activation, naïve OT-I $CD8^+$ T-cells were incubated with irradiated EG7 cells at a 3:2 ratio in 24-well plates for 3 days. Each well contained 2.4×10^6 OT-I $CD8^+$ T-cells plus 1.6×10^6 EG7 cells in 2 ml of RPMI 1640 medium supplemented with 10 % FBS, penicillin, streptomycin, and 50 μmol/l 2-mercaptoethanol. Activated OT-I $CD8^+$ T-cells were separated from EG7 cells using anti-CD8a magnetic beads before adoptive transfer.

IFN-γ ELISPOT assay

The BD™ ELISPOT set (BD Biosciences, San Jose, CA, USA) was used for an interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. CTLs were used as effector cells, and tumor cell lines with or without a peptide pulse (10 μg/ml for 1 h) were used as target cells. Effector cells (1×10^3/well) were incubated with target cells (1×10^4/well) in 200 μl of RPMI 1640 medium supplemented with 10 % FBS, penicillin, and streptomycin for 20 h at 37 °C in 5 % CO_2. The number of spots, indicating an antigen-specific CTL response, was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay

The Terascan VPC system (Minerva Tech) was used for cytotoxicity assays. Target cells were labeled with Calcein-AM (Dojindo Laboratories, Kumamoto, Japan) solution for 30 min at 37 °C, washed three times, distributed to 96-well culture plates in duplicate, and incubated with effector cells for 4 h. Fluorescence intensity was measured before and after the 4-h culture, and antigen-specific cytotoxic activity was calculated as described previously [16].

Intratumoral peptide injection

In in vivo studies, tumors implanted on the backs of mice were injected with 50 μg peptide mixed with an equal volume of incomplete Freund's adjuvant (IFA, Montanide ISA-51VG; SEPPIC, Paris, France). The total volume of solution injected was 100 μl in all experiments.

Tumor excision and isolation of tumor cells

To investigate whether the injected peptide was loaded onto HLA class I molecules of tumor cells in a solid mass, an IFN-γ ELISPOT assay was performed using these isolated tumor cells as target cells. Mice were killed and their dorsal tumors were dissected, cut into small pieces, and digested with collagenase (1.5 mg/ml) for 20 min at 37 °C.

In vivo tumor growth inhibition assay

In a peptide vaccine model, H-2 K^b-restricted $OVA_{257–264}$ peptide emulsified with IFA (50 μg/100 μl) was intradermally injected at the base of the tail of C57BL/6 mice, five times at 7-day intervals as described previously [13]. After vaccination, the induction of H-2 K^b-restricted $OVA_{257–264}$ peptide-specific CTLs was detected by IFN-γ ELISPOT assay (data not shown). In an adoptive transfer model, activated OT-I CTL (1×10^7 cells/500 μl) was intravenously injected.

SW620 cells (5×10^6 cells/100 μl) were subcutaneously implanted into the backs of BALB/c nude mice; SK-Hep-1/vec, SK-Hep-1/GPC3, or HepG2 cells (5×10^6 cells/100 μl) were implanted into NOD/SCID mice, and RMA cells (5×10^4 or 5×10^5 cells/100 μl) were implanted into C57BL/6 mice. Tumor volume was monitored twice a week and calculated using the following formula: tumor volume (mm^3) $= a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. Mortality and morbidity were checked daily, and the mice were maintained until each mouse showed signs of morbidity or the length or width of the tumors exceeded 30 mm, at which point they were killed for reasons of animal welfare.

Tetramer staining and flow cytometry analysis

For the analysis of local accumulation of antigen-specific CTLs, isolated tumor cells, including tumor-infiltrating lymphocytes, were stained with H-2 K^b OVA Tetramer-PE ($OVA_{257–264}$ [SIINFEKL]; MBL, Nagoya, Japan) for 20 min at room temperature and anti-mouse CD8-FITC (rat monoclonal, clone KT15; MBL) for 20 min at 4 °C. Flow cytometry analysis was carried out using a FACSCanto II flow cytometer (BD Biosciences).

Immunohistochemistry

To investigate whether $CD8^+$ T-cells infiltrated normal tissues due to intratumoral peptide injection in a murine adoptive cell transfer model, we performed immunohistochemical staining of CD8 in tissue specimens from C57BL/6 mice using monoclonal anti-CD8 antibody (dilution 1:20, BioLegend, San Diego, CA, USA).

Statistical analysis

Comparisons of spot numbers and tumor volume at the last time point were performed using the Mann–Whitney U test. Survival was analyzed according to the Kaplan–Meier estimate, and differences between groups were compared using the log-rank test. Differences were considered

significant at $P < 0.05$. Data were analyzed with the statistical package, Dr. SPSS II (SPSS Japan, Tokyo, Japan).

Results

In vitro CTL activity against peptide-pulsed targets

To evaluate the antigen-specific CTL response in vitro, IFN-γ ELISPOT and cytotoxicity assays were performed. In both assays, the two types of effector cells were the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTL clone, which was established from peripheral blood mononuclear cells (PBMCs) of an HCC patient who had received the $GPC3_{144–152}$ peptide vaccine [16], and the HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTL clone, which was established from PBMCs of a healthy volunteer. The target cells were tumor cell lines with or without antigenic peptide pulses.

As shown in Fig. 1a, in an IFN-γ ELISPOT assay, the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs produced IFN-γ in the presence of GPC3-expressing tumor cells, HepG2 and SK-Hep-1/GPC3, without peptide pulse. These effector cells recognized $GPC3_{144–152}$ antigen peptide, which is endogenously presented on the cell surface of the non-peptide-pulsed target cells. The number of IFN-γ-producing cells increased dramatically after the pulse of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide. In contrast, $GPC3_{144–152}$ peptide-specific CTLs did not produce IFN-γ against GPC3-negative tumor cells, SW620 and SK-Hep-1/vec, without peptide pulse. However, a marked increase in IFN-γ-producing cells was detected against these cell lines after the pulse of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide. The IFN-γ-producing cells did not increase after the pulse of HLA-A*24:02-restricted $GPC3_{298–306}$ or HLA-A*02:01-restricted $HIV_{77–85}$ peptide (Fig. 1a). Similarly, HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTLs produced IFN-γ only in the presence of HLA-A*02:01-restricted $CMV_{495–503}$ peptide-pulsed target cells (Fig. 1b).

