

## HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: Preclinical study using mice

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**Abstract.** We previously reported that glypican-3 (GPC3) is uniquely overexpressed in human hepatocellular carcinoma and melanoma and that it is an ideal tumor antigen for immunotherapy in mouse models. We recently identified both HLA-A24 (A\*2402) and H-2K<sup>d</sup>-restricted GPC3<sub>298-306</sub> (EYILSLEEL) and HLA-A2 (A\*0201)-restricted GPC3<sub>144-152</sub> (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs). The present study was a preclinical study in a mouse model that was conducted in order to design an optimal schedule for clinical trial of GPC3-derived peptide vaccine. When BALB/c mice were intradermally vaccinated at the base of the tail with K<sup>d</sup>-restricted GPC3<sub>298-306</sub> peptide mixed with incomplete Freund's adjuvant (IFA), the peptide-specific CTLs were induced. But the peptide alone could not induce peptide-specific CD8<sup>+</sup> T cells. Furthermore, proteomic analyses showed that IFA protected the peptide against degradation in the human serum. Peptide-reactive CTLs were induced by peptide vaccine in a dose-dependent manner. In addition, at least two vaccinations with a single dose >10 µg were needed for the induction of GPC3<sub>298-306</sub>-specific CTLs. But repeated vaccination with a lower dose of GPC3<sub>298-306</sub> did not induce peptide-specific CTLs. Similarly, induction of an Ag-specific immune response by HLA-A2

GPC3<sub>144-152</sub> depended on the dose administered. The results of this study suggested that IFA is one of the indispensable adjuvants for peptide-based immunotherapy, and that the immunological effect of peptide vaccines depends on the dose of peptide injected.

### Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide, especially in Asian and Western countries (1). Despite advances in diagnosis and treatment, the overall survival of patients with HCC has not significantly improved in the last two decades (2). The effective treatments currently available are only indicated in a relatively small proportion of early stage cases. When patients presents with clinical manifestations of HCC, the tumor is usually advanced, and there are few treatment options. Many HCC patients have type B or C hepatitis or cirrhosis, so patients treated surgically or by other therapies are also at high risk for recurrence. Furthermore, the liver function of such patients is often very poor, so treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor and new therapies for cancer development and recurrence, i.e., adjuvant therapy, are urgently needed.

We previously reported that glypican-3 (GPC3), glycosylphosphatidylinositol (GPI)-anchored membrane protein, is specifically overexpressed in human HCC and melanoma, and that among normal tissues it is slightly expressed in placenta and embryonic liver (3). We found that GPC3 is useful not only as a novel tumor marker, but also as a target antigen for immunotherapy in several studies with mice (4,5). In addition, we identified CTL epitope peptides: HLA-A24-restricted GPC3<sub>298-306</sub> (EYILSLEEL) and HLA-A2-restricted GPC3<sub>144-152</sub> (FVGEFFTDV) (6). To design the schedule for the phase I clinical study of these GPC3-derived peptide vaccines, many factors need to be taken into consideration: the adjuvant, dosage, number of doses, vaccination interval, etc. Many investigators have reported various vaccination schedules (7,8). There is no world-wide consensus concerning the schedule to use for administration of peptide

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*Abbreviations:* BM-DC, bone marrow-derived dendritic cell; mAb, monoclonal antibody; M/W, molecular/weight

*Key words:* cancer immunotherapy, GPC3, peptide vaccine, hepatocellular carcinoma

vaccines. In this study, we attempted to identify a more effective vaccine regimen that would induce a strong cell-mediated immune response. Ten years ago Salgaller *et al* reported that they did not observe any dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). The results of our present study, however, showed dose-dependency in the immunizing effect of a peptide vaccine.

## Materials and methods

**Mice.** Female BALB/c mice at 6-8 weeks of age were obtained from Japan SLC (Hamamatsu, Japan) or Charles River Laboratories. *HLA-A2.1* (HHD) Tgm; *H-2D<sup>b</sup>-h2m<sup>-/-</sup>* double knockout mice transfected with a human h2m-*HLA-A2.1* ( $\alpha 1 \alpha 2$ )-*H-2D<sup>b</sup>* ( $\alpha 3$  transmembrane cytoplasmic) (HHD) monochain construct gene were prepared in the Department SIDA-Retrovirus, Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, France (10), and kindly provided by Dr F.A. Lemonnier. The mice were maintained under specific-pathogen-free conditions. The mouse experiments were approved by the Animal Research Committee of the National Cancer Center Hospital East.

