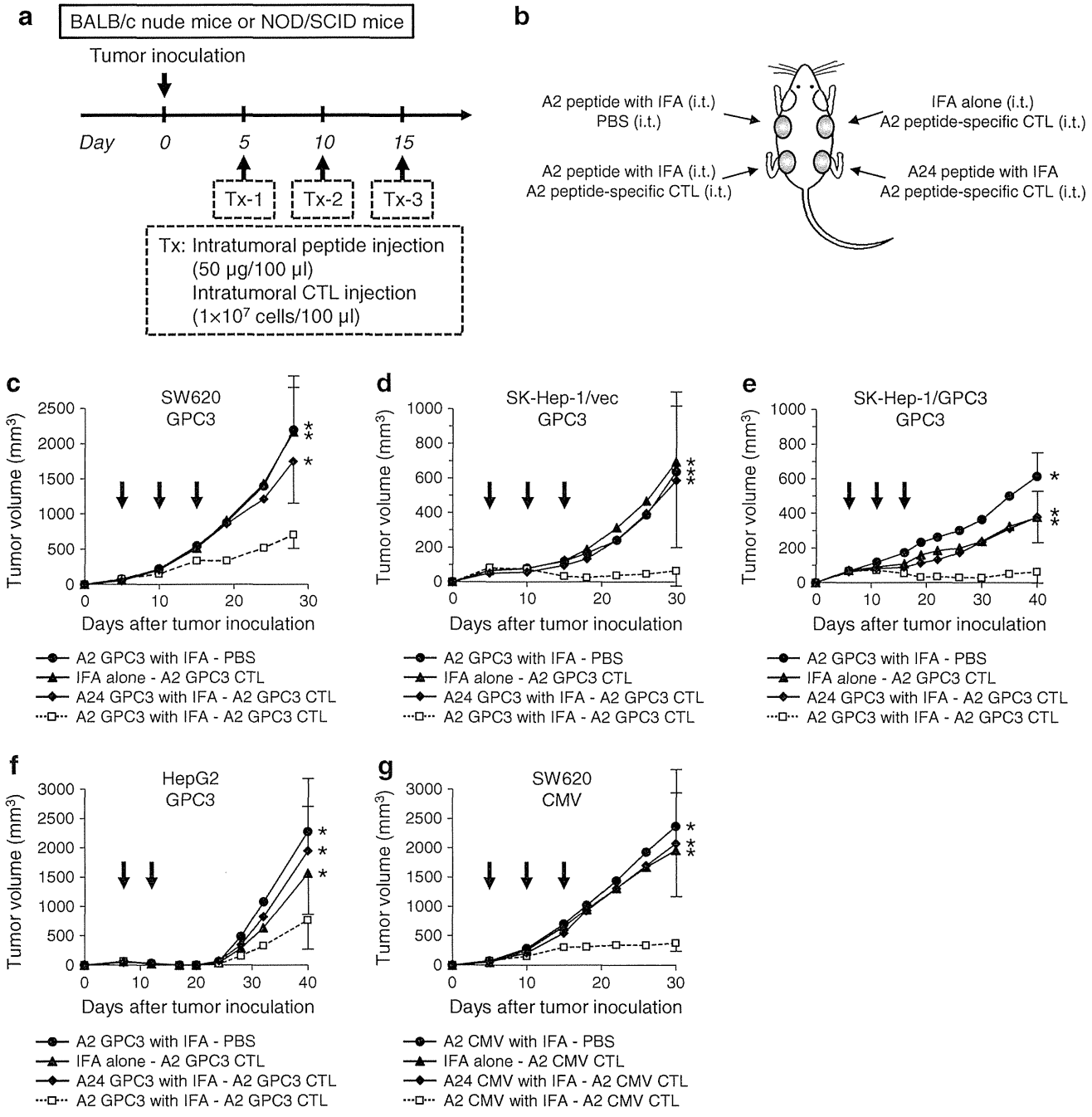

Fig. 3 Antitumor effect of intratumoral peptide injection in an immunodeficient mouse model. Intratumoral injection of a combination of antigen peptide and its specific CTLs had a significant antitumor effect. **a** Treatment schedule. **b** Experimental schematic representation. BALB/c nude mice or NOD-SCID mice were inoculated subcutaneously on their *back* with SW620, SK-Hep-1/vec, SK-Hep-1/GPC3, or HepG2 tumor cells. Four tumors were implanted per mouse, and HLA-A*02:01-restricted $GPC3_{144-152}$ or $CMV_{495-503}$ peptide emulsified with IFA (50 μg/100 μl) and HLA-A*02:01-restricted $GPC3_{144-152}$ or $CMV_{495-503}$ peptide-specific human CTLs (1×10^7 cells/100 μl) were injected into each tumor. (**c**, **d**, **e**, **f**, and **g**) Tumor volume. Tumor growth was expressed by mean tumor volume; *bars* (SD). Seven mice were used in each experiment. *Arrows* indicate the days when treatment was performed. $^*P < 0.05$ compared with treatment group (Mann–Whitney U test)

mice that had intratumoral injection with IFA alone. In mice treated with intratumoral injection of $OVA_{257-264}$ peptide, a larger number of $CD8^+$ T-cells had infiltrated the RMA tumor 24 days after the transfer of OT-I CTLs and 10 days after the last intratumoral injection of $OVA_{257-264}$ peptide. However, the simultaneous infiltration of normal tissues by

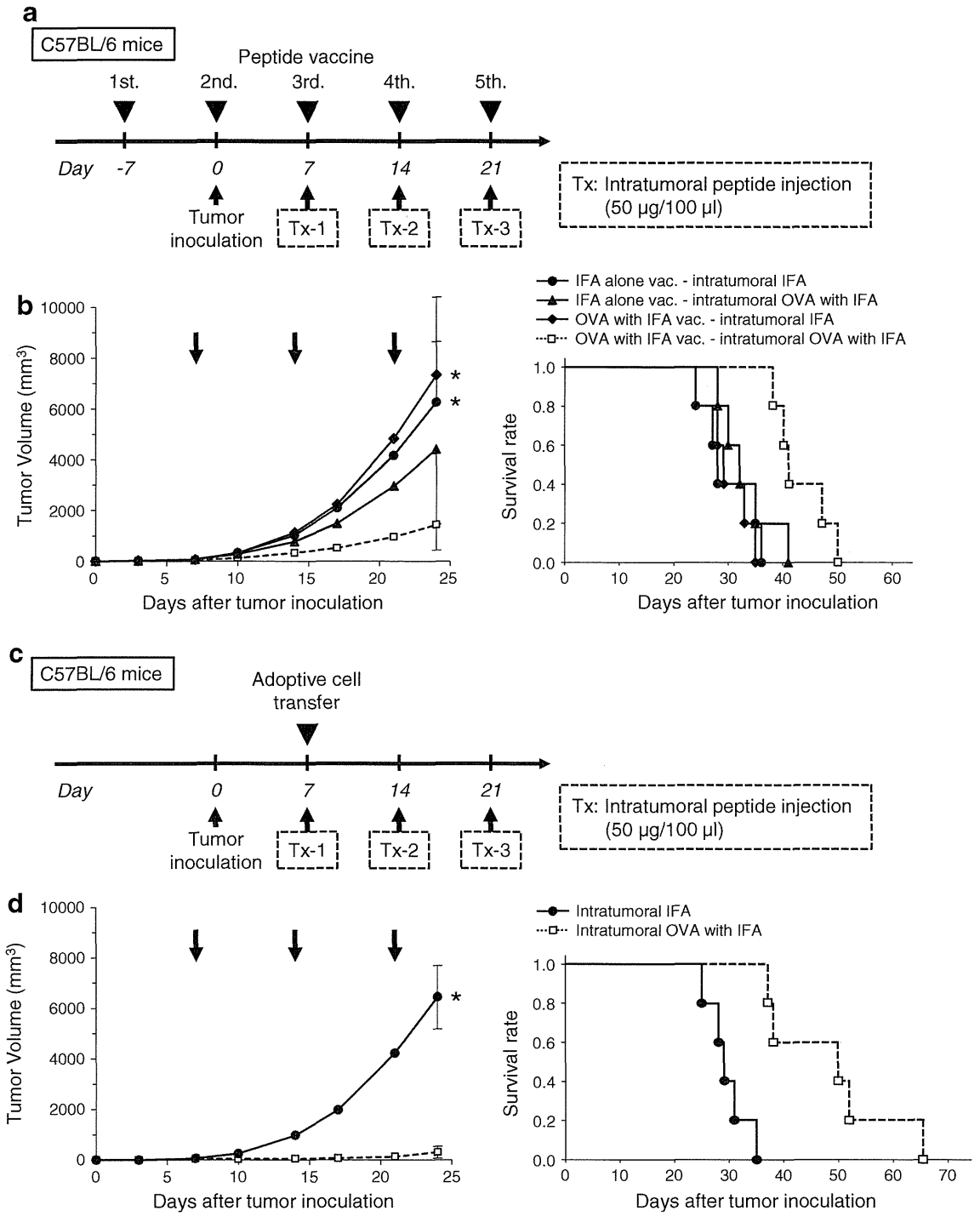

Fig. 4 Therapeutic advantage of intratumoral peptide injection as an option for antigen-specific cancer immunotherapy. (**a** and **b**) Peptide vaccine model. (**c** and **d**) Adoptive cell transfer model. (**a** and **c**) Treatment schedule. (**b** and **d**) Tumor growth and Kaplan–Meier survival *curves*. Tumor growth was expressed by mean tumor volume; *bars* (SD). $*P < 0.05$ compared with the treatment group (Mann–Whitney U test). The survival of mice in the treatment group was significantly better than that in the control groups ($P < 0.05$) (log-rank test). Five mice were used in each group. **e** Schedule for analysis of local accumulation of OVA-specific CTLs in an adoptive cell transfer model. **f** Experimental schematic representation. Two tumors were implanted per mouse (5×10^4 cells/100 μl). One tumor was injected with the OVA peptide plus IFA, and the other with IFA alone. **g** OVA tetramer assay. Local accumulation of OVA-specific CTLs was confirmed in a tumor injected with the OVA peptide plus IFA. Data are representative of three independent experiments. **h** Immunohistochemical staining of CD8 in tumor and normal tissues. Spleen was used as positive control. *Scale bars*, 50 μm