In a cytotoxicity assay, HLA-A*02:01-restricted $GPC3_{144–152}$ and $CMV_{495–503}$ peptide-specific CTLs showed antigen-specific killing activity according to the peptide density on tumor cells. HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs showed specific cytotoxicity against HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-pulsed SW620 and T2 targets, whereas they did not show cytotoxicity against HLA-A*02:01-restricted $HIV_{77–85}$ peptide-pulsed targets (Fig. 1c). In addition, HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs showed apparent but weak cytotoxicity (13–44 %) against non-peptide-pulsed HepG2 and SK-Hep-1/GPC3 cells, but the cytotoxicity was markedly strengthened (55–99 %) against all examined cell lines after the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide pulse (Fig. 1d). Similarly, HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTLs showed $CMV_{495–503}$ peptide-specific cytotoxicity against all examined cell lines pulsed with $CMV_{495–503}$ peptide (Fig. 1e).

The peptide-specific CTLs showed strong activity against all peptide-pulsed cell lines, regardless of whether the tumor cells expressed the antigen. The density of the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide endogenously presented on tumor cells was not enough to induce strong CTL activity.

Loading of injected peptide onto HLA class I molecules of tumor cells in vivo

As shown in Fig. 2a, BALB/c nude mice were inoculated subcutaneously on their backs with SW620 ($GPC3^-$) tumor cells. When tumor diameters reached 5–7 mm, 50 μg HLA-A*02:01-restricted $GPC3_{144–152}$ peptide was injected into the tumor. After 2–96 h, the tumors were dissected, cut into small pieces, and digested with collagenase (1.5 mg/ml) for 20 min at 37 °C. To investigate whether the injected HLA-A*02:01-restricted $GPC3_{144–152}$ peptide was loaded onto HLA class I molecules of tumor cells in a solid mass, an IFN-γ ELISPOT assay was performed in duplicate using these isolated tumor cells as target cells and HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs as effector cells.

Loading of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide onto HLA class I of tumor cells was detected (Fig. 2b). Without IFA, the density of loaded peptide gradually decreased after intratumoral peptide injection, whereas the loaded peptide density remained for 96 h after injection with IFA, suggesting that IFA is required for long-term stability of the injected peptide (Fig. 2c). Similar data were obtained with a combination of the HLA-A*02:01-restricted $CMV_{495–503}$ peptide and its specific CTLs (data not shown).

Antitumor effect of intratumoral peptide injection in an immunodeficient mouse model

We planned and executed the experimental schedule shown in Fig. 3a. Four tumors were implanted per mouse, and each tumor received a different combination of injections, as shown in Fig. 3b. From 5–7 days after tumor inoculation, mice were treated two or three times in 5-day intervals. The treatment regime was as follows: HLA-A*02:01-restricted $GPC3_{144–152}$ or $CMV_{495–503}$ peptide emulsified with IFA (50 μg/100 μl) was injected into a tumor, and, 2 h later, 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 the tumor.

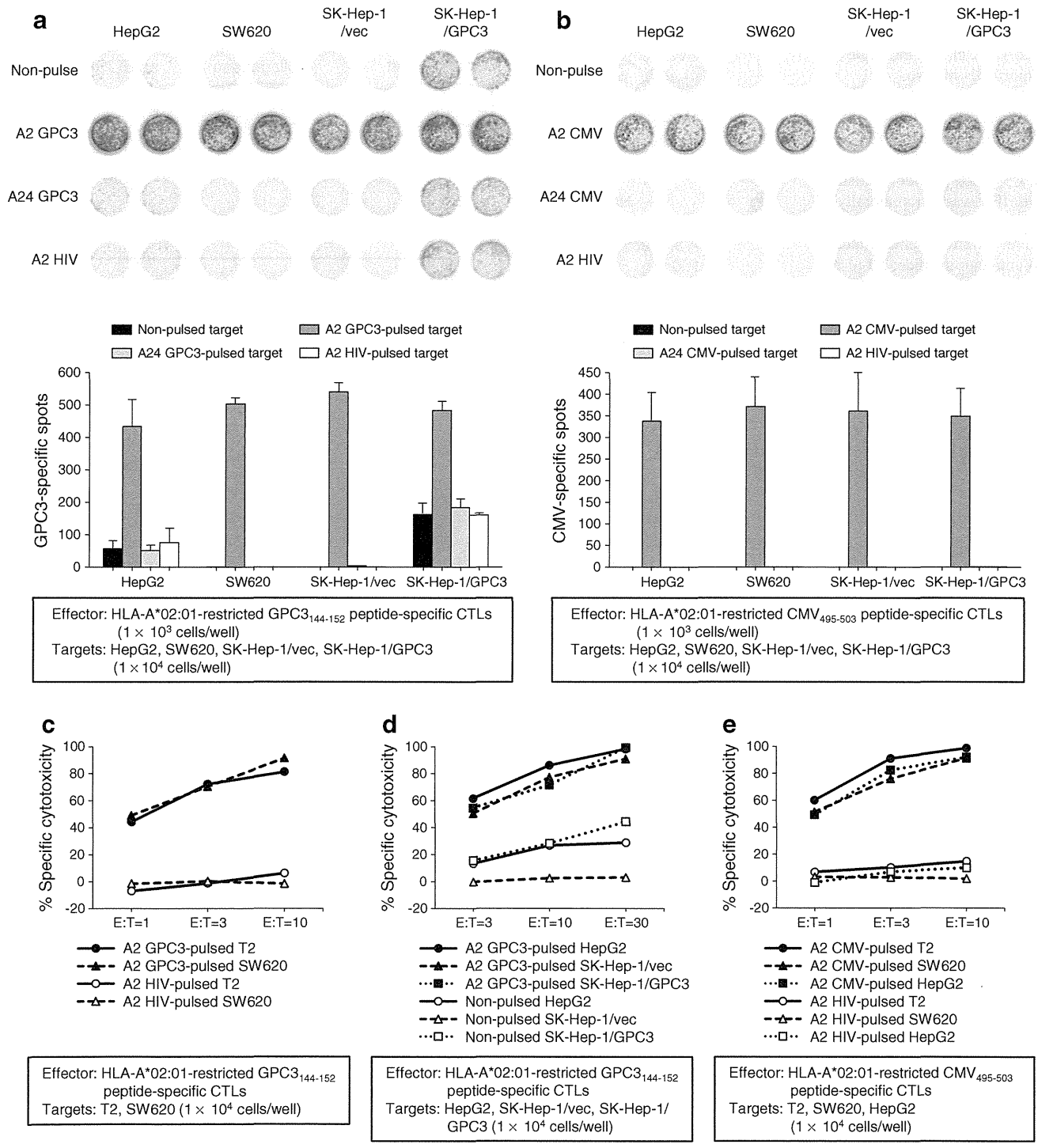

Fig. 1 In vitro CTL activity against the peptide-pulsed targets. (**a** and **b**) IFN-γ ELISPOT assay. (**c**, **d**, and **e**) Cytotoxicity assay. HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs (**a**, **c**, and **d**) and HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTLs (**b** and **e**) showed activity depending on the peptide density of tumor cells. Data are representative of three independent experiments, and bar graphs represent mean values of three independent experiments (SD) in (**a** and **b**)

