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Identification of glypican-3-derived long peptides activating both CD8$^+$ and CD4$^+$ T cells; prolonged overall survival in cancer patients with Th cell response

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Abbreviations: APCs, antigen-presenting cells; DC, dendritic cell; ELISPOT, enzyme-linked immunospot assay; GM-CSF, granulocyte macrophage colony-stimulating factor; GPC3, glypican-3; HCC, hepatocellular carcinoma; HLA, human histocompatibility leukocyte antigen; IEDB, Immune-Epitope Database; IL, interleukin; LP, long peptide; mAb, monoclonal antibody; OS, overall survival; PBMCs, peripheral blood mononuclear cells; SP, short peptide; Tgm, transgenic mice; Th, CD4$^+$ helper T cells; Th1, T-helper type 1.

In a recent phase I clinical trial, a vaccine consisting of glypican-3 (GPC3)-derived CTL epitopes was found to be safe and induced measurable immune and clinical responses in patients with hepatocellular carcinoma (HCC). The aim of this study was to identify GPC3-derived long peptides (GPC3-LPs) carrying promiscuous HLA class II-restricted T helper (Th) cell epitopes. Using a computer algorithm, we predicted GPC3-LPs that can bind to promiscuous HLA class II molecules. Their antigenicity for induction of specific CD4$^+$ T cells in healthy donors or patients with HCC, before and after vaccination with GPC3-SPs, was proven by IFNγ enzyme-linked immunospot assays. Natural processing of these epitopes was confirmed by the immune response of helper T cells to dendritic cells (DCs) loaded with GPC3 proteins. Cross-presentation capacity was assessed *in vitro* using human DCs and LPs encapsulated in liposomes and *in vivo* in HLA-A2 transgenic mice (Tgm). All five LPs could induce Th1 cells and were presented by several frequently occurring HLA class II molecules *in vitro*. Four of them were likely to be naturally processed. One of the LPs encapsulated in liposomes was well cross-presented *in vitro*; it cross-primed CTLs in HLA-A2 Tgm. LP-specific and HLA class II-restricted CD4$^+$ T-cell responses were observed in 14 of 20 HCC patients vaccinated with GPC3-SPs. Repeated vaccinations enhanced GPC3-LP-specific responses in 8 of 13 patients with HCC. Moreover, the presence of the specific Th cell was correlated with prolonged overall survival (OS). GPC3-LPs can be useful for cancer immunotherapy.

Introduction

Current treatments of advanced HCC are at best minimally effective; the multikinase inhibitor sorafenib, considered the first-line treatment in patients with HCC,[1] extends the life span by only 3 months.[2] The efficacy against HCC recurrence after a liver transplant is weak,[3] and a high incidence of adverse drug reactions is observed in elderly patients.[4] These observations underscore the need for novel therapies for HCC, and combination therapies including an immunotherapy may fit the bill.

Tumor-associated antigens (TAA), especially cancer testis or oncofetal antigens, which are recognized by adaptive immune system, can be used as immunotherapeutic targets.

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One of such novel oncofetal antigens is membrane-bound heparan sulfate proteoglycan GPC3 previously identified by us using genome-wide cDNA microarray analysis;[5] GPC3 is overexpressed in HCC[6] and promotes the growth of such tumors through stimulating canonical Wnt signaling.[7] A phase I clinical trial of cancer immunotherapy for advanced HCC using two short peptides (SPs), A2-GPC3$_{144–152}$ (A2-GPC3-SP) and A24-GPC3$_{298–306}$ (A24-GPC3-SP),[8,9] showed that these peptide vaccines were well tolerated, induced measurable immune responses, and exhibited some antitumor efficacy. The clinical trial also showed that high frequency of GPC3-specific CTLs was correlated with prolonged OS in patients vaccinated with GPC3-SPs. A phase II clinical trial of adjuvant cancer immunotherapy (using the two above-mentioned GPC3-SPs) is underway for patients with HCC who received curative surgical treatment.

Inoculation with a GPC3-SP emulsified with incomplete Freund's adjuvant (IFA) could elicit a peptide-specific CTL response in patients;[8] however, OS was not convincingly prolonged. One possibility is that GPC3-SP can bind directly to HLA class I molecules expressed not only on professional antigen-presenting cells (APCs) but also on all nucleated cells; therefore, SPs may induce T-cell tolerance or anergy.[10,11] To overcome this drawback, an extended long peptide (LP) consisting of more than 23 amino acid residues can be used.[12] LPs may form a tertiary structure that may protect from exopeptidase-mediated degradation and are too long to be presented directly by HLA class I molecules; thus, they are internalized and cross-presented by professional APCs such as DCs obligated to be recognized by CTLs.[13]

Depletion of $CD4^+$ T cells abrogates protective immune response induced by a vaccine,[14] and an adoptive transfer therapy with $CD4^+$ T cells specific to NY-ESO-1 antigen was shown to induce antitumor protection.[15] Moreover, induction of HIV-1-specific $CD4^+$ T-cell responses augments HIV-specific $CD8^+$ T-cell reactivity in patients with HIV-1 infection.[16]

T helper type 1 (Th1) cells play a pivotal role in enhancing CTL-mediated antitumor immunity.[17] Th1 cells also help to induce and maintain lasting immune responses through multiple interactions with CTLs.[18] Direct antitumor or anti-angiogenic effects were also reported to be mediated by IFNγ-secreting Th1 cells.[19] Furthermore, Th1 cells make it possible for CTLs to enter a tumor site.[20] Therefore, for induction of effective antitumor immunity, it is important to identify the epitopes that can activate tumor-specific Th1 cells.