**Cells lines.** A subline of BALB/c-derived colorectal adenocarcinoma cell line Colon 26, C26 (C20) (11) was provided by Dr Kyoichi Shimomura (Astellas Pharmaceutical Co., Tokyo, Japan). Colon 26/GPC3 (C26/GPC3) is an established stable GPC3-expressing cell line (4). RMA-HHD cells were kindly provided by Dr Masanori Matsui of Saitama Medical School, Saitama, Japan. A human  $\beta 2m$ -*HLA-A2.1* ( $\alpha 1 \alpha 2$ )-*H-2D<sup>b</sup>* ( $\alpha 3$  transmembrane cytoplasmic) (HHD) monochain construct was transfected into RMA lymphoma cells [transporter associated with antigen presentation (TAP) positive] to establish RMA-HHD cells (10). The cells were cultured in RPMI-1640 medium supplemented with 10% FCS. To obtain GPC3-expressing RMA-HHD (RMA-HHD-GPC3) cells, RMA-HHD cells were transfected with pCAGGS-GPC3-internal ribosomal entry site (IRES)-puromycin-resistant gene with Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA), selected with puromycin, and then subjected to cloning by limiting dilution in drug-free medium in 96-well culture plates (12,13). Dendritic cells were obtained from bone marrow cells (BM-DCs) as described previously (4). Irradiated BM-DCs pulsed with peptide were used for *in vitro* CTL culture or as target cells for Elispot assays.

**Vaccination.** HLA-A24- and K<sup>d</sup>-restricted GPC3<sub>298-306</sub> (EYILSLEEL) and HLA-A2-restricted GPC3<sub>144-152</sub> (FVGEFFTDV) were dissolved in 7% NaHCO<sub>3</sub> and the solution was diluted with saline. For peptide vaccination, mice were intradermally injected at the base of the tail with peptide solution emulsified in incomplete Freund's adjuvant (IFA). Different doses of peptide were administered at 7-day intervals, and mice were sacrificed to obtain inguinal lymphocytes and spleen cells seven days after the final vaccination.

**IFN- $\gamma$  Elispot assays.** Female BALB/c mice were intradermally vaccinated with GPC3<sub>298-306</sub> or GPC3<sub>144-152</sub>/IFA.

Their inguinal lymphocytes were stimulated with peptide-pulsed BM-DCs *in vitro* for five days. The proportion of cells producing IFN- $\gamma$  against target cells (BM-DCs pulsed with or without GPC3 peptide) was assessed by an Elispot assay as described previously (14). The spots were automatically counted and subsequently analyzed with the Eliphoto system (Minerva Tech, Tokyo, Japan).

**Analysis of peptide degradation.** GPC3<sub>298-306</sub> was mixed with human serum, and the solution was applied to the surfaces of a Q10 (strong anion exchange) ProteinChip (Bio-Rad Laboratories, CA). We sequentially examined the solution with a SELDI-TOF mass spectrometer (Bio-Rad). Female BALB/c mice were intradermally vaccinated with GPC3<sub>298-306</sub>/IFA at the base of the tail. A week later, we collected the residual peptide vaccine at the base of the tail into buffer in a tube, and the tube was centrifuged at 4°C at 10,000 rpm for 15 min. The supernatant was applied to a ProteinChip and the surface of the chip was examined with the spectrometer.

**Induction of GPC3-specific CTLs and cytotoxicity assay.** Mice were intradermally vaccinated twice with 50  $\mu$ g GPC3 peptide/IFA 7 days apart. Seven days after the second vaccination, inguinal lymph nodes were excised and the lymphocytes were cultured in 24-well culture plates (5x10<sup>6</sup> per well) with GPC3 peptide-pulsed BM-DCs (1x10<sup>5</sup> per well) in RPMI medium supplemented with 10% horse serum, recombinant human interleukin (IL)-2 (100 units/ml), and 2-mercaptoethanol (50  $\mu$ mol/l). After culture for 5 days, the cells were recovered and analyzed for their cytotoxic activity against target cells with the TERASCAN VPC system (Minerva Tech) as previously described (15). Briefly, C26, C26/GPC3, RMA-HHD and RMA-HHD-GPC3 cells were used as target cells and labeled with calcein-AM solution for 30 min at 37°C. The labeled cells were washed three times and distributed to the 96-well culture plate (1x10<sup>4</sup> per well), and they were then incubated with effector cells for 5-6 h. Fluorescent intensity was measured before and after the 5-6-h culture, and Ag-specific cytotoxic activity was calculated by using this formula: cytotoxicity (%) = [(sample release) - (spontaneous release)] / [(maximum release) - (spontaneous release)] x 100. In some experiments, CD8<sup>+</sup> T cells were isolated from effector cell preparations with a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Positively selected CD8<sup>+</sup> T cells were 95% pure as determined by flow cytometry.