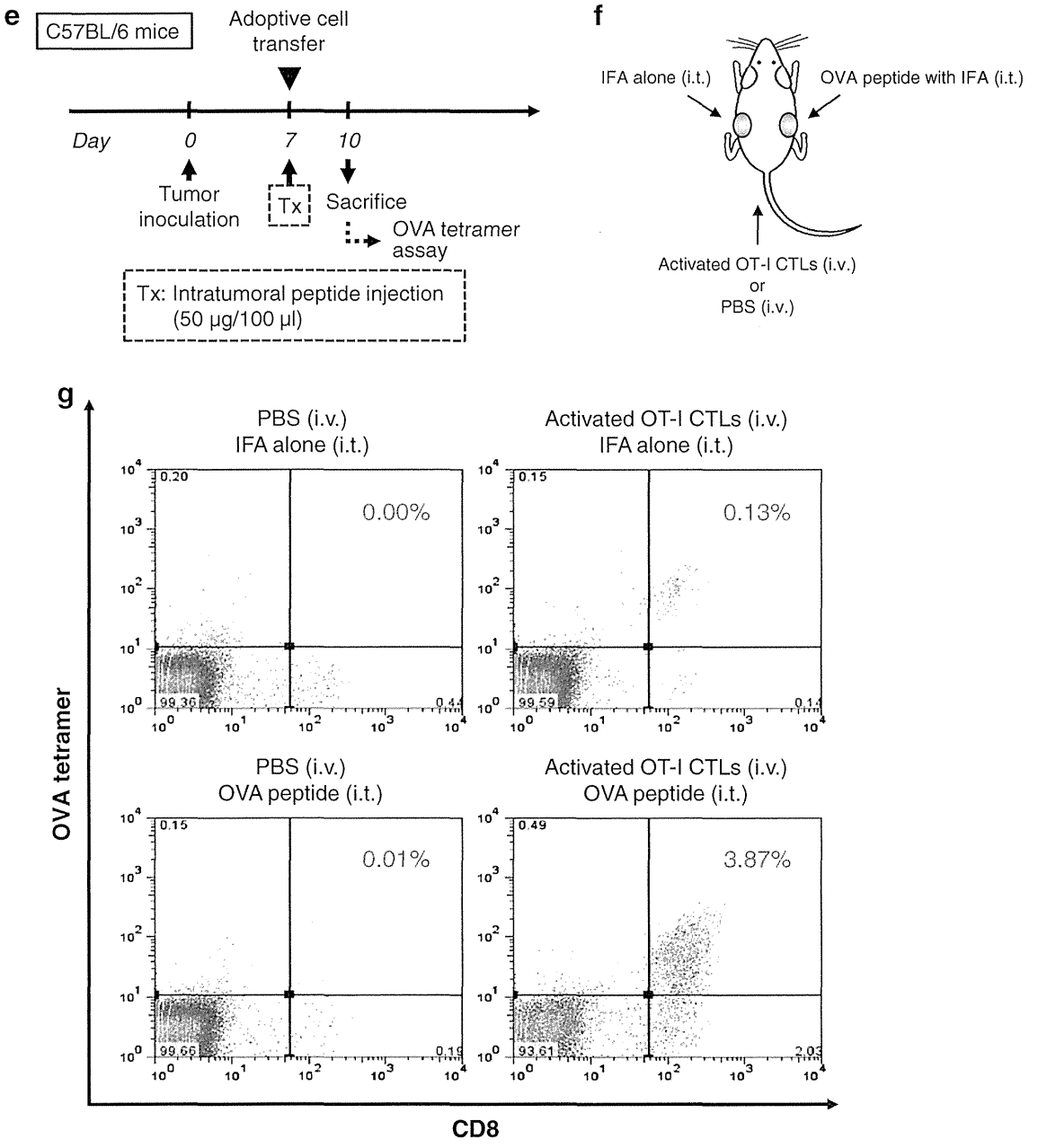

Fig. 4 continued

$CD8^+$ T-cells was not observed (Fig. 4h). These results suggest that peptide from intratumoral injection did not spread into normal tissues.

The effect of antigen spreading to another tumor after intratumoral peptide injection

Using an adoptive cell transfer model, we assessed the possibility of antigen-spreading effect after intratumoral peptide injection, as depicted in Fig. 5a. Two RMA tumors were bilaterally and metachronously implanted per mouse, and only the first tumors received intratumoral injection of the $OVA_{257–264}$ peptide. The sizes of the second tumors were compared with those from mice that received intratumoral injection of IFA alone (Fig. 5b). Whereas the second tumors were established 14 days after the second tumor inoculation in three out of four control mice, all four peptide-loaded mice that had received intratumoral $OVA_{257–264}$ peptide injection into their first tumor completely rejected the challenge of the second tumor, which did not receive intratumoral $OVA_{257–264}$ peptide injection itself (Fig. 5c).

To confirm the hypothesis of antigen spreading, an IFN-γ ELISPOT assay was performed. RMA tumor-bearing

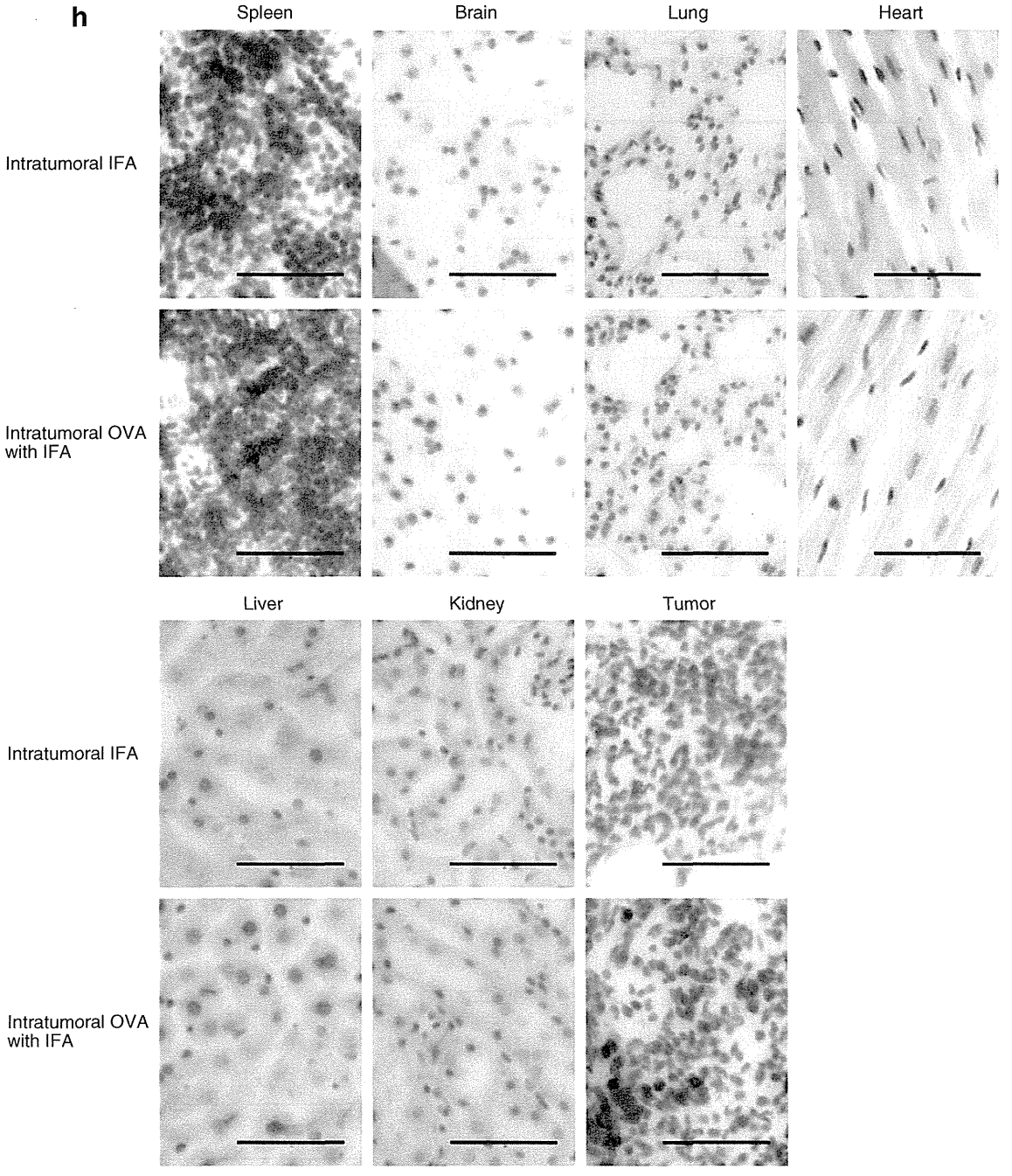

Fig. 4 continued

C57BL/6 mice that had received adoptive transfer of OT-I CTLs and intratumoral injection of $OVA_{257–264}$ peptide were killed, and their spleens were obtained 21 days after adoptive transfer and 7 days after the last intratumoral injection. $CD8^+$ T-cells, isolated from the spleen cells using anti-CD8a magnetic beads, were incubated with irradiated RMA cells for 3 days. $CD8^+$ T-cells were separated from RMA cells using anti-CD8a magnetic beads before the assay. An IFN-γ ELISPOT assay was performed in duplicate using $CD8^+$ T-cells as effector cells and RMA cells as target cells (Fig. 5d). The mice that had received intratumoral injection of $OVA_{257–264}$ peptide showed a significant response to OVA-negative RMA tumor cells compared with control mice that had received intratumoral injection of IFA alone ($P < 0.05$). The observed induction of RMA-derived antigen-specific CTLs provides evidence that antigen spreading occurred by treatment with intratumoral $OVA_{257–264}$ peptide and intravenous OT-I CTLs (Fig. 5e).