Intratumoral injection of a combination of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide and its specific CTLs resulted in statistically significant tumor growth inhibition ($P < 0.05$) (Fig. 3c). Similarly, this treatment was effective against SK-Hep-1/vec (Fig. 3d), SK-Hep-1/GPC3 (Fig. 3e), and HepG2 (Fig. 3f) tumors. Intratumoral injection of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs alone against GPC3-expressing tumors, SK-Hep-1/GPC3 and HepG2, was only partially effective, suggesting that the HLA-A*02:01-restricted $GPC3_{144–152}$

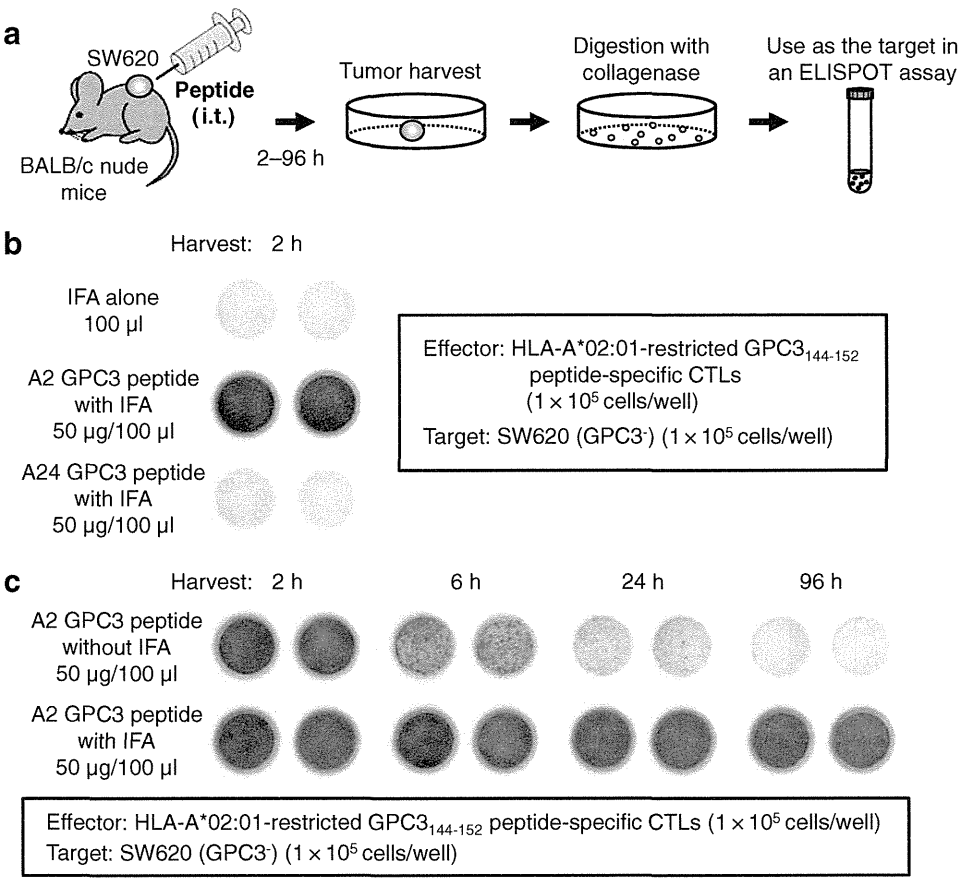

Fig. 2 IFN-γ ELISPOT assay for loading of injected peptide onto HLA class I molecules of tumor cells in vivo. **a** Experimental schematic representation. **b** HLA-A*02:01-restricted $GPC3_{144–152}$ or -A*24:02-restricted $GPC3_{298–306}$ peptide emulsified with IFA was intratumorally injected, and the tumors were harvested after 2 h. IFA alone: no antigenic peptide; 50 µl of 7 % $NaHCO_3$ was mixed with an equal volume of IFA. **c** HLA-A*02:01-restricted $GPC3_{144–152}$ peptide with or without IFA was injected, and tumors were harvested at various times. Data are representative of three independent experiments

peptide endogenously presented on SK-Hep-1/GPC3 and HepG2 tumor cells was not sufficiently dense. However, intratumoral injection of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide increased the peptide density and markedly enhanced CTL activity. Similarly, intratumoral injection of HLA-A*02:01-restricted $CMV_{495–503}$ peptide followed by its specific CTLs resulted in statistically significant tumor growth inhibition ($P < 0.05$) (Fig. 3g). Intratumoral injection of a combination of antigen peptide and its specific CTLs had a significant antitumor effect.

Therapeutic advantage of intratumoral peptide injection as an option for antigen-specific cancer immunotherapy

After the induction of $OVA_{257–264}$ peptide-specific CTLs by peptide vaccination (Fig. 4a) or after the adoptive transfer of $OVA_{257–264}$ peptide-specific CTLs (Fig. 4c), intratumoral injection of $OVA_{257–264}$ peptide was effective against RMA cells, which are OVA-negative tumor cells. The RMA tumors cells that were injected intratumorally with $OVA_{257–264}$ peptide demonstrated significant tumor growth inhibition, compared with mice without intratumoral injection of $OVA_{257–264}$ peptide ($P < 0.05$). The survival rate in the treatment group was significantly better than that in the control groups ($P < 0.05$) (Fig. 4b, d). The group that did not receive $OVA_{257–264}$ peptide vaccine but that received intratumoral peptide injection showed a partial treatment effect (Fig. 4b).

To obtain direct evidence that intratumoral peptide injection leads to local accumulation of antigen-specific CTLs, an OVA tetramer assay was performed using an adoptive cell transfer model (Fig. 4e). Two RMA tumors were bilaterally implanted per mouse. One tumor was injected with the $OVA_{257–264}$ peptide plus IFA, and the other tumor with IFA alone (Fig. 4f). As shown in Fig. 4g, the tumor that underwent both adoptive cell transfer of activated OT-I CTLs and intratumoral injection of the OVA peptide contained more OVA-specific CTLs than the other tumors. Local accumulation of OVA-specific CTLs after intratumoral injection of the $OVA_{257–264}$ peptide was confirmed by OVA tetramer assay.