**Histologic and immunohistochemical analysis.** Mice were injected twice with GPC3 peptide vaccine, and seven days later tumor cells were subcutaneously implanted in their shaved backs. Seven days after the tumor challenge, frozen sections of tumor tissue were prepared. The frozen tissue sections were immunohistochemically analyzed using monoclonal antibody (mAb) specific for CD4 (L3T4; BD Pharmingen, San Diego, CA) or CD8 (Ly-2; BD Pharmingen) as described previously (4).

**Statistical analysis.** The 2-tailed Student's t-test was used to determine the statistical significance of differences in the

data obtained by ELISPOT assay.  $P < 0.05$  was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

## Results

*IFA is an effective adjuvant for the peptide vaccine that induced strong immune responses and maintained the stability of the peptide.* We attempted to verify whether emulsions of GPC3 peptide in IFA could induce a peptide-specific immune response in mouse models. The results showed that only GPC3 peptide emulsified in IFA elicited a T-cell-mediated immune response, whereas vaccination with peptide alone failed to induce any detectable immune response (Fig. 1A). In addition, we investigated the stability of GPC3 peptide alone in human serum, with the Surface Enhanced Laser Desorption/Ionization SELDI system (Bio-Rad). We applied the sample of GPC3 peptide mixed with human serum to a ProteinChip and detected the peak of GPC3 peptide. The peak value of GPC3<sub>298-306</sub> in buffer was about 1,000, whereas the peak of GPC3<sub>298-306</sub> in serum had decreased to 200, 3 min after mixing it with the serum (Fig. 1B). This finding indicated that GPC3 peptide was immediately degraded in serum. Moreover, we collected the white residue of peptide/IFA emulsions that were still present at the base of the tail of the vaccinated mice, and after applying the peptide/IFA emulsions to a ProteinChip, quantified the peak of GPC3<sub>298-306</sub>. Mass spectrometric analysis demonstrated that the peptide was still present in a stable form in the peptide/IFA emulsions (Fig. 1C). IFA not only induced a potent immune response, but protected the peptide from various enzymes in the serum.

*Dose-dependent effects of GPC3-derived peptide vaccine emulsified in IFA.* Next we examined whether a more peptide-specific response was induced, when a higher dose of peptide was used. The proportion of peptide-specific CTLs among  $5 \times 10^4$  CD8<sup>+</sup> T cells was evaluated by IFN- $\gamma$  Elispot assays, when mice were vaccinated with GPC3<sub>298-306</sub> K<sup>d</sup>-restricted peptide doses of 5, 10, 20, or 50  $\mu$ g. Peptide-specific CD8<sup>+</sup> T cell responses were observed when vaccinated with GPC3<sub>298-306</sub> K<sup>d</sup>-restricted peptide doses above 10  $\mu$ g (Fig. 2A). Additionally, as the peptide dose increased, peptide reactive CTLs were detected more frequently (Fig. 2A). We vaccinated A2 Tg mice with HLA-A2-restricted GPC3<sub>144-152</sub> peptide in the same manner. The results indicated that the higher doses of the peptide induced a greater peptide-specific immune response (Fig. 2B). We therefore, concluded that the higher the dose of peptide injected, the more peptide-specific CD8<sup>+</sup> T cells were induced. But Elispots by vaccinations of  $>5$   $\mu$ g GPC3<sub>144-152</sub> been seen to reach a plateau level.