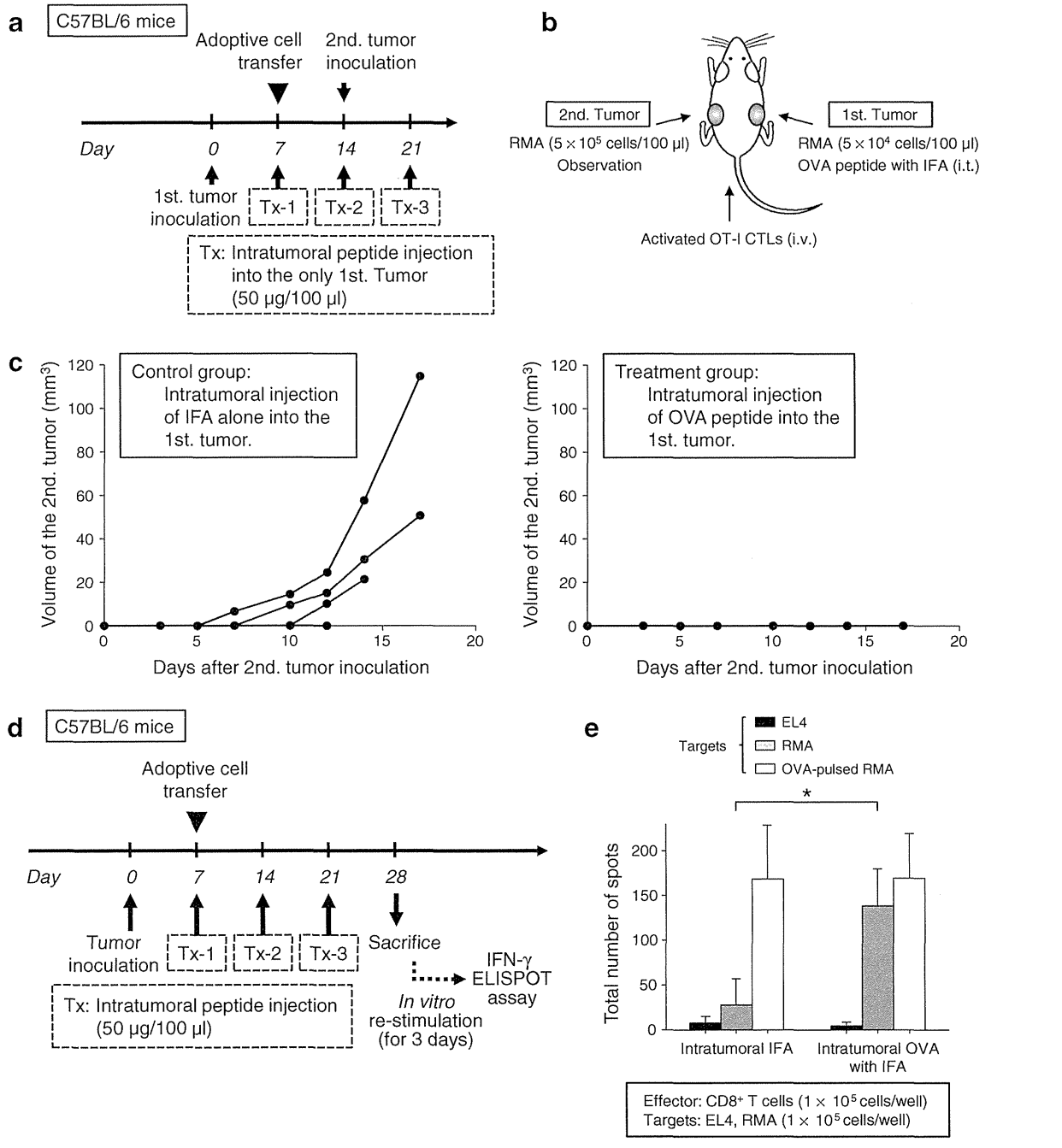

Fig. 5 Effect of antigen-spreading to another tumor after intratumoral peptide injection. **a** The schedule for the experiment on antigen-spreading effect in an adoptive cell transfer model. **b** Experimental schematic representation. Two tumors were metachronously implanted per mouse (first tumor: 5×10^4 cells/100 μl, second tumor: 5×10^5 cells/100 μl), and only the first tumor (*right back*) received intratumoral peptide injection. The second tumor (*left back*) was not treated, but was observed. **c** The growth of the second inoculated RMA tumor. *Four lines* indicate the tumor growth of each mouse. All four mice in the treatment group completely rejected the second tumor challenge. **d** The experiment schedule to confirm antigen spreading. **e** IFN-γ ELISPOT assay. EL4 cells were used as negative control targets. The data are expressed as mean values of three mice (SD). $^*P < 0.05$ compared with control (Mann–Whitney U test)

Discussion

We demonstrated that intratumoral peptide injection leads to additional peptide loading onto MHC class I molecules of tumor cells, causing enhanced CTL recognition of tumor cells. It is likely that a larger number of antigen-specific CTLs infiltrate the tumors after this procedure, and tumor cells are killed more easily because CTL activity depends

on the peptide density of tumor cells in an HLA class I-restricted manner. In other words, intratumoral peptide injection enhances the antigenicity of tumor cells, regardless of whether the tumor cells originally expressed the antigen. To the best of our knowledge, this is the first study to show the efficacy of intratumoral peptide injection in detail. A previous report demonstrated that peptide injection around a tumor assisted the activity of low-avidity CTLs in an immunodeficient mouse model [21]. In addition, we demonstrated the advantage as a therapeutic modality combined with antigen-specific cancer immunotherapy without any adverse reactions associated with this procedure in mice. Intratumoral peptide injection can strengthen the efficacy of every kind of antigen-specific cancer immunotherapy and may be a useful therapeutic option.

This is the first study to describe anticancer treatment with CMV-derived peptide-specific CTLs. Virus-derived antigens, which are exogenous antigens, usually have stronger antigenicity than tumor-associated autoantigens. Therefore, virus-derived antigen-specific CTLs are easier to induce [22]. Theoretically, every kind of antigen is applicable to our procedure unless it is expressed in healthy human cells. However, it is unclear whether post-CMV-infected lesions are safe from CMV-specific CTL cytotoxicity. Further investigations are necessary regarding the possible clinical use of exogenous antigens, such as CMV-derived peptides.

We used $NaHCO_3$, which is known to have therapeutic effects against tumors [23, 24], as a peptide diluent. However, our data demonstrated the efficacy of intratumoral peptide injection, because control animals which underwent intratumoral injection of IFA alone or IFA plus an irrelevant peptide also received $NaHCO_3$.

In an in vivo tumor growth inhibition assay using a peptide vaccine model, the group that did not receive the $OVA_{257–264}$ peptide vaccine but that received intratumoral peptide injections showed a partial treatment effect. This indicates that intratumoral or peritumoral antigen-presenting cells recognized intratumorally injected $OVA_{257–264}$ peptide and induced $OVA_{257–264}$ peptide-specific CTLs after three intratumoral peptide injections. However, we showed in this study that intratumoral peptide injection attracted more $OVA_{257–264}$ peptide-specific CTLs and was more effective when combined with peptide vaccines or adoptive cell transfer therapies.

A limitation of intratumoral peptide injection is its delivery method. First, immunotherapy is expected to contribute toward cancer therapy especially in the early stages or in the prevention of recurrence, in which cancer sites, the so-called "micro lesions," are undetectable by imaging modalities. However, intratumoral peptide injection must be limited to the tumors, which are detectable by imaging modalities, and can be approached with a needle. Second, it is difficult to spread the peptides over the whole tumor by intratumoral injection, especially against large tumors. Moreover, it is difficult to approach all of the multiple tumors. This procedure might limit the ability of immunotherapy as a systemic therapy. If a novel method of delivering peptides to tumor cells selectively through a systemic route is established in the future due to advances

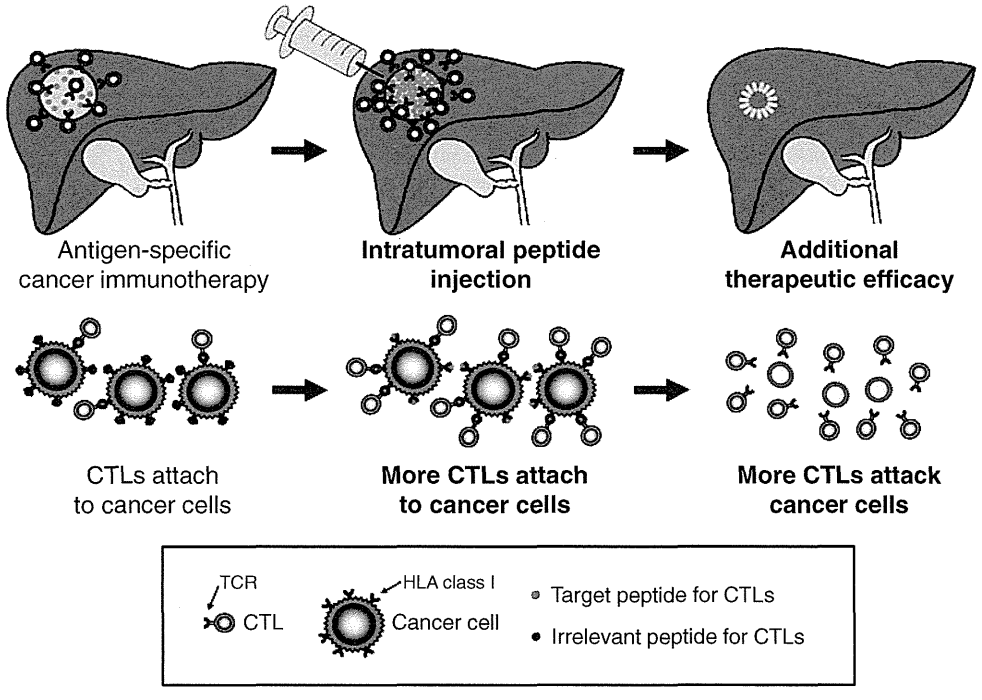

Fig. 6 A proposed mechanistic model of intratumoral peptide injection for improvement in antigen-specific cancer immunotherapy of solid tumors

in drug-delivery technologies, this method will become more suitable for clinical application.

Another limitation is that it requires the presence of MHC class I molecules. The potential loss of MHC class I expression in tumors would lead theoretically to the failure of this approach. Previous reports have indicated that 61–85 % of breast cancers had loss of or decreased HLA class I expression [25–27]. On the other hand, the down-regulation of HLA class I was less frequently observed in other cancers [27–30]. Before clinical application, it is necessary to select cancers in which HLA class I expression is sufficiently high.

Antigen-spreading effects have been observed following anticancer immunotherapy [31–34]. The second tumor challenge is easily rejected due to immunological memory. Therefore, we fixed the number of implanted tumor cells as the second tumors could be established. In this study, we report evidence of an antigen-spreading effect after intratumoral peptide injection. If this antigen-spreading effect is sufficiently steady and reliable, intratumoral peptide injection may even be effective against imaging-invisible or unapproachable tumors.

In conclusion, intratumoral peptide injection is an attractive strategy for enhancing tumor cell antigenicity. It can induce additional peptide loading onto tumor cells, making tumor cells more antigenic for antigen-specific CTL activity against tumor cells. Moreover, it may be a useful option for improvement in antigen-specific cancer immunotherapy against solid tumors (Fig. 6).

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Conflict of Interest The authors declare that they have no conflicts of interest.

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Remarkable tumor lysis in a hepatocellular carcinoma patient immediately following glypican-3-derived peptide vaccination

An autopsy case

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Keywords: peptide vaccine, glypican-3, CTL, HCC, tumor necrosis

Abbreviations: GPC3, glypican-3; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; CTL, cytotoxic T-lymphocyte; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cells; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin

We recently reported the safety, immunological and clinical responses to a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced hepatocellular carcinoma (HCC). We conducted a subsequent trial in advanced HCC to assess the histopathological findings before and after vaccination with the GPC3 peptide. Here, we present the clinical course and the pathological study including the autopsy of a patient with advanced HCC in the ongoing clinical trial. A 62-year old patient suffering from HCC refractory to sorafenib therapy received the GPC3 peptide vaccine. The patient had fever and remarkably impaired liver function twice after vaccination. Contrast-enhanced CT after the second vaccination showed multiple low-density areas in the liver tumor, indicating tumor necrosis. In contrast, the tumor thrombus in the right atrium increased. The patient discontinued protocol treatment due to disease progression and died 30 days after the second vaccination. An autopsy was performed to determine the main cause of death and to evaluate the antitumor effect of the vaccination. A histological examination showed central necrosis in most of the intrahepatic tumor. The main cause of death was circulatory failure due to tumor thrombus, which occupied most of the right atrium. An immunohistochemical analysis revealed infiltration of CD8-positive T cells in the residual carcinoma, but not within the cirrhotic area. Ex vivo IFN-γ enzyme-linked immunospot analysis revealed vaccine-induced immune-reactivity against the GPC3 peptide. A histopathological examination at the estimated time of a strong immunological response demonstrated a GPC3 peptide vaccination-induced cytotoxic T-lymphocyte response with an anti-tumor effect.