Neither toxic signs nor death due to intratumoral injection of the $OVA_{257–264}$ peptide was observed. Moreover, to evaluate the risk of autoaggression by intratumoral peptide injection, the tissues of treated mice in an adoptive cell transfer model were pathologically examined. The spleen, brain, lung, heart, liver, kidney, and tumor were critically scrutinized, and the findings were compared with those from

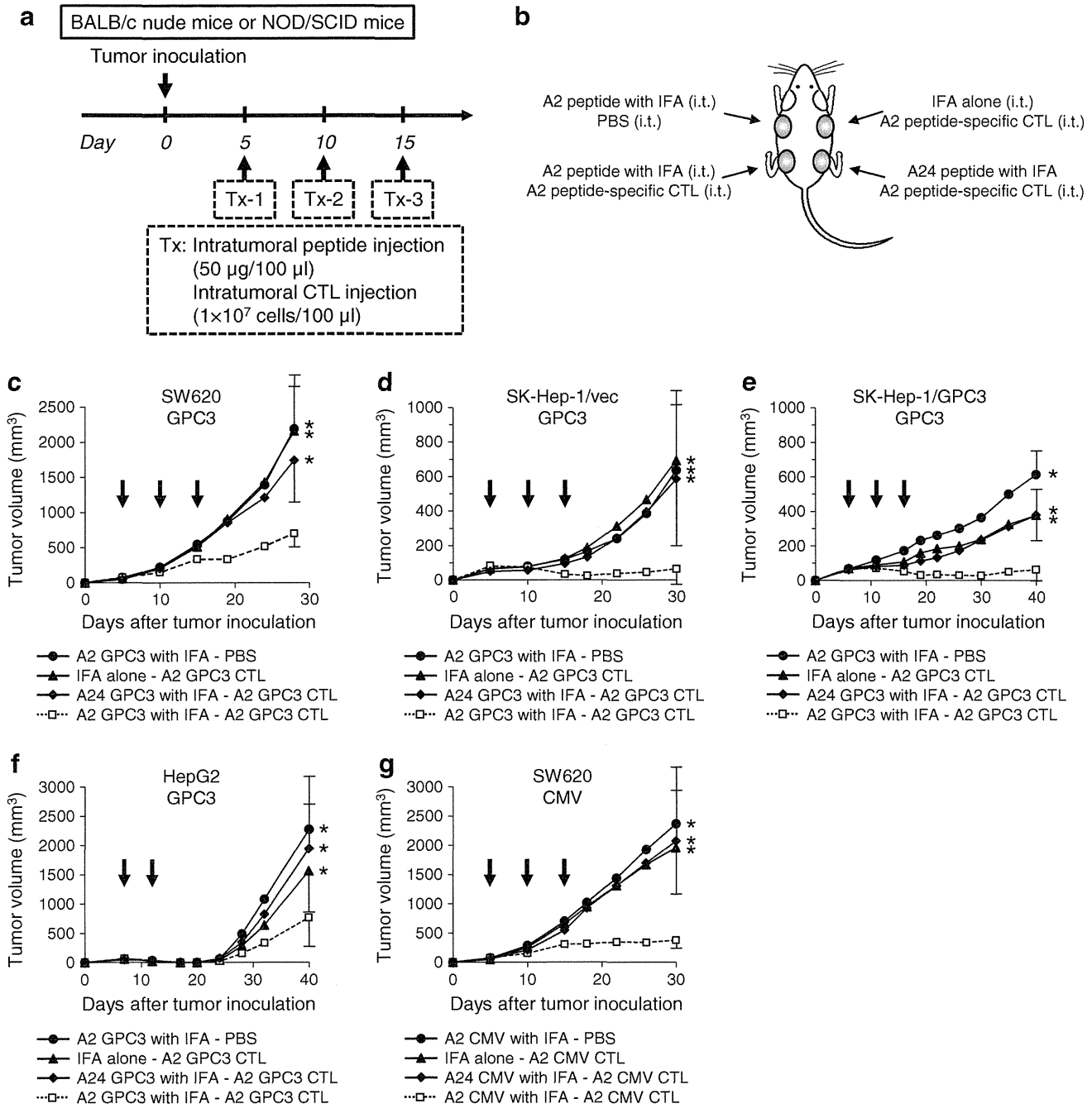

Fig. 3 Antitumor effect of intratumoral peptide injection in an immunodeficient mouse model. Intratumoral injection of a combination of antigen peptide and its specific CTLs had a significant antitumor effect. **a** Treatment schedule. **b** Experimental schematic representation. BALB/c nude mice or 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