*Marked infiltration of subcutaneous tumor tissue by CD8<sup>+</sup> T cells in mice vaccinated with the 50  $\mu$ g dose of GPC3 peptide.* Immunohistochemical analysis of the tumor tissue specimens showed more intense infiltration by CD8<sup>+</sup> T cells, but not by CD4<sup>+</sup> T cells, in and/or around C26/GPC3 (Fig. 3D) or RMA-

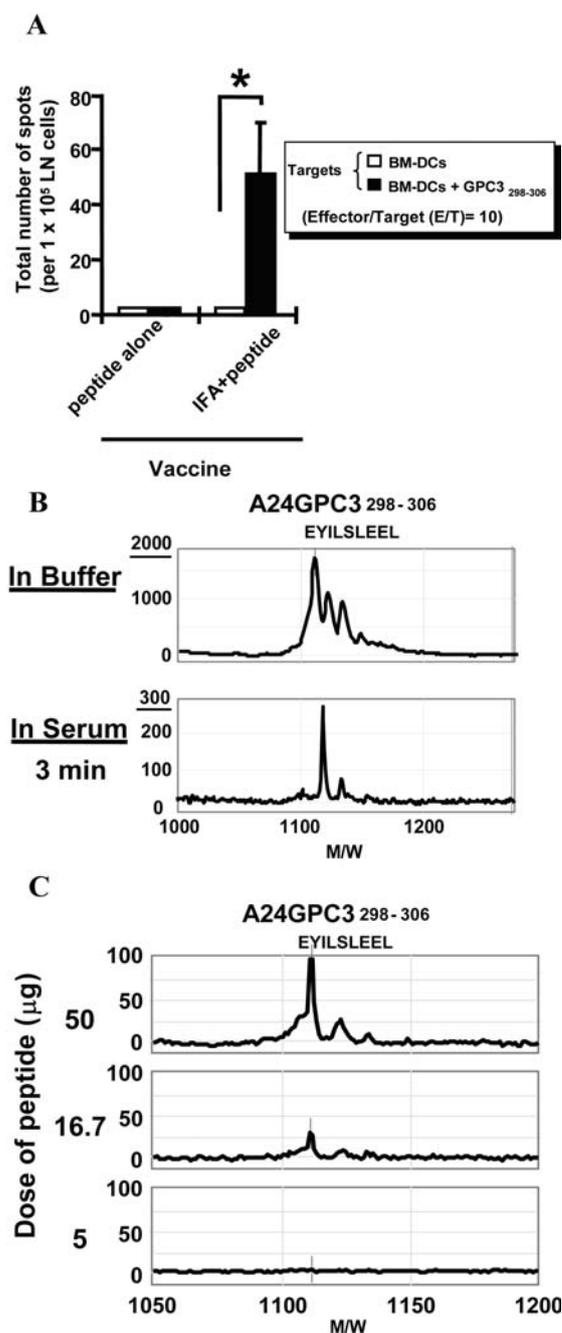


Figure 1. IFA is an appropriate adjuvant for the peptide vaccine. (A) The GPC3<sub>298-306</sub>-specific immune response was induced by peptide emulsified in IFA. Female BALB/c mice were vaccinated twice by intradermal injection of 50  $\mu$ g of GPC3<sub>298-306</sub> (EYILSLEEL) with IFA. Seven days after the second injection, inguinal lymph node cells were isolated and cultured with GPC3<sub>298-306</sub>-pulsed BM-DCs for 5 days. The cells were recovered, and their Ag-specific activity was analyzed by IFN- $\gamma$  Elispot assays against BM-DCs pulsed or not pulsed with GPC3<sub>298-306</sub>. \* $P < 0.05$ , statistically significant difference in response. (B) Stability of GPC3<sub>298-306</sub> (EYILSLEEL) in human serum. We measured GPC3<sub>298-306</sub> in the human serum 3 min after the mixture of GPC3<sub>298-306</sub> and human serum by SELDI-TOF mass spectrometry. The discriminating peaks of M/W 1111 represent GPC3<sub>298-306</sub>. When GPC3 peptide was mixed with human serum, the peptide was immediately degraded by various proteases. (C) Detection of GPC3 peptide in emulsions collected from vaccinated mice. BALB/c mice were intradermally injected at the base of the tail with GPC3<sub>298-306</sub> emulsified in IFA. A week later we collected peptide/IFA emulsions at the base of the tail and measured GPC3 peptide contained in the emulsions. When injected with 50  $\mu$ g of peptide, GPC3 peptide was detected clearly. Data are representative of 3 independent experiments with similar results in (A-C).