Introduction

Cancer vaccine targeting hepatocellular carcinoma (HCC) tumor antigens have been tested in clinical trials.[1,2] However, cancer vaccines using tumor-antigen-derived peptides have not demonstrated adequate antitumor efficacy in clinical trials for advanced HCC.[1-3] Glypican-3 (GPC3), a carcinoembryonic antigen, is an ideal target for immunotherapy against HCC because it is overexpressed specifically in HCC (72–81%) and correlates with a poor prognosis.[4-10] GPC3 forms a complex with Wnt molecules and promotes the growth of HCC by stimulating canonical Wnt signaling.[10] We identified HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which induce GPC3-reactive cytotoxic T-lymphocytes (CTLs) without inducing autoimmunity.[8,9] We recently reported the safety, immunological, and clinical responses of a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced HCC.[11] The results of that trial showed that GPC3 peptide-specific CTLs increased in peripheral blood, and that many CD8-positive T cells infiltrated the tumors in some patients, demonstrating a correlation between the CTL response and overall survival following GPC3 peptide vaccination. Based on these results, we conducted a trial in patients with advanced HCC to assess the clinical outcome and whether tumor-infiltrating lymphocytes with an antitumor effect increased. In all cases, liver biopsies were performed before and after GPC3 peptide vaccination according to the protocol. This trial was approved by the Ethics Committee of

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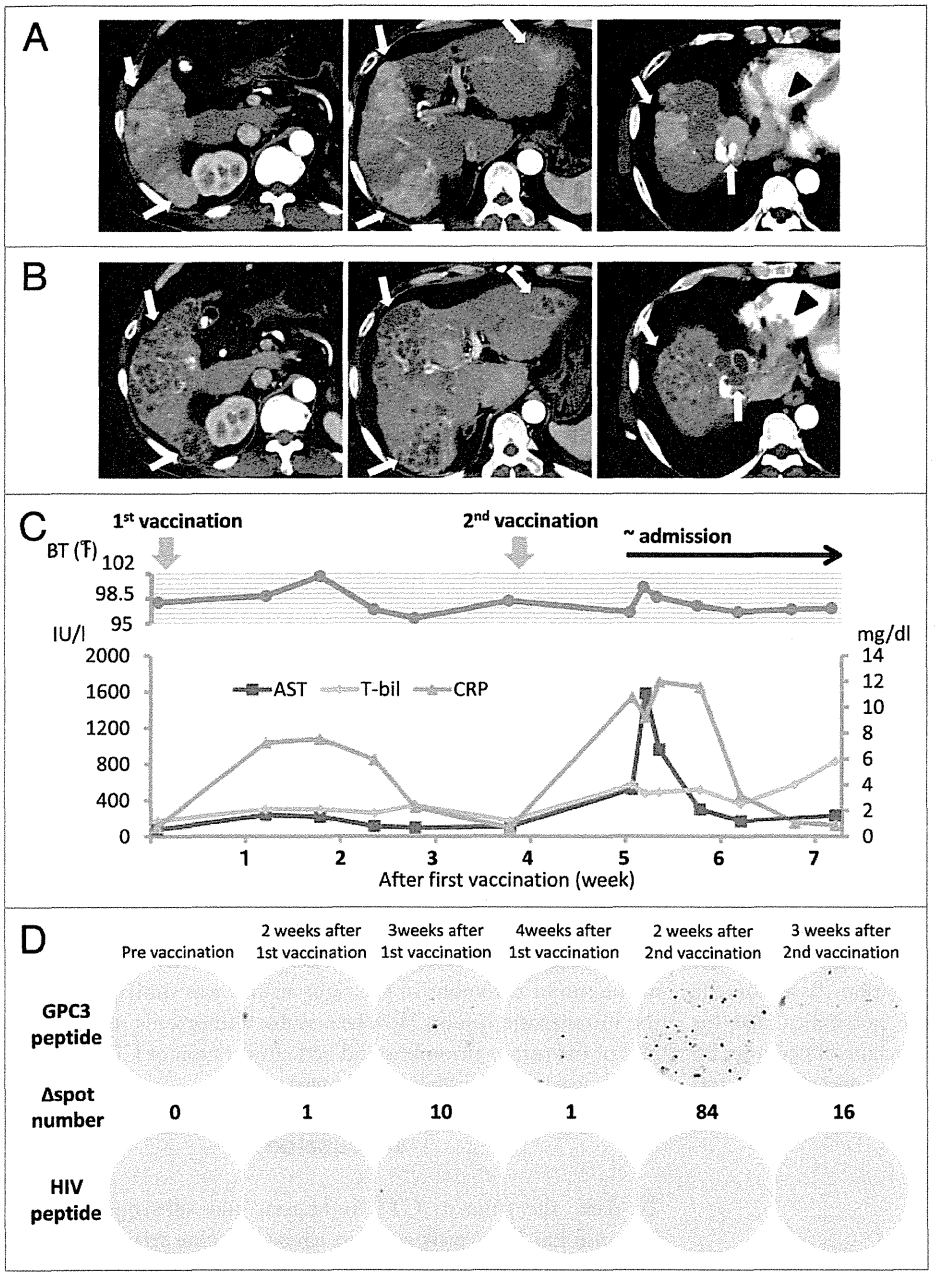

Figure 1. Findings of an early-phase contrast-enhanced CT (CT) scan. (**A**) Contrast-enhanced CT scan before vaccination shows a 68 × 51-mm tumor with multiple intrahepatic tumors (arrow) and a 44 × 30-mm tumor invading the right atrium (arrowhead). (**B**) Contrast-enhanced CT after the second vaccination showing multiple low-density areas in the liver, indicating extensive tumor necrosis (arrow). By contrast, a tumor thrombus in the right atrium increased to a 83 × 50-mm tumor (arrowhead). (**C**) Clinical course from the beginning of GPC3 peptide vaccination. Approximately 1 week after the first vaccination, the patient began reporting general fatigue and showed intermittent fever. Inflammatory and hepatic parameters were elevated (CRP: pink line, AST: red line, T-bil: green line). The abnormal laboratory parameters improved after observation. On day 9 after the second vaccination, the patient was admitted to our hospital as an emergency due to fever and general fatigue, which were similar to his previous symptoms. One day after hospitalization, the inflammatory and hepatic parameters were remarkable. Inflammatory and hepatic parameters improved 1 week after hospitalization. However, his status gradually worsened, and he died on day 30 after the second vaccination. (**D**) Immunological monitoring of the GPC3 peptide-specific T cell responses. Ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assays against GPC3 in 5 × 10^5 peripheral blood mononuclear cells (PBMCs) were performed before and after vaccination. The Δ spot number indicates the number of GPC3 peptide-specific cytotoxic T-lymphocytes (CTLs). The number of interferon (IFN)-γ positive spots increased from 0 to 84 after the second vaccination.

the National Cancer Center and registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number 000005093). The patient described herein was the first case examined pathologically using autopsy specimens. Here, we present the clinical course and pathological study, including an autopsy, of a patient with advanced HCC who revealed remarkable tumor lysis immediately after the second vaccination in an ongoing clinical trial of a GPC3 peptide vaccine.

Patient presentation. A 62-year-old male had a history of asymptomatic chronic hepatitis C. In September 2009, he was diagnosed with HCC. Laboratory data disclosed no abnormalities. Abdominal CT (CT) scans showed four lesions in the liver, and the patient was treated four times with hepatic artery chemoembolization. In December 2010, CT scans revealed a new lesion indicative of a tumor thrombus extending into the inferior vena cava. The patient was treated with sorafenib. However, the sorafenib treatment was discontinued in January 2011 due to progressive multiple intrahepatic tumors.

As no established therapeutic regimens exist for this condition, he was offered participation in a clinical trial of a GPC3 peptide vaccine for advanced HCC. HLA-typing revealed an HLA-A2 phenotype. The patient had a performance status of 0, and Child-Pugh class B disease. The patient did not have active HBV infection or rapidly progressive tumor thrombus before enrollment, met the eligibility criteria, and was enrolled after providing informed consent. Early-phase contrast-enhanced CT before treatment showed a maximum 68 × 51-mm tumor with multiple intrahepatic tumors and a 44 × 30-mm tumor invading the right atrium (**Fig. 1A**). Pretreatment tumor markers were as follows: α fetoprotein (AFP), 852 ng/mL and des-gamma-carboxy prothrombin (DCP), 1346 mAU/mL. A liver biopsy was performed 1 week prior to GPC3

peptide vaccination according to the protocol. In April 2011, 3 mg of HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (American Peptide Co.) emulsified with incomplete Freund's adjuvant (Montanide ISA-51VG; SEPPIC) was injected intradermally as the vaccine following Good Manufacturing Practice guidelines. The patient had a low-grade fever on day 6 following the first vaccination, and inflammatory and hepatic parameters were elevated on day 12 (**Fig. 1C**). The abnormal laboratory findings improved later. Therefore, he received the second vaccination on day 26 after the first vaccination. On day 9 after the second vaccination, the patient was admitted to our hospital with a high fever and general fatigue. On admission, the patient's C-reactive protein (CRP) level (10.76 mg/dL) and laboratory hepatic parameters were elevated. One day after hospitalization, aspartate aminotransferase and alanine aminotransferase and levels were elevated to 1,580 IU/L and 1,112 IU/L, respectively. The prothrombin time-international normalized ratio increased from 1.18 to 1.51. But the patient did not have ammonemia or asterixis. As seen by early-phase contrast-enhanced CT scan, most tumors in the liver were not contrast enhanced. Findings of the CT scan indicated tumor necrosis and regression. In contrast, the size of the tumor thrombus in the right atrium increased to a maximum of 83 × 50 mm (**Fig. 1B**). Levels of the tumor markers AFP and DCP decreased temporarily to 634 ng/mL and 777 mAU/mL, respectively. He was infused with a liver-supporting agent (monoammonium glycyrrhizinate, glycine, and L-cysteine hydrochloride hydrate). The inflammatory and hepatic parameters improved 1 week after hospitalization (**Fig. 1C**). We did not perform a liver biopsy when the hepatic parameters were elevated because they improved promptly. Nevertheless, his status worsened gradually. Protocol treatment was discontinued due to progressive disease and he died 30 days after the second vaccination. Based on the clinical course, we could not rule out the possibility that his condition had worsened as a result of the vaccine. Therefore, an autopsy was performed to determine the main cause of death and the elevated hepatic parameters, and to evaluate the anti-tumor effect of vaccination.

Results

Immunological analysis and autopsy. Generally, CTLs specific for tumor antigens cannot be detected directly ex vivo; they can be detected only after expansion by repeated in vitro stimulation with the antigenic peptide in conjunction with appropriate antigen-presenting cells. This is attributed to the sensitivity of the assay and the low frequency of tumor-antigen-specific CTLs.[12] GPC3 peptide-specific CTLs in PBMCs, which can be detected directly ex vivo without in vitro stimulation, can provide strong immunological evidence. An ex vivo IFN-γ ELISPOT assay was performed, as described previously.[13] The number of GPC3 peptide-specific CTLs increased from 0 to 84 in 5×10^5 PBMCs after the second vaccination (**Fig. 1D**). This result led us to anticipate a good clinical response because the increased number of CTLs and the specific CTL number correlated with the clinical response in a previous trial of the GPC3 peptide vaccine.[11]

A liver biopsy was performed before vaccination according to protocol. Histological examination of the specimen revealed well-differentiated HCC. Immunohistochemical staining showed expression of GPC3 and HLA class I in the cytoplasm and membranes of the carcinoma cells and a few CD8-positive T cells in the carcinoma tissue before vaccination (**Fig. 2A**).

A general autopsy (with the exception of the brain) was performed 2 h following death. Macroscopic findings of the liver revealed multiple macro-nodular lesions with central necrosis mainly in the right lobe (**Fig. 2B**, left). As the tumor occupied most of the right atrium, the main cause of death was circulatory failure due to progressive tumor thrombus (**Fig. 2B**, right). We judged that his condition had worsened as a result of the tumor thrombus. A histological examination showed central necrosis in most of the tumor in the right lobe, and viable carcinoma cells remained around the necrotic tissue, whereas a cirrhotic nodule adjacent to the carcinoma tissue was not necrotic (**Figs. 2C and 3A**). Immunohistochemical staining revealed GPC3-positive carcinoma cells (**Fig. 3A**). There was infiltration of CD8-positive T cells (brown) in the residual carcinoma, but not within the cirrhotic area (**Figs. 2C and 3A**). We did not detect degeneration or necrosis of the hepatocytes in the non-tumor liver parenchyma of the left lobe. These findings suggest that the elevated hepatic parameters in our patient were due to an antitumor effect. We diagnosed that the cause of death was unlikely to be related to vaccine-induced liver injury. We focused on the necrotic area around the cirrhotic nodules, in which CD68-positive macrophages (brown) aggregated (**Fig. 3B**). CD8-positive T cells also infiltrated the marginal zone between the necrotic area and noncancerous cirrhotic nodule, suggesting that carcinoma cells were attacked by CD8-positive T cells, which may have resulted in necrosis (**Fig. 3B**). The histology of the tumor thrombus in the right atrium was similar to that of the intrahepatic tumor. However, viable tumor cells remained in half of the tumor thrombus and little infiltration of CD8-positive T cells was detected (data not shown).