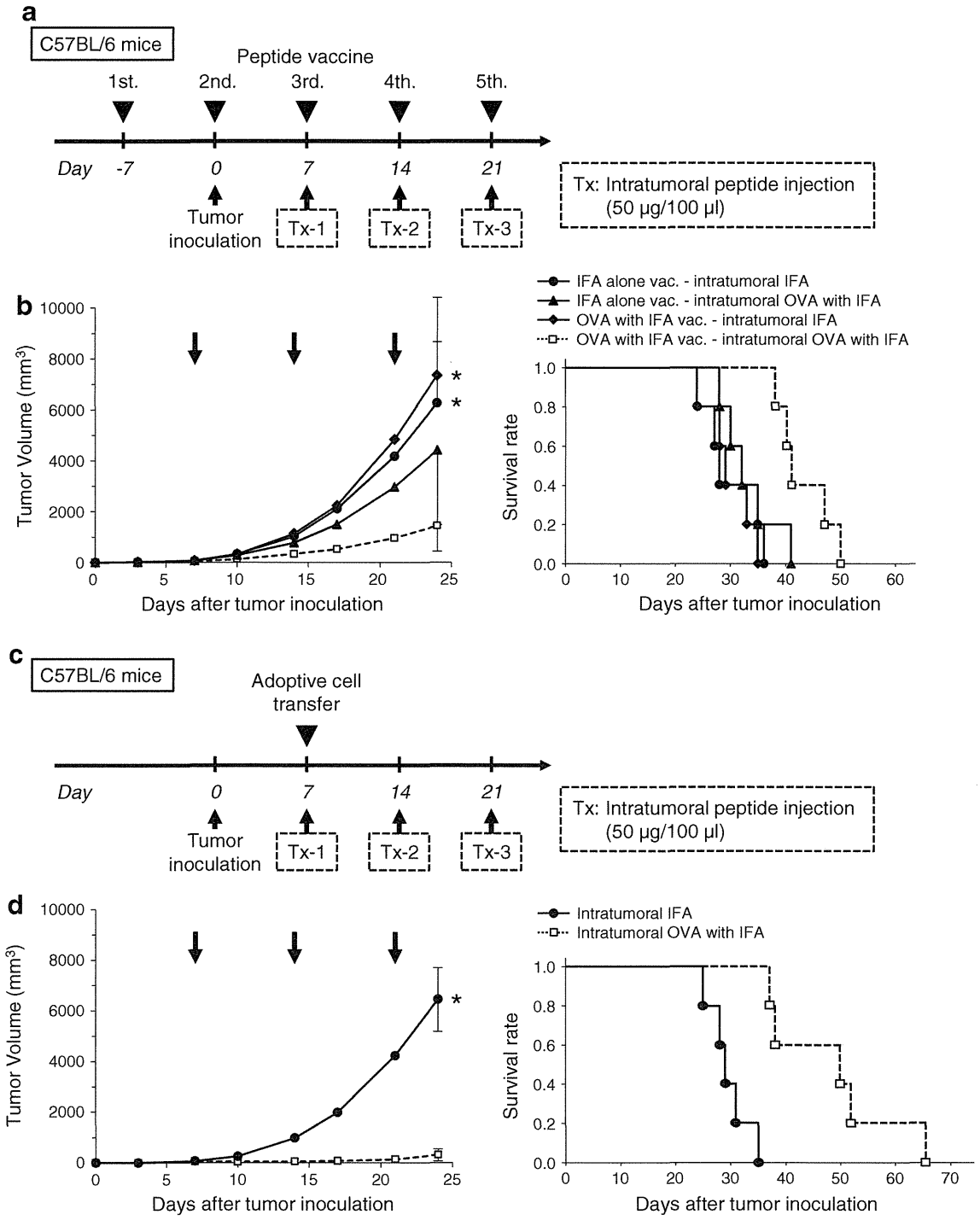

Fig. 4 Therapeutic advantage of intratumoral peptide injection as an option for antigen-specific cancer immunotherapy. (**a** and **b**) Peptide vaccine model. (**c** and **d**) Adoptive cell transfer model. (**a** and **c**) Treatment schedule. (**b** and **d**) Tumor growth and Kaplan–Meier survival *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