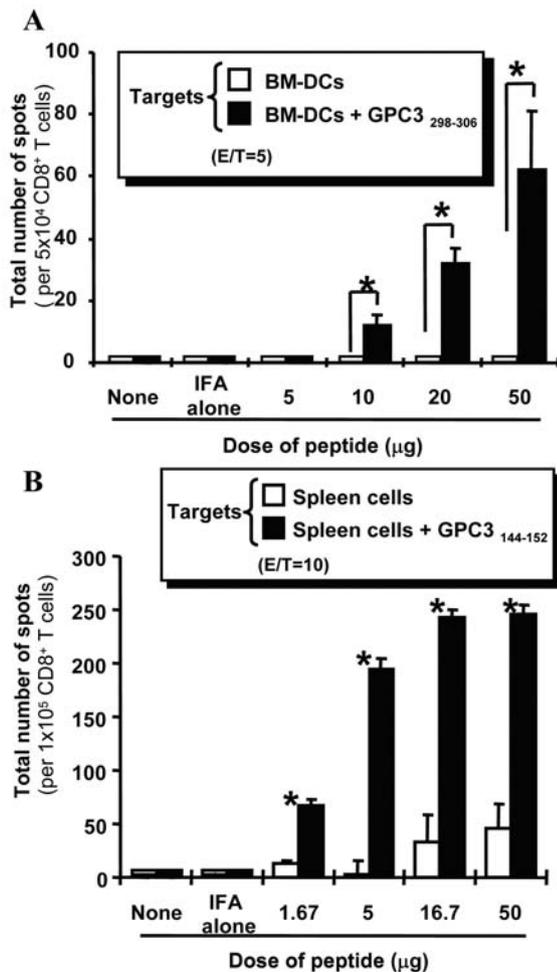


Figure 2. The higher the dose of peptide injected, the more peptide-specific CD8<sup>+</sup> T cells were induced. Groups of mice were vaccinated twice at 7-day intervals with one of the dose levels of H-2K<sup>b</sup>-restricted GPC3<sub>298-306</sub> peptide (A) or HLA-A2-restricted GPC3<sub>144-152</sub> peptide (B) emulsified with IFA (each group; n=3). Inguinal lymphocytes were restimulated *in vitro* with each GPC3 peptide-pulsed BM-DCs for 5 days. The recovered cells were sorted to the CD8<sup>+</sup> T cells fraction by MACS and IFN- $\gamma$  Elispot assays were performed. Peptide-specific CD8<sup>+</sup> T cells were induced dose-dependently. Data are representative of 3 independent experiments with similar results. \*P<0.05, difference in response was statistically significant.

HHD-GPC3 (Fig. 3H) tumor tissue of mice vaccinated with the 50  $\mu$ g dose of GPC3 peptide than with IFA alone, and the 1.67  $\mu$ g dose of GPC3 peptide (Fig. 3). This phenomenon was also observed in metastatic inguinal lymph nodes tissues (data not shown). These results also suggest that the higher the dose of peptide, the more peptide-specific CD8<sup>+</sup> T cells were induced and infiltrated the GPC3-expressing tumor. However, 1.67  $\mu$ g dose of GPC3<sub>144-152</sub> had seemed to induce a few peptide-specific CD8<sup>+</sup> T cells, which corresponded to the result shown in Fig. 2B.

*A second vaccination is needed to induce a peptide-specific response.* Next we attempted to determine how many vaccinations were required to induce a peptide-specific immunological response. BALB/c mice were vaccinated with a 1.67, 5, 16.7, 50  $\mu$ g dose of peptide once a week for 1-4 weeks, respectively. A single vaccination did not elicit a peptide-specific immune response at any of the