Discussion

To date, the time to CTL induction and subsequent tumor response has been prolonged in cancer vaccine trials.[14] By contrast, no discrepancy regarding the time between CTL induction and tumor response was observed in our phase I trial of a GPC3 peptide vaccine.[11] In this case, central necrosis of each intra-hepatic tumor was observed at the time of a strong immunological response against the GPC3 peptide, immediately after the second vaccination.

We did not perform a liver biopsy when the hepatic parameters were elevated. A biopsy may be necessary to rule out vaccine-induced liver injury when the hepatic parameters are elevated. However, the clinical course and autopsy results suggested that the elevated hepatic parameters in our patient were due to an antitumor effect.

Treatment-induced necrosis is included in the modified RECIST assessment for HCC.[15] Therefore, a positive radiographic response following vaccination, suggesting tumor necrosis, could be evaluated as a treatment response.

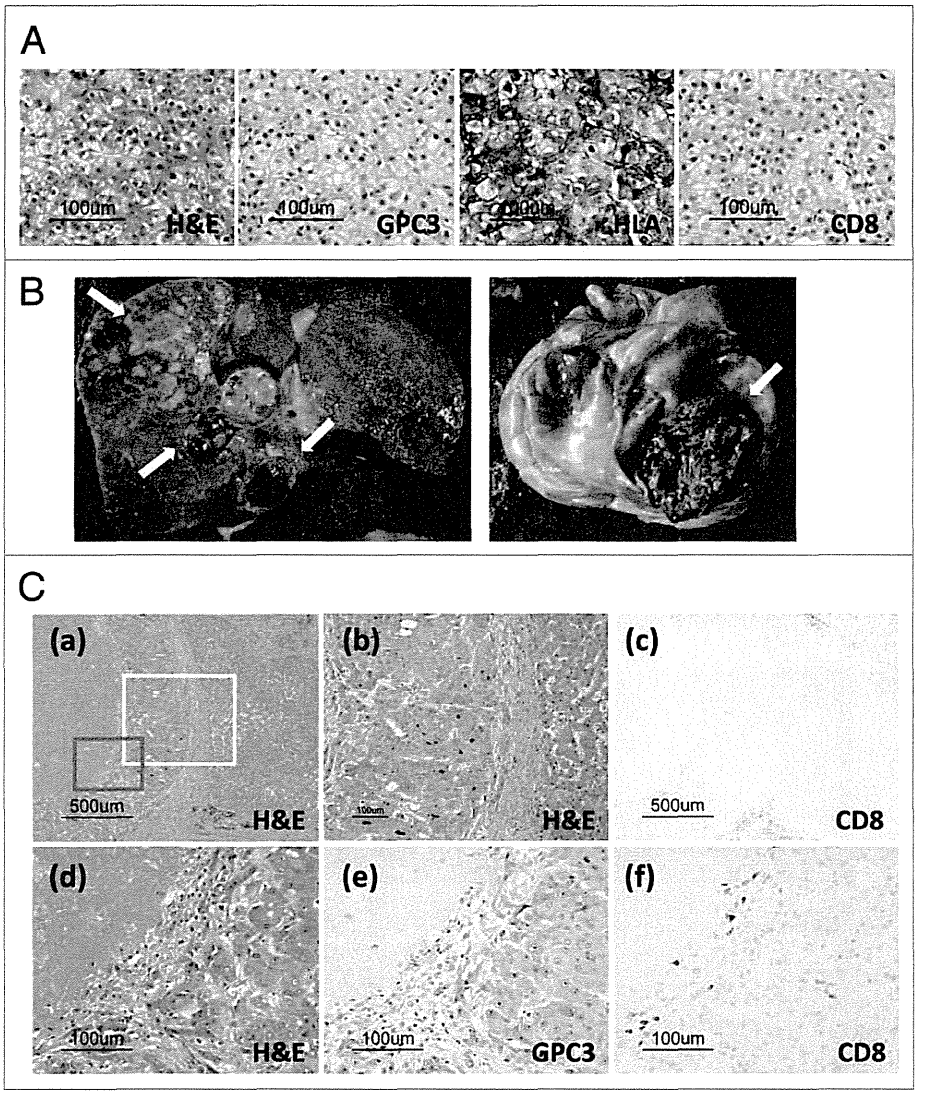

Figure 2. (**A**) Pathological findings of liver biopsy specimens before vaccination. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows well-differentiated hepatocellular carcinoma (HCC). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the cytoplasm and membranes of carcinoma cells, respectively. No CD8-positive T cells were observed in carcinoma tissue before vaccination. (**B**) Macroscopic findings of the liver and heart before formalin fixation at the time of autopsy. Most liver tumors had a necrotic area (arrow). A tumor thrombus occupied most of the right atrium (arrow). (**C**) Pathological findings of the autopsy specimen. (**a**) Microscopic images of H&E-stained sections showing central necrosis of carcinoma tissue, whereas a cirrhotic nodule adjacent to the carcinoma tissue was not necrotic. (**b**) Magnified image of the area enclosed within the white box in (**a**) showing that cancer cells exhibited a morphology (left) different from that of cirrhotic cells (right). (**c**) CD8-positive T cells (brown) infiltrated the carcinoma cells accompanied by necrosis. In contrast, no infiltration of CD8-positive T cells was detected within the cirrhotic nodule. (**d**) Magnified image of the area enclosed within the red box in (**a**) showing necrosis and viable carcinoma cells. (**e**) Positive immunohistochemical GPC3 staining was observed in only the cytoplasm of carcinoma cells. (**f**) CD8-positive T cells infiltrated the necrotic area and carcinoma tissue.

Necrosis was found in the center of each tumor; therefore, the central necrosis caused by ischemia, in addition to CD8-positive T cells attacking tumor cells, may have led to tumor necrosis. Three findings support the hypothesis that tumor necrosis was caused by CD8-positive T cells, as follows: (1) the necrotic changes determined by CT after vaccination, accompanied by clinical laboratory data; this was consistent with an immune response, although no tumor necrosis was evident on the CT before vaccination; (2) no necrosis was evident in the left lobe (no tumors) of the autopsy liver specimen, but it was present in the right liver lobe (tumors present); and (3) CD8-positive T cells infiltrated residual viable tumor cells. The analyses used in this study may contribute to identifying the pathological state after vaccination.

We detected infiltration of CD8-positive T cells into the hepatic tumors, but little infiltration of CD8-positive T cells into

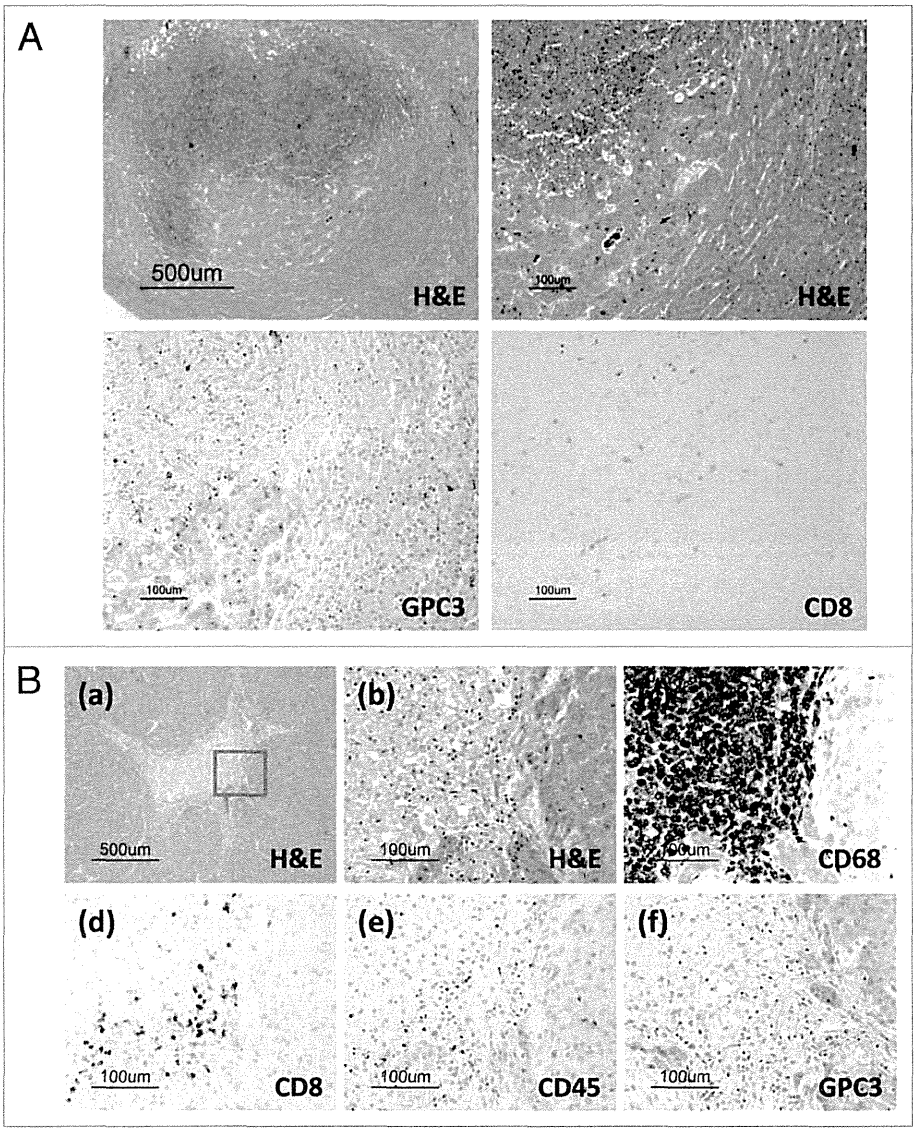

Figure 3. Pathological findings in the autopsy specimen. (**A**) Carcinoma in a cirrhotic nodule. CD8-positive T cells (brown) infiltrated only the carcinoma area, accompanied by necrosis. No infiltration of CD8-positive T cells was detected in the cirrhotic nodule. Only carcinoma cells were GPC3-positive by immunohistochemical staining. (**B**) Necrotic area surrounded by cirrhotic nodules. (**a**) Necrosis was surrounded by viable cirrhotic cells. (**b**) The margin between the necrosis and the cirrhotic nodule. This portion is enclosed by the red box in (**a**). (**c**) CD68-positive macrophages (brown) aggregated in the necrotic area around the cirrhotic nodule. (**d**) CD8-positive T cells (brown) infiltrated the necrotic area but not the cirrhotic nodule. (**e**) CD45-positive lymphocytes infiltrated the necrotic area. Based on the image in (**d**), most of the lymphocytes were CD8-positive T cells. (**f**) Cirrhotic cells did not express GPC3.

the tumor thrombus. This discrepancy may have been caused by the heterogeneity associated with immune-escape mechanisms in tumor cells.