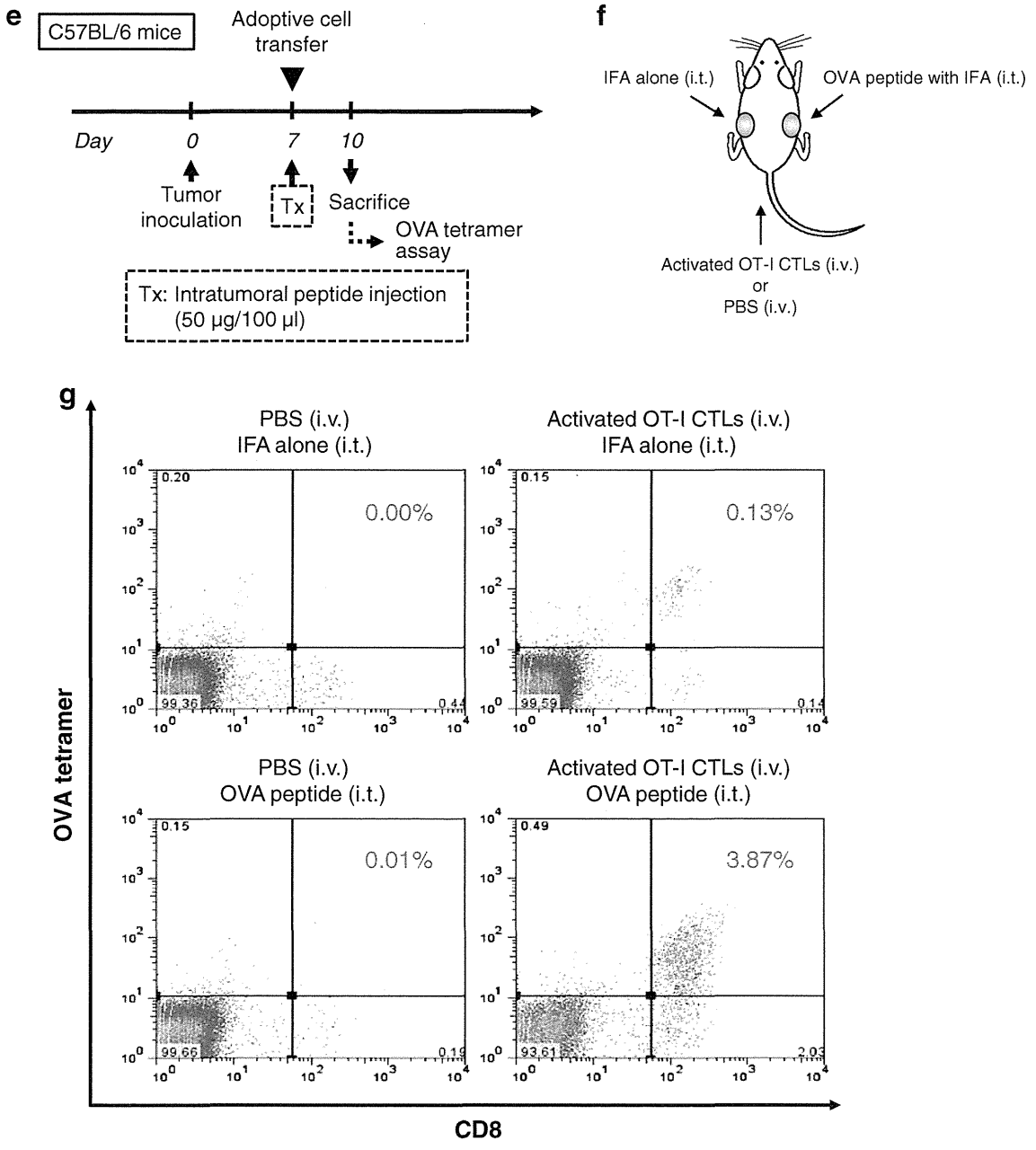

Fig. 4 continued

$CD8^+$ 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

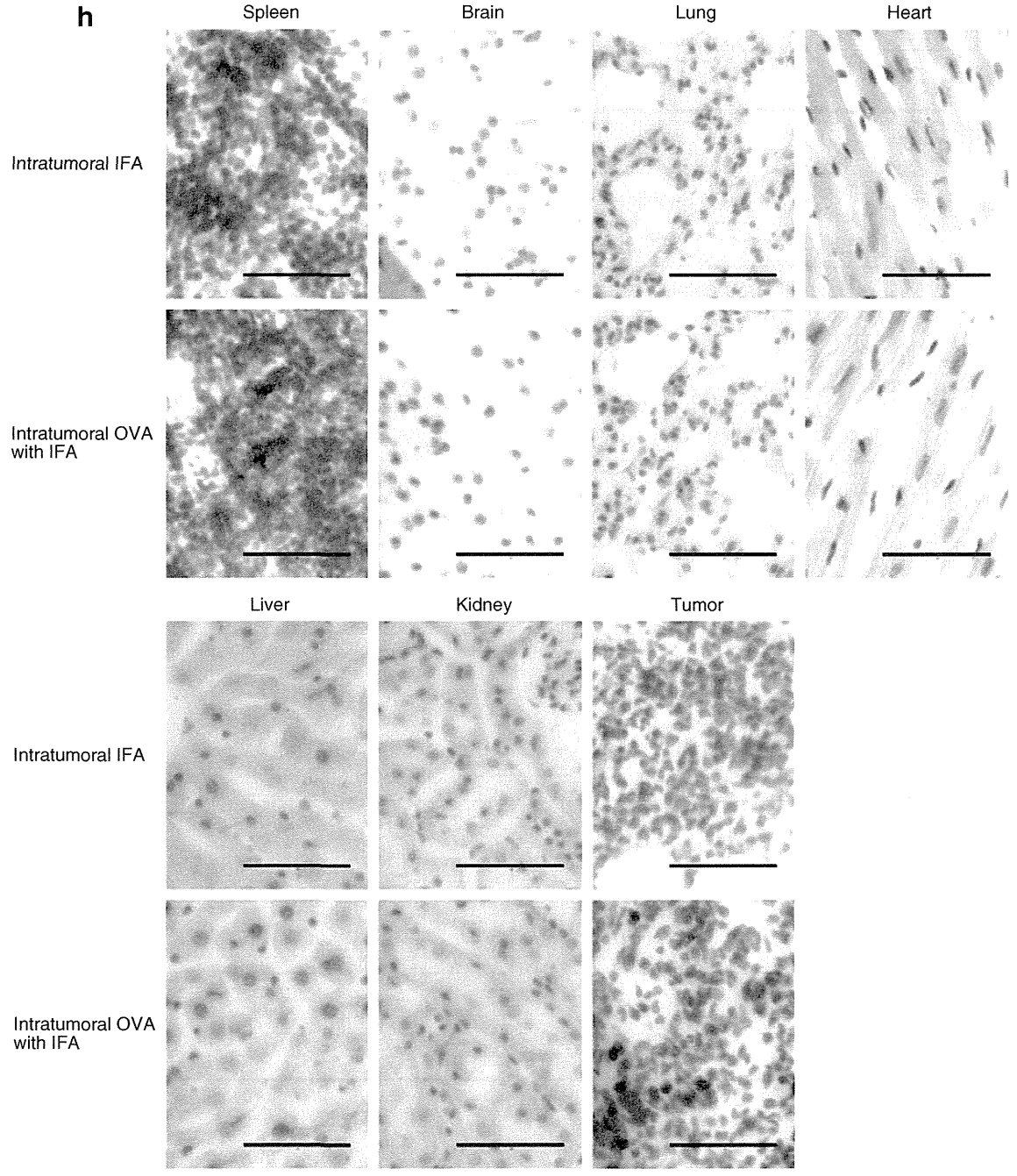

Fig. 4 continued

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).

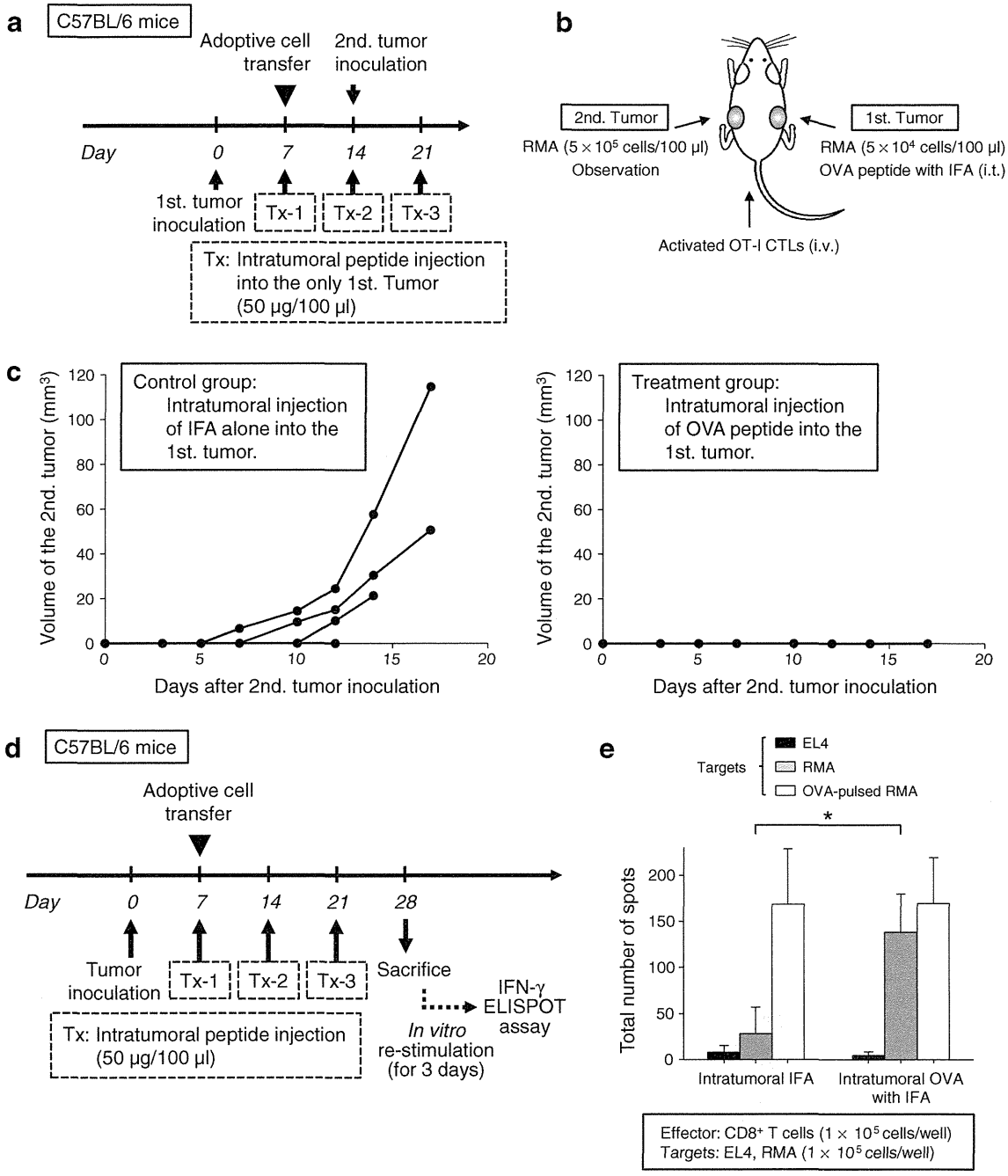

Fig. 5 Effect of antigen-spreading to another tumor after intratumoral peptide injection. **a** The schedule for the experiment on antigen-spreading effect in an adoptive cell transfer model. **b** Experimental schematic representation. Two tumors were metachronously implanted per mouse (first tumor: 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