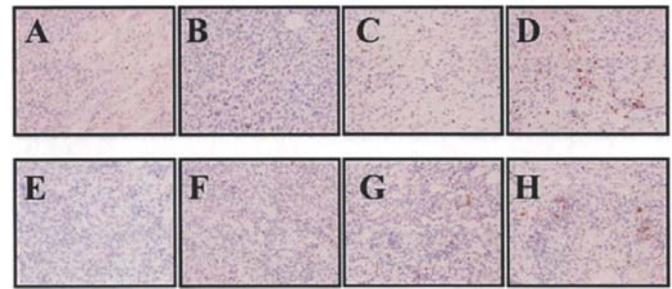


Figure 3. Infiltration by CD8<sup>+</sup> T cells around and into subcutaneous C26/GPC3 (A-D) or RMA-HHD-GPC3 (E-H) tumor tissue is shown. We estimated infiltration by CD8<sup>+</sup> T cells immunohistochemically; no treatment (A and E), IFA (B and F), 1.67  $\mu$ g (C), 50  $\mu$ g (D) of GPC3<sub>298-306</sub>, 1.67  $\mu$ g (G), 50  $\mu$ g (H) of GPC3<sub>144-152</sub>. In mice treated with 50  $\mu$ g GPC3 peptide, a larger number of CD8<sup>+</sup> T cells had clearly infiltrated into and/or around the tumor (D and H). But even in 1.67  $\mu$ g dose of GPC3<sub>144-152</sub>, a few peptide-specific CD8<sup>+</sup> T cells were induced (original magnification, x200).

GPC3<sub>298-306</sub> dose levels (Fig. 4A). Induction of a peptide-specific T-cell response required at least two vaccinations and >16.7  $\mu$ g dose of peptide, and no expansion of peptide-specific T cells occurred after repeated vaccinations with lower doses of peptide (Fig. 4B-D). We also compared immunological responses induced by two and five vaccinations, with 1.67 and 50  $\mu$ g doses of HLA-A2 GPC3<sub>144-152</sub>, but the same as with HLA-A24 GPC3<sub>298-306</sub>, five vaccinations did not increase a peptide-specific response (Fig. 5).

*Cytotoxicity of CD8<sup>+</sup> T cells primed with GPC3 peptide vaccines.* We analyzed the cytotoxicity of CD8<sup>+</sup> T cells primed with GPC3 peptide vaccines. Their killing activity against target cells that expressed or did not express GPC3 was analyzed. The effector cells primed with the GPC3 vaccines showed a significantly higher killing activity against C26/GPC3 cells than against C26 cells, and significantly higher killing activity against RMA-HHD-GPC3 cells than against untransfected RMA-HHD cells (Fig. 6). These results suggest that the CD8<sup>+</sup> T cells induced by GPC3 peptide vaccinations have cytotoxic activity against tumor cells that express GPC3 naturally.

## Discussion

The stability of antigens and the immunogenicity of ISA 720 based on Western blot experiments (16) have been verified, and in the present study we showed that IFA is one of the indispensable adjuvants for peptide vaccines.

We previously reported that vaccination with GPC3<sub>298-306</sub> peptide-pulsed BM-DCs induced complete rejection of a C26/GPC3 tumor challenge in a mouse model (4), but in the present study, C26/GPC3 tumors in a prophylactic model were not rejected after two intradermal vaccinations with GPC3 peptide/IFA at the base of the tail even though CD8<sup>+</sup> T cells by GPC3 peptide vaccine was demonstrated by immunological and immunohistological analysis (data not shown). Comparison of the capacity of peptide-pulsed BM-DCs vaccine to induce peptide-specific CTLs with the