This case report of central necrosis in a patient with HCC might be regarded as spontaneous regression correlated with circulatory failure due to a massive tumor embolism. It was not known whether the tumor necrosis was induced by CTLs, ischemia, or other factors. However, the infiltration of CD8-positive T cells into tumor cells supports immune-related necrosis.

The rate of spontaneous partial regression among patients with HCC is 0.406% compared with the control arm of a randomized clinical trial.[16] In contrast, three of 33 patients who received GPC3 peptide vaccination in the phase I trial had suspicious tumor necrosis on CT scans. In one report, massive infiltration of CD8-positive T cells in the remaining liver tumor and tumor necrosis were identified by histological examination of a biopsy specimen after vaccination.[11] Indeed, on-going clinical trials of the GPC3 peptide vaccine will provide additional information and further demonstrate the antitumor effect.[17,18] Histological results at the estimated time of a strong GPC3-specific CTL response suggest that GPC3 peptide vaccination may be a promising approach to treat HCC.

Materials and Methods

Ex vivo interferon-γ (IFN-γ) enzyme-linked immunospot assay. An ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed to evaluate the antigen-specific CTL response, as described previously.[13] Briefly, peripheral blood (30 mL) was obtained from the patient before the first vaccination and 2 weeks after each vaccination and centrifuged on a Ficoll-Paque gradient. Non-cultured peripheral blood mononuclear cells (PBMCs) (5×10^5/well) were added to plates in the presence of 10 μg/mL peptide antigens and incubated for 20 h. The GPC3 antigen used was the HLA-A2-restricted $GPC3_{144-152}$(FVGEFFTDV) peptide. PBMCs with the HLA-A2-restricted HIV19–27 (TLNAWVKVV) peptide (ProImmune) were used as negative controls. Assays were performed in duplicate.

Immunohistochemical analysis. Immunohistochemical staining with monoclonal antibodies against GPC3 (clone, 1G12; Biomosaics), HLA class I (clone, EMR8/5; Hokudo), CD8 (clone, 1A5; Novocastra), CD45 (cloned 2B11 and PD7/26; Ventana), and CD68 (clone, KP-1; Ventana) was performed according to the manufacturer's protocol.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to declare with regard to this study.

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Analysis of cytotoxic T lymphocytes from a patient with hepatocellular carcinoma who showed a clinical response to vaccination with a glypican-3-derived peptide

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Abstract. Glypican-3 (GPC3), which is a carcinoembryonic antigen, is overexpressed in human hepatocellular carcinoma (HCC). Previously, we performed a phase I clinical trial of GPC3-derived peptide vaccination in patients with advanced HCC, and reported that GPC3 peptide vaccination is safe and has clinical efficacy. Moreover, we proposed that a peptide-specific CTL response is a predictive marker of overall survival in patients with HCC who receive peptide vaccination. In this study, we established GPC3-derived peptide-specific CTL clones from the PBMCs of an HLA-A*02:07-positive patient with HCC who was vaccinated with an HLA-A2-restricted GPC3 peptide vaccine and showed a clinical response in the phase I clinical trial. Established CTL clones were analyzed using the IFN-γ ELISPOT assay and a cytotoxicity assay. GPC3 peptide-specific CTL clones were established successfully from the PBMCs of the patient. One CTL clone showed cytotoxicity against cancer cell lines that expressed endogenously the GPC3 peptide. The results suggest that CTLs have high avidity, and that natural antigen-specific killing activity against tumor cells can be induced in a patient with HCC who shows a clinical response to vaccination with the $GPC3_{144-152}$ peptide.

Introduction

Primary liver cancer, which is frequently hepatocellular carcinoma (HCC), is the sixth most common cancer and third most frequent cause of cancer-related death worldwide, and it is becoming more prevalent not only in East Asia, South-East Asia, and Africa but also in Western countries (1-3). Recently, the multikinase inhibitor sorafenib was demonstrated to prolong overall survival (OS) in patients with advanced HCC, and it has become the standard drug for first-line systemic treatment (4-6). However, based on the Response Evaluation Criteria in Solid Tumors (RECIST), the response rate for sorafenib is rather low, and the incidence of adverse events is relatively high, especially in elderly patients (7). Therefore, the generation of a novel effective therapy for HCC is a priority.

Immunotherapy is an attractive option for treating HCC. Many of the tumor antigens associated with HCC are potential candidates for peptide vaccines (8,9). The carcinoembryonic antigen Glypican-3 (GPC3), which is a 65-kDa protein of 580 amino acids, belongs to the family of glycosyl-phosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans (HSPG) (10,11). GPC3 is specifically overexpressed in HCC (72-81% of cases) and correlates with poor prognosis (12-16). This suggests that GPC3 is an ideal target for anti-HCC immunotherapy.

We have previously demonstrated the antigenicity of GPC3, and that the HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) peptide and the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide can induce GPC3-reactive CTLs without inducing autoimmunity (17-21).

HLA-A2 is the most frequent HLA-A type in all ethnic groups (22). HLA-A2 is also expressed in about 40% of Japanese persons (23,24) and in about 50% of Caucasians (25). Among Caucasians, >90% of HLA-A2-positive individuals carry the HLA-A*02:01 allele (25), whereas among the Japanese, there are multiple common and well-documented (CWD) allelic variants, including HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 (26). The frequencies of the HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 alleles in the Japanese population are 19, 14 and 7%, respectively (26). Therefore, we confirmed that the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide could also bind to HLA-A*02:06 and HLA-A*02:07 using a binding assay (unpublished data).

On the basis of these results, we conducted a phase I clinical trial of a GPC3-derived peptide vaccine in 33 patients with advanced HCC. The HLA-A*24:02-restricted $GPC3_{298-306}$ peptide was used for HLA-A*24:02-positive patients and

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Key words: glypican-3, peptide vaccine, CTL clone

the HLA-A*02:01-restricted $GPC3_{144-152}$ peptide was used for HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07-positive patients. We found that GPC3 vaccination was well-tolerated, and that the GPC3 peptide vaccine induced a GPC3-specific CTL response in almost all of the patients (27-30). Moreover, the vaccination-induced GPC3-specific CTL response correlated with overall survival (OS); the OS was significantly longer in patients with high GPC3-specific CTL frequencies than in those with low GPC3-specific CTL frequencies (27). In terms of clinical responses, one patient showed a partial response (PR) and 19 patients showed stable disease 2 months after initiation of treatment. One patient with HCC who showed a PR was HLA-A*02:07-positive. In addition, several HLA-A*02:01-restricted GPC3 peptide-specific CTL clones with cytotoxic activities against GPC3 were established from the peripheral blood mononuclear cells (PBMCs) of patients vaccinated in this trial (27).

The aims of the present study were: i) to establish GPC3-derived, peptide-specific CTL clones from the PBMCs of an HLA-A*02:07-positive patient with HCC who showed a PR in the phase I clinical trial; and ii) to analyze the functions of these CTL clones.

Materials and methods

Ethics information. 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. All the patients gave written informed consent before entering the study at the National Cancer Center Hospital East (Chiba, Japan). The trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR no. 000001395).

PBMCs collection. Peripheral blood samples were obtained pre- and post-vaccination from the patient with HCC who was HLA-A*02:07-positive. Post-vaccination, blood samples were collected from the patient every 2 weeks. The GMP-grade peptide $GPC3_{144-152}$ (FVGEFFTDV) (American Peptide Co., Sunnyvale, CA, USA) was emulsified in IFA (Montanide ISA-51 VG; SEPPIC, Paris, France) and injected intradermally at 30 mg/body three times at 14-day intervals (27,28). PBMCs were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and frozen in liquid nitrogen until use.

Cell lines. The human lung cancer cell line 1-87 ($GPC3^-$, HLA-A*02:07$^+$/A*11:01$^+$) and hepatitis B virus (HBV)-integrated human hepatocellular carcinoma cell line JHH-7 ($GPC3^+$, HLA-A*24:02$^+$/A*31:01$^+$) were conserved in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Company, St. Louis, MO, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA).

Plasmids and transfection. The expression vectors pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pcDNA3.1 that contained the HLA-A*02:07 cDNA were used for the transfection experiments. The pcDNA3.1 construct that contained HLA-A*02:07 was kindly provided by Dr Ryo Abe (Tokyo University of Science, Chiba, Japan). The JHH-7/HLA-A*02:07 cell line was obtained by transfection of JHH-7 cells with the expression vector using FuGENE HD (Roche Applied Science, Mannheim, Germany). JHH-7/mock and JHH-7/HLA-A*02:07 cells were cultured in DMEM that was supplemented with 10% heat-inactivated FBS and 1 mg/ml G418 (Calbiochem, Darmstadt, Germany).

Induction of $GPC3_{144-152}$ peptide-specific CTLs from PBMCs. The PBMCs were cultured ($2x10^6$ cells/well) with the $GPC3_{144-152}$ peptide in RPMI-1640 (Sigma Chemical Company) that was supplemented with 10% heat-inactivated FBS, 100 IU/ml recombinant human IL-2 (Nipro, Osaka, Japan), and 10 ng/ml recombinant human IL-15 (PeproTech Inc, Rocky Hill, NJ, USA) for 14 days.

CD107a staining and flow cytometry analysis. $CD8^+$ T cells were isolated using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs that were stimulated with the $GPC3_{144-152}$ peptide for 14 days. The $CD8^+$ T cells were incubated with $GPC3_{144-152}$-pulsed or HIV_{19-27}-pulsed 1-87 cells at a ratio of 2:1 for 3.5 h at 37°C. CD107a-specific antibodies (BD Biosciences, San Jose, CA, USA) were included in the mixture during the incubation period.

Generation of CTL clones. $CD8^+$ $CD107a^+$ cells were sorted using a FACSAria cell sorter (BD Biosciences). Sorted CTLs were stimulated and the CTL clones were established as previously described (28).

Cytotoxicity assay. Cytotoxic capacity was analyzed with the Terascan VPC system (Minerva Tech, Tokyo, Japan). The CTL clone was used as the effector cell type. Target cells were labeled in calcein-AM solution for 30 min at 37°C. The labeled cells 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 previously described (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 or without peptide for 1.5 h at room temperature and then washed three times. Responder cells were incubated with stimulator cells for 20 h. The resulting spots were counted using an ELIPHOTO counter (Minerva Tech).

Determination of recognition efficiency. Calcein-AM-labeled target cells were pulsed with various concentrations of peptide, starting at 10^{-6} M and decreasing in log steps to 10^{-14} M. The CTL clones were incubated with the target cells at an effector:target (E/T) ratio of 10:1 for 4 h. The recognition efficiencies of the CTL clones were defined as previously described (28).

RNA interference. Human GPC3-specific siRNAs were chemically synthesized as double-strand RNA (Invitrogen). A non-silencing siRNA, AllStras Neg. Control siRNA, was obtained from Qiagen (Valencia, CA, USA). The following

GPC3-specific siRNA sequences were used: GPC3-siRNA (#4149), 5'-UUAUCAUUCCAUCACCAGAGCCUCC-3'; GPC3-siRNA (#4150), 5'-GGAGGCUCUGGUGAUGGAAU GAUAA-3': and GPC3-siRNA (#4151), 5'-UAUAGAUGACUG GAAACAGGCUGUC-3'. Synthetic siRNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocols.