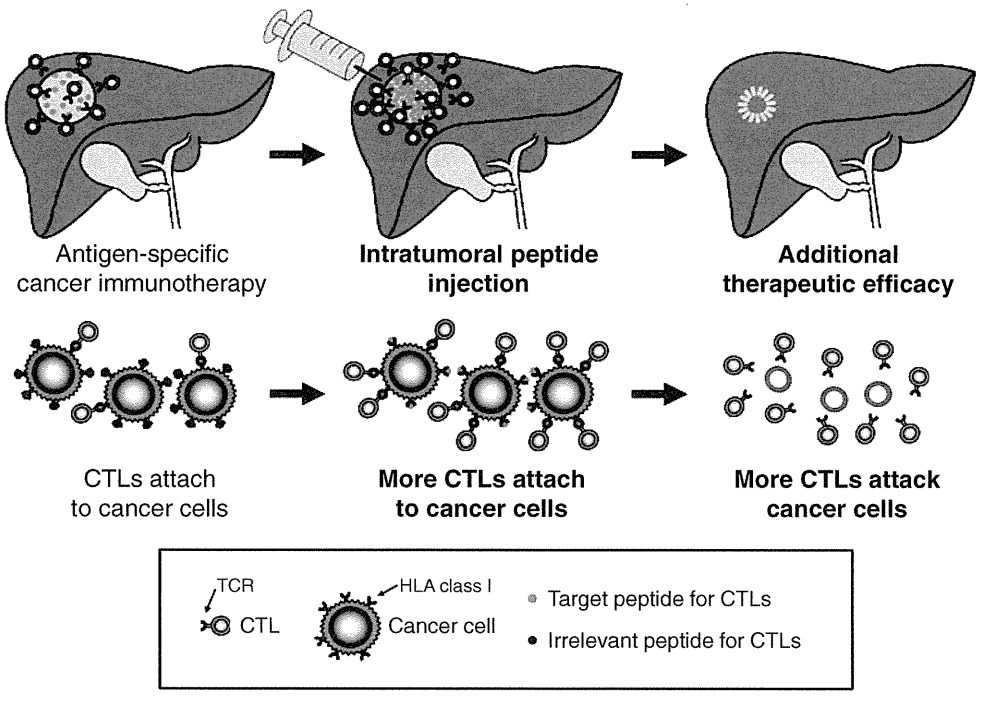

Fig. 6 A proposed mechanistic model of intratumoral peptide injection for improvement in antigen-specific cancer immunotherapy of solid tumors

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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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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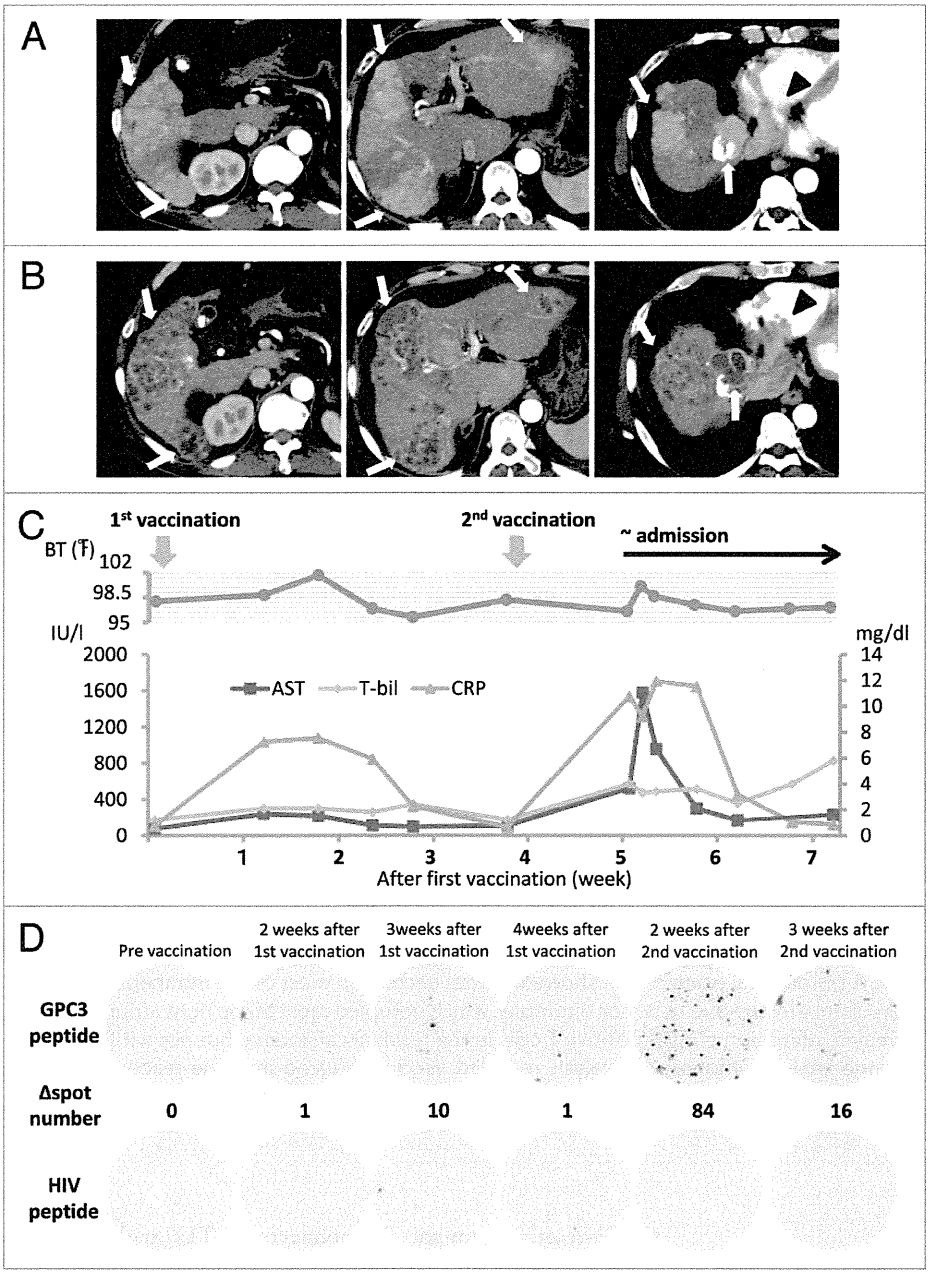

Figure 1. Findings of an early-phase contrast-enhanced CT (CT) scan. (**A**) Contrast-enhanced CT scan before vaccination shows a 68 × 51-mm tumor with multiple intrahepatic tumors (arrow) and a 44 × 30-mm tumor invading the right atrium (arrowhead). (**B**) Contrast-enhanced CT after the second vaccination 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.