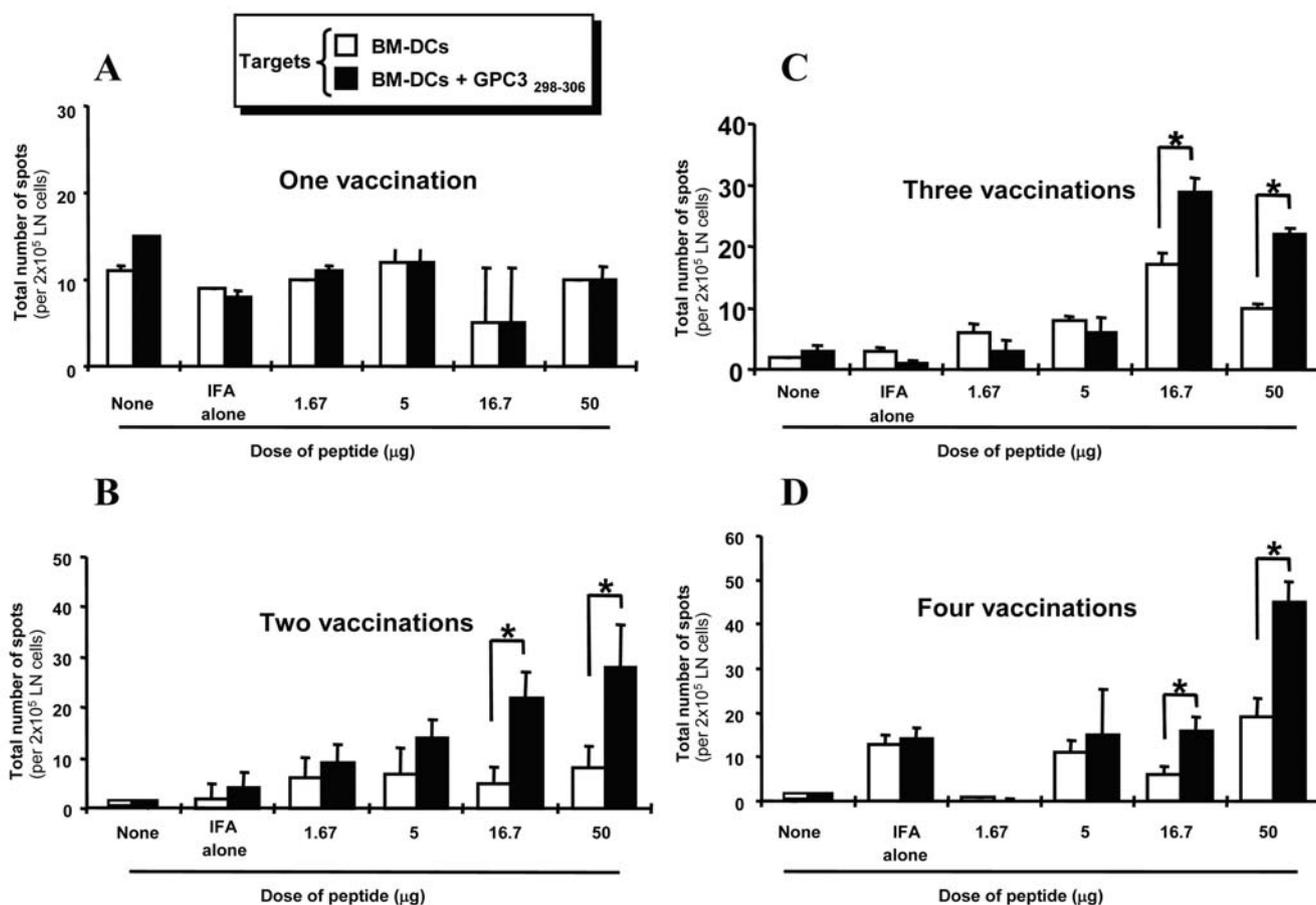


Figure 4. A second vaccination is needed to induce peptide-specific T cells. The immune responses to one (A), two (B), three (C) and four (D) vaccinations with each dose of peptide are shown. BALB/c mice (each group: n=3) were vaccinated with 1.67, 5, 16.7, or 50 μg GPC3<sub>298-306</sub>. Seven days after the final vaccination, bilateral inguinal LNs were excised. Each lymphocyte was restimulated *in vitro* with GPC3<sub>298-306</sub> peptide-pulsed BM-DCs for 5 days, and IFN-γ Elispot assays were then performed against BM-DCs pulsed or not pulsed with GPC3<sub>298-306</sub> to count GPC3<sub>298-306</sub> peptide-specific CTLs. Data are representative of 3 independent experiments with similar results (A-D).

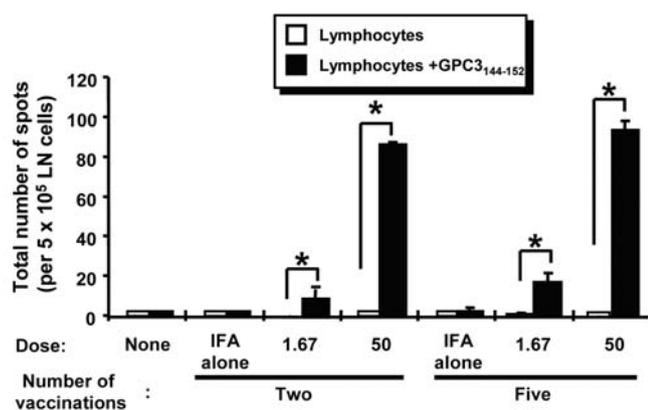


Figure 5. Comparison of immune responses after two or five vaccinations with a 1.67 μg or 50 μg dose of GPC3<sub>144-152</sub>. A2 Tg mice (n=3) were vaccinated with 1.67 μg or 50 μg GPC3<sub>144-152</sub> in the same manner in Fig. 3, but the IFN-γ Elispot assay was performed using whole lymph node cells without *in vitro* culture. Data are representative of 3 independent experiments with similar results. \*P<0.05, difference in response was statistically significant.