RT-PCR. Using the TRIzol reagent (Invitrogen), we extracted total cellular RNA from untreated or siRNA (GPC3-siRNA or negative-siRNA)-treated JHH-7/HLA-A*02:07. cDNA was synthesized using the PrimeScript II 1st Strand cDNA Synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The cDNA was added to a reaction mix that contained 10X Ex Taq Buffer (Takara), 2.5 mM dNTP mixture (Takara), 5 units Ex Taq (Takara), and 10 μM of the *GPC3*- or *β-actin*-specific PCR primers. The following primer sequences (sense and antisense, respectively) were used: for *GPC3*, 5'-AGCCAAAAGGCAGCAAGGAA-3' and 5'-AAGA AGAAGCACACCACCGA-3'; and for *β-actin*, 5'-CCTCGCCT TTGCCGATCC-3' and 5'-GGATCTTCATGAGGTAGTC AGTC-3'. PCR was performed using the 96-well Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). PCR was performed for 20 cycles of 98°C for 10 sec, 64°C for 30 sec and 72°C for 30 sec, followed by a step of 72°C for 10 sec.

Sequence analysis of TCR-β gene. Using the TRIzol reagent (Invitrogen), total cellular RNA was extracted from established CTL clones. The cDNA of the *TCR-β* gene was synthesized using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions, with the modification that we used 200 nM of the primer specific for the TCR-β chain constant region. The cDNA products were subjected to 2-step PCR, as previously described by Yukie Tanaka-Harada (35,36), and the PCR products were purified and sequenced in the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). The *TCR-β* variable (*TRBV*) gene, *TCR-β* joining (*TRBJ*) gene, *TCR-β* diversity (*TRBD*) alleles, and complementarity-determining region 3 (*CDR3*) sequences were identified using the IMGT databases (http://www.imgt.org/).

Results

$GPC3_{144-152}$ peptide-specific CTLs in the peripheral blood of the patient exert a clinical effect. We analyzed the immune responses of the patient who showed a PR following $GPC3_{144-152}$ peptide vaccination. In this patient, the supraclavicular lymph node metastases markedly regressed, two liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (27). The levels of DCP decreased in the patients over the 2-month period. We evaluated the $GPC3_{144-152}$-specific immune responses in the peripheral blood using the *ex vivo* IFN-γ ELISPOT assay. For the HLA-A*02:07-positive patient with advanced HCC, the number and area of the spots increased after two rounds of vaccination, as compared with the pre-vaccination values, and the peak values were noted 10 weeks after the start of the treatment (Fig. 1A).

Establishment of $GPC3_{144-152}$-specific CTL clones from the PBMCs of the patient. To investigate the ability of the $GPC3_{144-152}$-specific CTLs induced by peptide vaccination to recognize antigen, we established CTL clones from the PBMCs of this patient 10 weeks after the start of treatment. The PBMCs were stimulated with the $GPC3_{144-152}$ peptide *in vitro* for 14 days. $CD8^+$ T cells were isolated from the stimulated PBMCs, and then incubated with peptide-pulsed 1-87 cells. $CD8^+$ $CD107a^+$ cells that reacted with the $GPC3_{144-152}$-pulsed 1-87 cells were sorted to the single-cell level. Thus, we established $GPC3_{144-152}$ peptide-specific CTL clones.

Three established CTL clones were analyzed for function using the IFN-γ ELISPOT assay and cytotoxicity assay. All of the CTL clones released IFN-γ in response to the $GPC3_{144-152}$-pulsed 1-87 cells, but not in response to non-pulsed 1-87 cells (Fig. 1B). Moreover, these CTL clones showed cytotoxicity against $GPC3_{144-152}$-pulsed 1-87 cells, but not against non-pulsed or HIV19-27-pulsed 1-87 cells (Fig. 1C). These results indicate that the CTL clones 24-4-2, 24-4-7 and 24-2-10 have specificity for the $GPC3_{144-152}$ peptide.

Functional avidity of the $GPC3_{144-152}$-specific CTL clones. We evaluated the cytotoxicity profiles of the CTL clones for 1-87 cells pulsed with a decreasing concentration series (from 10^{-6} to 10^{-14} M) of the $GPC3_{144-152}$ peptide. The peptide concentration at which the curve reached 50% cytotoxicity was defined as the recognition efficiency of the clone. The recognition efficiencies of CTL clones 24-4-2, 24-4-7 and 24-2-10 were 10^{-11}, 10^{-9} and 10^{-8} M, respectively (Fig. 2). This result suggests that CTL clone 24-4-2 has a higher avidity than the other two clones and, conversely, that CTL clone 24-2-10 has a lower avidity than the other two clones.

A $GPC3_{144-152}$-specific CTL clone recognizes cancer cells that endogenously express GPC3. Next, we tested the reactivities of these CTL clones against cancer cell lines that expressed GPC3 and HLA-A*02:07. We used the JHH-7/mock ($GPC3^+$, HLA-A*02:07-) and JHH-7/HLA-A*02:07 ($GPC3^+$, HLA-A*02:07$^+$) transfectants as the target cells (Fig. 3A). The CTL clone 24-4-2 (with high avidity) produced IFN-γ and was cytotoxic for JHH-7/HLA-A*02:07 cells but not for JHH-7/mock cells (Fig. 3B and C). The other clones did not produce IFN-γ and did not exhibit cytotoxicity for the two target cell lines. These results suggest that only high-avidity CTLs recognize cancer cells that express GPC3 peptide endogenously.

CTL clone 24-4-2 shows specificity for GPC3. To ascertain the GPC3 antigen-specific response of CTL clone 24-4-2, we created a GPC3 knockdown via siRNA treatment of the JHH-7/HLA-A*02:07 cells. GPC3 expression by the JHH-7/HLA-A*02:07 cells was clearly decreased by the GPC3-siRNA, as assessed by RT-PCR (Fig. 4A). We examined the IFN-γ production levels of CTL clone 24-4-2 against JHH-7/HLA-A*02:07 cells treated with GPC3-siRNA. IFN-γ production by CTL clone 24-4-2 was significantly decreased by the GPC3-siRNA (Fig. 4B). These results indicate that the HLA-A2-restricted $GPC3_{144-152}$ peptide is processed naturally by cancer cells, and that both HLA-A*02:07 and HLA-A*02:01 can present the $GPC3_{144-152}$ peptide on the surfaces of cancer cells.

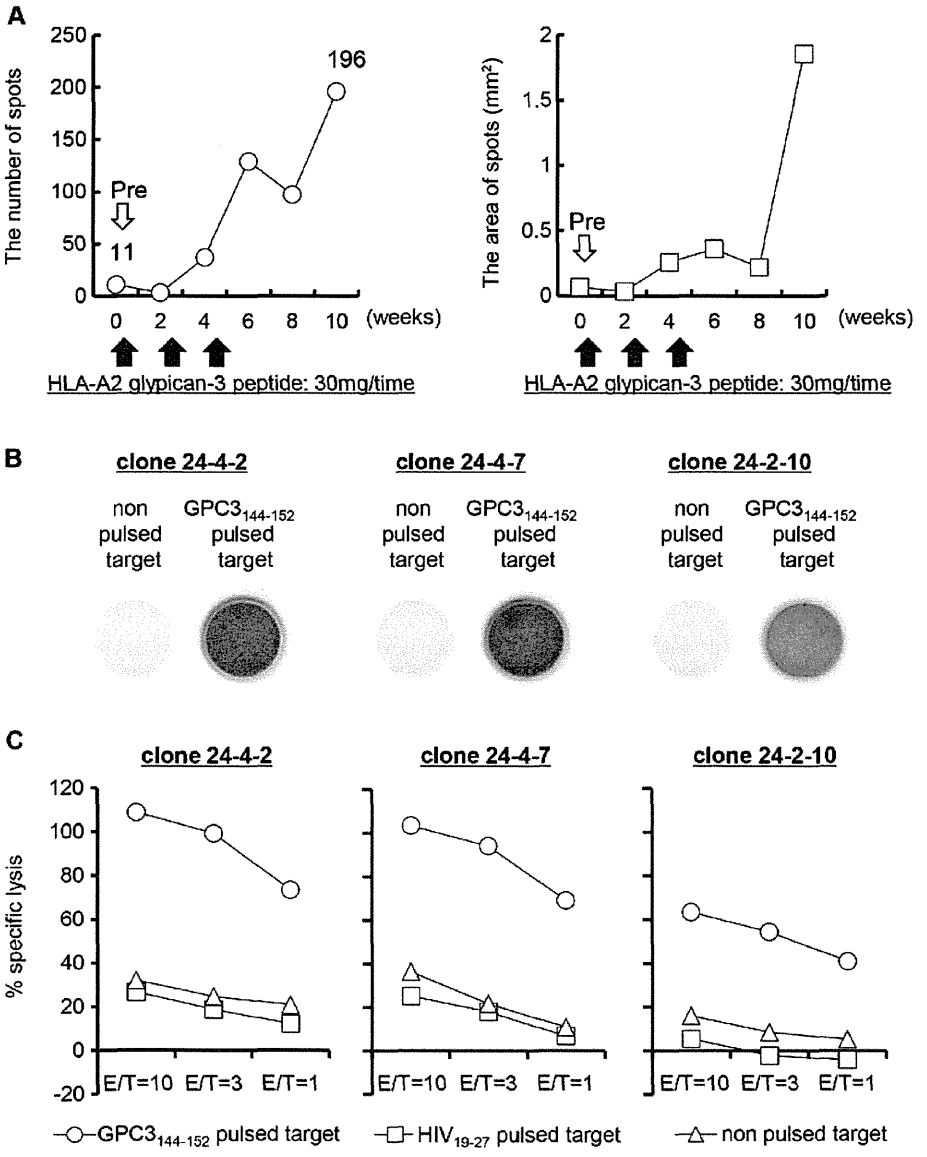

Figure 1. GPC3 peptide-specific CTL clones established from the PBMCs of a patient following GPC3 peptide vaccination. (A) Changes in the frequencies of $GPC3_{144-152}$ peptide-specific CTLs before and after vaccination in a patient who showed a PR post-vaccination. Changes in the GPC3 peptide-specific CTLs are observed as differences in the number (left) and the area (right) of spots in an *ex vivo* IFN-γ ELISPOT assay. (B) Results of the IFN-γ ELISPOT assay against peptide-pulsed target. HLA-A*02:07+ cancer cell line 1-87 was used as the target. The target was pulsed with the $GPC3_{144-152}$ peptide. A non-pulsed target was used as the negative control. The ratio of effector cells to target cells (E/T) is 1. (C) Results of the cytotoxicity assay against peptide-pulsed target. The 1-87 cells were used as the target. Non-pulsed and HIV_{19-27} peptide-pulsed targets were used as negative controls. E/Ts are 10, 3 and 1, respectively. A representative of three experiments is shown.

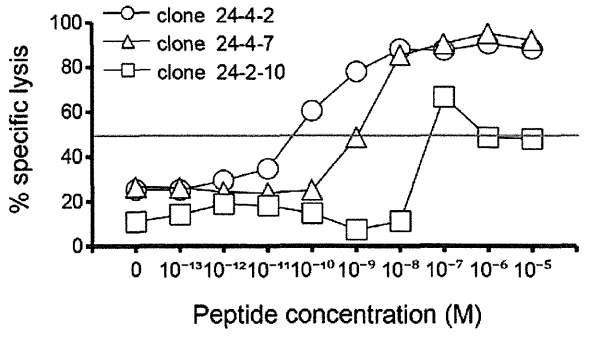

Figure 2. $GPC3_{144-152}$ peptide-specific avidity of the established CTL clones. The established CTL clones were tested for avidity using 1-87 cells that were pulsed with various concentrations of the $GPC3_{144-152}$ peptide. The peptide concentration at which the curve crossed the 50% cytotoxicity mark was defined as the recognition efficiency of that clone. E/T is 10. A representative of three experiments is shown.