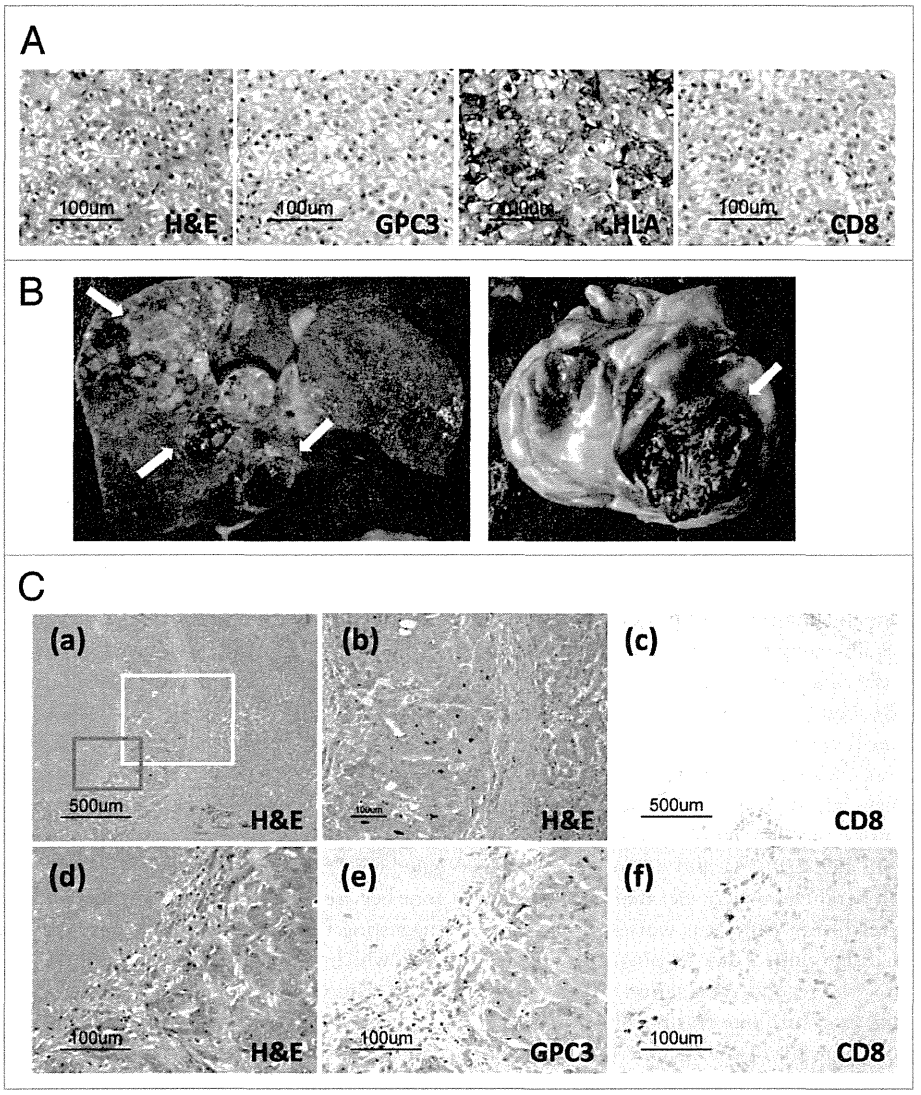

Figure 2. (**A**) Pathological findings of liver biopsy specimens before vaccination. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows well-differentiated hepatocellular carcinoma (HCC). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the cytoplasm 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

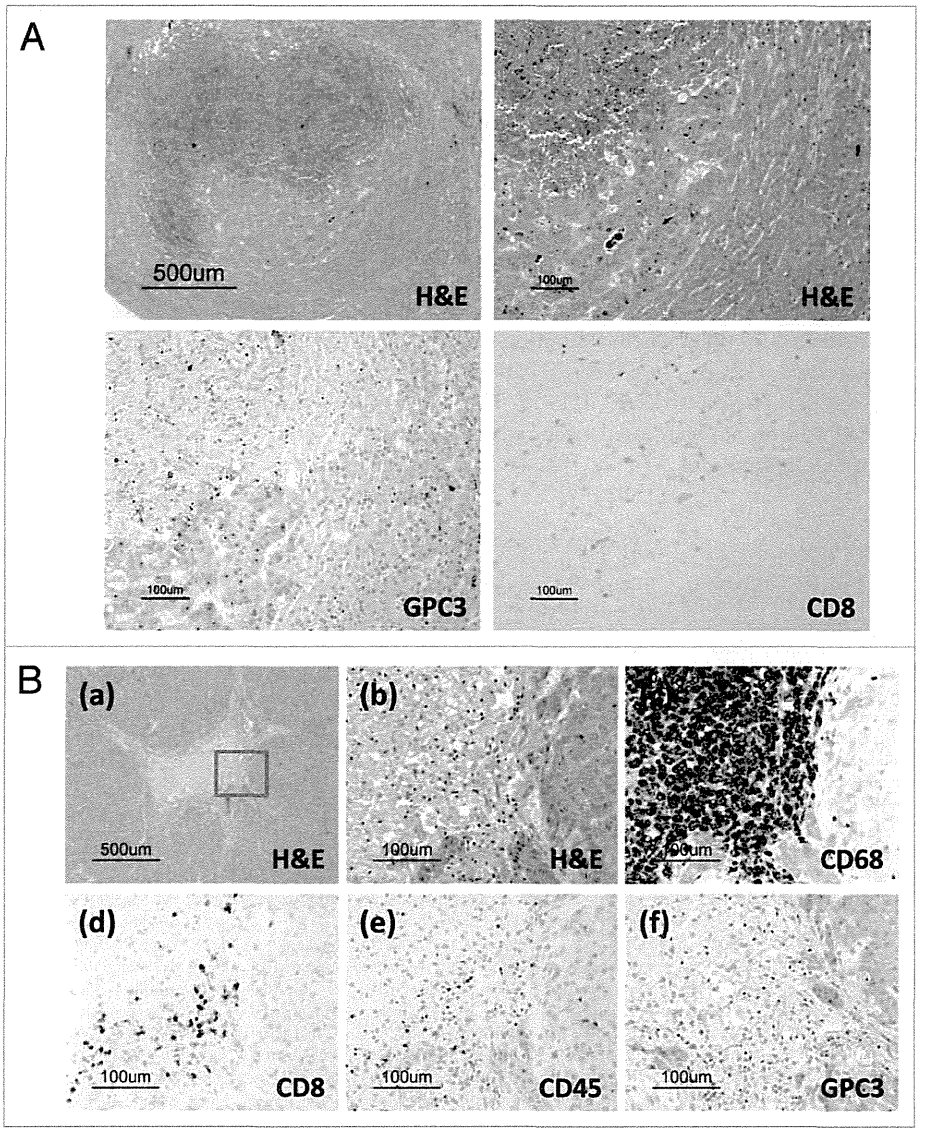

Figure 3. Pathological findings in the autopsy specimen. (**A**) Carcinoma in a cirrhotic nodule. CD8-positive T cells (brown) infiltrated only the carcinoma area, accompanied by necrosis. No infiltration of CD8-positive T cells was detected in the cirrhotic nodule. Only carcinoma cells were GPC3-positive 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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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