capacity of peptide/IFA vaccine by *in vitro* IFN-γ Elispot assays demonstrated that peptide-pulsed BM-DCs vaccine

induced more peptide-specific CTLs (data not shown). There have been few reports of induction of tumor regression *in vivo* by peptide vaccine. Pilar *et al* recently reported finding that a combination of peptide vaccine and CpG induced stronger anticancer responses not only in a prophylactic model, but also in a therapeutic model. They reported in the same study that vaccination with peptide p66 in IFA in the absence of CpG resulted in insignificant CTL responses (17). Although other adjuvants, including CpG, were not effective in the present study (data not shown), peptide/IFA with CpG may be effective. Further study is needed.

The results of the present study showed that at least two vaccinations were necessary to elicit immunological effects. A comparison between HLA-A2-restricted GPC3<sub>144-152</sub>/IFA and K<sup>d</sup>-restricted GPC3<sub>298-306</sub> showed that GPC3<sub>144-152</sub> induced more peptide-specific CTLs at a lower dose. Moreover, HLA-A2-restricted GPC3<sub>144-152</sub> specific CTLs were induced without *in vitro* stimulation with GPC3<sub>144-152</sub> peptide. That may have been attributable to the difference in mouse strain. It is usually said that C57BL/6 and BALB/c mice are a prototypical Th1-type strain and a prototypical Th2-type mouse strain, respectively (18,19) and the difference in genetic background seemed to affect their susceptibility to each of the peptide vaccines.

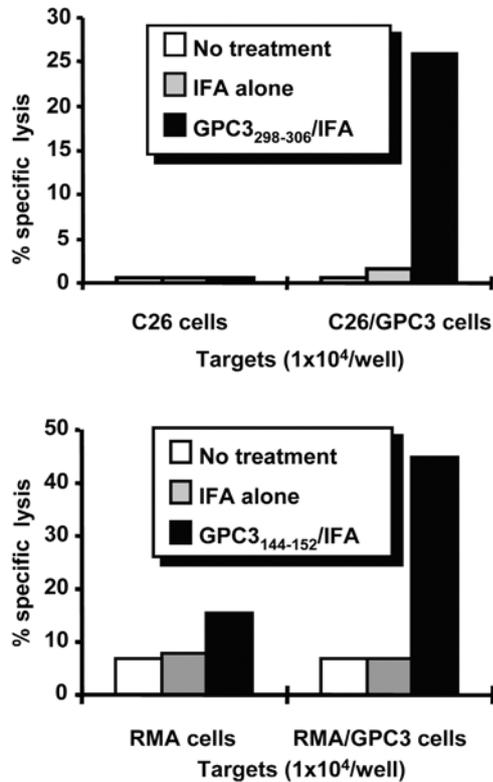


Figure 6. Cytotoxicity of CD8<sup>+</sup> T cells primed with GPC3 peptide vaccine. BALB/c or C57BL/6 mice (n=2-3) were vaccinated with IFA alone or 50  $\mu$ g peptide/IFA in the same manner as described above. The inguinal LN cells were cultured with  $1 \times 10^5$  peptide-pulsed BM-DCs for 5 days. The cells obtained were sorted to the CD8<sup>+</sup> T cell fraction with microbeads. Cytotoxic assays were performed with the cells to evaluate their capacity to kill  $1 \times 10^4$  C26, C26/GPC3, RMA-HHD, or RMA-HHD-GPC3 cells. Data are representative of 3 independent experiments with similar results.

In 1996, Salgaller *et al* reported that they did not detect dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). A dose-dependent effect of peptide vaccine was shown in the present study. We are conducting a phase I clinical trial of GPC3-derived peptide vaccine in patients with advanced HCC at National Cancer Center Hospital East, and we are waiting for the results to determine whether a dose-dependent effect of peptide/IFA was shown in humans, the same as in the mouse model.

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