Established CTL clones have different sets of TCR-β alleles. We analyzed the *TCR-β* gene sequences of the established CTL clones. The *TRBV*, *TRBJ* and *TRBD* alleles were identified using the IMGT databases. Thus, we identified the *TRBV*, *TRBD* and *TRBJ* alleles of the CTL clones (Table I). Each of the established CTL clones had different allele sets.

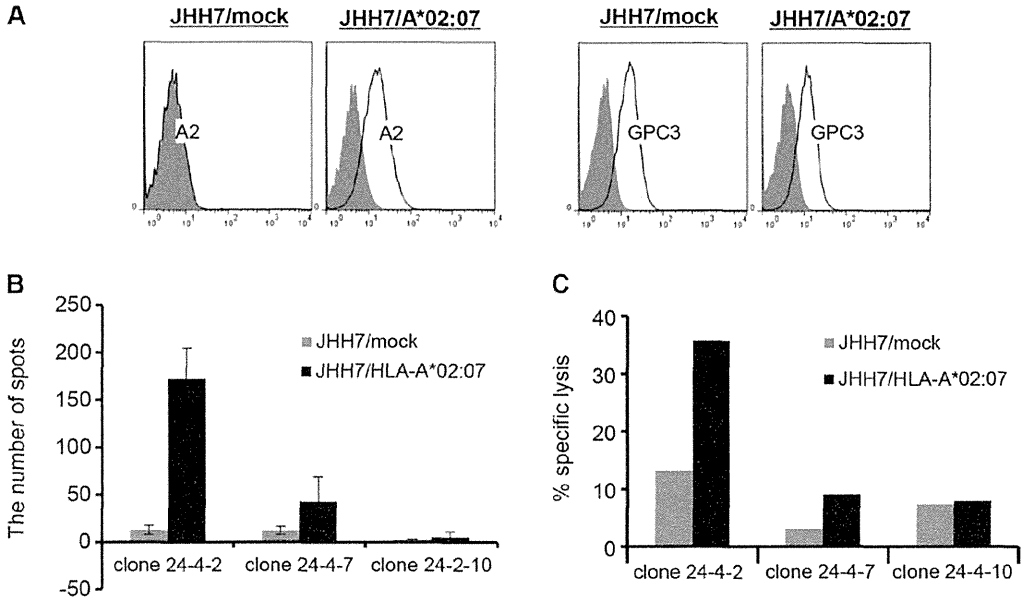

Figure 3. Recognition of GPC3$^+$ cancer cells by the established CTL clones. (A) Expression of HLA-A2 (left panel) and GPC3 (right panel) on established GPC3$^+$ HLA-A*02:07$^+$ cancer cells and control cells. (B) Results of the IFN-γ ELISPOT assay for the GPC3$^+$ cancer cell line. The HLA-A*02:07-overexpressing GPC3$^+$ cancer cell line, JHH7/HLA-A*02:07, was established and used as the target. JHH7/mock cells were used as the negative control. E/T ratio, 1. Data are presented as mean ± SD of three independent batches. (C) Results of the assay for cytotoxicity against the GPC3$^+$ cancer cell line. JHH7/HLA-A*02:07 cells were used as the target. JHH7/mock cells were used as the negative control. E/T is 3. A representative of three experiments is shown.

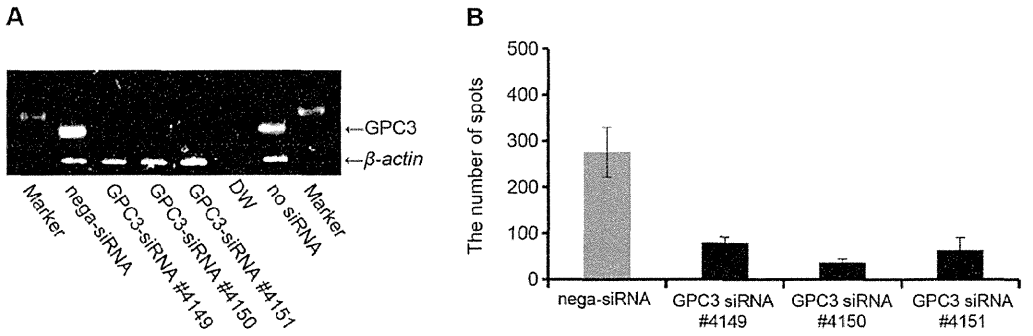

Figure 4. GPC3 specificity of CTL clone 24-4-2. (A) GPC3 expression levels on JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or negative (nega)-siRNA for 48 h, as determined by RT-PCR. (B) Results of the IFN-γ ELISPOT assay for JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or nega-siRNA. E/T is 1. Data are presented as mean ± SD of three independent batches.

*CTL clone 24-4-2 is subject to HLA-A*02:07 restriction.* We investigated whether CTL clone 24-4-2 recognized the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex and the $GPC3_{144-152}$ peptide-HLA-A*02:06 complex, as well as the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex. Healthy donor PBMCs with HLA-A*02:01, HLA-A*02:06, HLA-A*02:07 and HLA-A*24:02 were used as the targets, and an HLA-A*02:01-restricted, GPC3-specific CTL clone, which is a previously established CTL clone (26), was used as the control. The HLA-A*02:01-restricted CTL clone recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex, and CTL clone 24-4-2 recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex (Fig. 5). These outcomes indicate that CTL clone 24-4-2 has HLA-A*02:07 restriction.

Table I. TCR-β chain sequencing for established CTL clones.

No.	TRBV	TRBJ	TRBD
Clone 24-4-2	18*01	1-2*01	1*01
Clone 24-4-7	7-3*01	2-7*01	1*01
Clone 24-2-10	7-6*01	2-1*01	2*01

Discussion

Clinical trials of peptide-based vaccines are underway in several parts of the world. However, the monitoring of individual CTL post-vaccination has scarcely been reported in

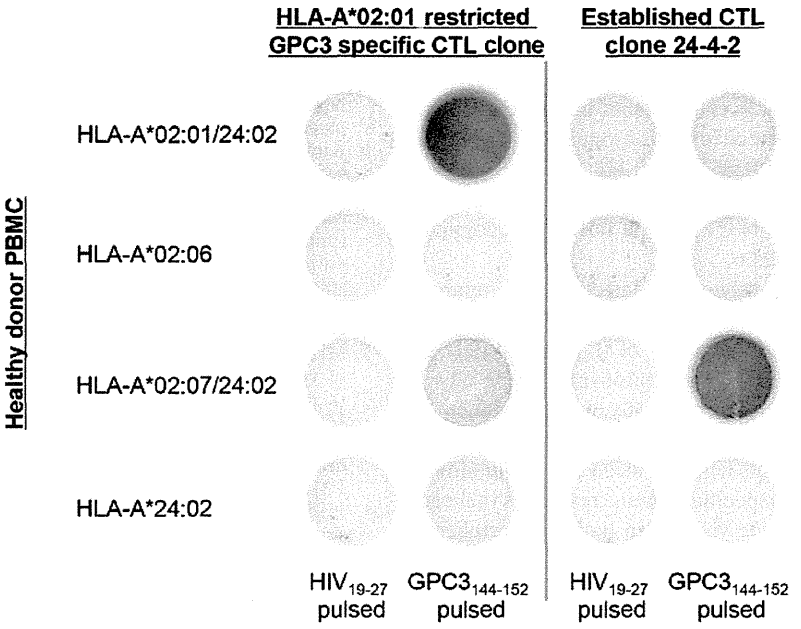

Figure 5. CTL clone 24-4-2 shows HLA-A*02:07 restriction. Results of the IFN-γ ELISPOT assay for healthy donor PBMCs with HLA-A2. The established CTL clone 24-4-2 and the HLA-A*02:01-restricted, GPC3-specific CTL clone were used as effectors. E/T is 0.2. A representative of two experiments is shown.

immunotherapy trials. In the present study, we established HLA-A*02:07$^+$ $GPC3_{144-152}$-specific CTL clones from the PBMCs of a patient who showed a PR following GPC3-derived peptide vaccination and we performed functional analyses against established CTL clones.

This patient showed an increase in the number of CTLs specific for the GPC3-derived peptide in the peripheral blood after vaccination (Fig. 1A) (27,28). Ten weeks after the start of treatment, the $GPC3_{144-152}$-specific CTL counts had increased approximately 18-fold, as compared with the pre-vaccination counts. In this case, analysis of the established CTL clones after vaccination could lend support to the notion that the vaccine-induced CTLs exert an antitumor effect, since few $GPC3_{144-152}$-specific CTLs were detected before vaccination.

In the present study, we confirmed that $GPC3_{144-152}$-specific CTL clones are cytotoxic for both $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that express GPC3 peptide endogenously. Confirming that the GPC3 peptide-specific CTL clones kill cancer cells that express endogenously the antigen peptide is important because antigen-derived and CTL-inducible peptides are not necessarily presented by cancer cells that endogenously express the antigen (31-33). Three established CTL clones showed cytotoxic activities related to their avidity for $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that expressed the GPC3 peptide endogenously. These results show that although CTLs with different avidity can be isolated, only those CTLs with high avidity can kill cancer cells that express the antigen peptide endogenously. Several investigators have demonstrated a correlation between T-cell avidity and target recognition by T-cell populations that recognize murine tumor models and human cancers (34). Our results strongly support this observation.

The TCR usage of antigen-specific T cells is thought to be influenced by the affinity of the TCR for the antigen peptide-HLA class I complex. Several studies on the TCR usage of tumor-associated antigen (TAA)-specific T cells have used the *TRBV* gene family (35-41). These studies mainly analyzed the frequencies of TAA tetramer positive CD8$^+$ T cells. Although it is important to examine quantitative aspects, such as the frequencies of TAA tetramer positive CD8$^+$ T cells, the cytotoxicity of these T cells against cancer cells that express the TAA peptide endogenously cannot be confirmed. Moreover, GPC3 dextramer positive CD8$^+$ T cells were not detected in the PBMCs of the patients with HCC before GPC3 peptide vaccination (27,28). To analyze biased usage of the *TCR* gene of GPC3 dextramer positive CD8$^+$ T cells in the patients with HCC before and after GPC3 peptide vaccination, a new detection system with greater sensitivity *ex vivo* will be required. In the present study, we analyzed the *TCR-β* genes of the established $GPC3_{144-152}$-specific CTL clones, to confirm that these CTL clones have different TCRs. Our experiments show that the established CTL clones have different TCR-β-chain allele sets, i.e., *TRBV*, *TRBD* and *TRBJ* alleles (Table I), and different CDR3 sequences (data not shown). These results suggest that various GPC3-specific CTLs are induced by $GPC3_{144-152}$ peptide vaccination.

A*HLA-A*02:07 differs from HLA-A*02:01 by a single non-conservative change (Y to C) at residue 99. X-ray crystallographic data have identified position 99 as one of the residues forming the D secondary pocket, which engages the residue at position 3 on peptide ligands (42-44). Although hHLA-A*02:07 was originally not included in the HLA-A2 supertype, cross-reactivity between HLA-A*02:07 and other A2 subtypes was detected at the functional level (44,45). Moreover, this HLA molecule indeed binds a subset of the peptide repertoire bound by other A2 subtypes (44). For these reasons, HLA-A*02:07 should also be included in the A2 supertype (46). Ito *et al* (47) and Nonaka *et al* (48) reported that an HLA-A2-restricted