After injection of an LP, DCs can take up the LP, process it, and present all possible epitopes in the context of various HLA class I and class II molecules.[21] In line with this finding, GV1001, a promiscuous telomerase-derived Th cell epitope vaccine, increases the survival of cancer patients when it is combined with radiotherapy and chemotherapy.[22,23] Given the important role of both $CD8^+$ and $CD4^+$ T cells in antitumor immunity, an LP encompassing CTL epitope(s) may be regarded as a promising component of a vaccine.[21]

In this study, we used a computer algorithm for prediction of HLA class II-binding GPC3-derived peptides and utilized this information for selection of promising promiscuous Th cell epitopes. We demonstrated that the predicted GPC3-LPs triggered Th1 responses in healthy donors carrying several frequent *HLA-DR* and *DP* alleles. GPC3-LPs-specific Th cell responses were observed in the majority of HCC patients vaccinated with GPC3-SPs, and prolonged OS was observed in patients with Th cell response.

Results

Prediction and selection of possible promiscuous HLA class II-binding GPC3-LPs

We selected five GPC3-LPs; GPC3$_{92–116}$ (LP1), GPC3$_{137–161}$ (LP2), GPC3$_{289–313}$ (LP3), GPC3$_{386–412}$ (LP4), and GPC3$_{556–576}$ (LP5), with overlapping high-consensus percentile ranks for multiple HLA class II molecules encoded by *DPB1*05:01, DRB1*07:01, DRB1*08:03, DRB1*09:01, DRB1*13:02*, or *DRB1*15:02* alleles (See "Materials and Methods," **Table 1** and **Fig. S1**). Two regions, GPC3-LP2 and GPC3-LP3, were identified proximal to known 9- or 10-mer CTL epitopes recognized by HLA-A2- or A24-restricted CTLs (**Table 1** and **Fig. S1B**) and predicted to have high binding affinity to HLA class II molecules. The other three LPs (GPC3-LP1, GPC3-LP4, and GPC3-LP5) were predicted to have high binding affinity to HLA class II molecules but did not include known CTL epitope sequences.

Identification of promiscuous GPC3-derived Th cell epitopes

To examine the immunogenicity of candidate peptide, $CD4^+$ T cells isolated from PBMCs of healthy donors (HD; Genotypes of the HDs were given in **Table 1** and **Table S1**) were stimulated with GPC3-LPs at weekly intervals. After at least 3 rounds of stimulation we found GPC3-LP1-specific and IFNγ producing cells were induced from healthy donor HD10 (*DRB1*07:01/13:02/DR53/DR52*) and, healthy donor HD5 (*DRB1*04:05/09:01/DR53*) and these Th cell responses were restricted by HLA-DR (**Fig. 1A**).

By doing similar experiments we are also able to generate GPC3-LP2 (**Fig. 1B**), LP3 (**Fig. 1C** and **Fig. S2A**), LP4 (**Fig. 1D**) and LP5 (**Fig. 1E**)-specific and IFNγ producing cells. GPC3-LP2-iduced Th cells were derived from HD3, HD4, HD5, HD10, and HD11. GPC3-LP3-iduced Th cells were derived from HD5, HD10, and HD11. GPC3-LP4-induced Th cells were derived from HD3 and HD10. GPC3-LP5-induced Th cells were derived from HD5 and HD10. GPC3-LP2-induced Th cells derived from HD5 were restricted by HLA-DP. Other GPC3-LPs-induced Th cells were restricted by HLA-DR. All the *HLA* allelic products that can present these five peptides are summarized in the **Table 1**. These peptides can be applicable to more than 70 % of the Japanese population (**Table S2**).

Exact identification of restriction HLA class II molecules of GPC3-specific Th cells

The bulk GPC3-LP1-specific Th cells from healthy donor HD10 (*DRB1*07:0/13:02/DR53/DR52*) specifically recognized

Table 1. Identification of glypican-3 (GPC3)-derived and promiscuous HLA class II-restricted $CD4^+$ T-cell epitopes encompassing cytotoxic T lymphocyte (CTL) epitopes

Designation of long peptide (LP)	a.a. residue position	Sequence	a.a. length	T-cell donor	[a]Donors' MHC class II	Immune response	Restriction MHC class II molecule	Natural processing[b]
GPC3-LP1	92-116	LLQSASMELKFLIIQNAAVFQEAFE	25	HD3	*DRB1*08:02/15:02*	negative		
				HD10	*DRB1*07:01/13:02*	positive	DR52b (*DRB3*02:02*) (L)	positive *in vitro*
				HD5	*DRB1*04:05/09:01*	positive	DR9 (*DRB1*09:01*) (L)	n.t.
GPC3-LP2	137-161	LTPQAFEFVGEFFTDVSLYILGSDI	25	HD3	*DRB1*08:02/15:02*	positive		
		[c]CTL epitope:A2-$GPC3_{144-152}$		HD10	*DRB1*07:01/13:02*	positive	DR52b (*DRB3*02:02*) (L, allo)	positive *in vitro*
				HD5	*DPB1*02:01/04:02*	positive	DP2 (*DPB1*02:01*) (L, allo)	n.t.
				HD4	*DRB1*08:03/14:05*	positive	DR8 (*DRB1*08:03*) (L, allo)	n.t.
				HD11	*DRB1*09:01/14:54*	positive	n.t.	
				[d]Tgm	*I-A*^{b}	positive	I-A^b	n.t.
GPC3-LP3	289-313	VVEIDKYWREYILSLEELVNGMYRI	25	HD3	*DRB1*08:02/15:02*	negative		
		[c]CTL epitope:A24-$GPC3_{298-306}$		HD10	*DRB1*07:01/13:02*	positive	n.t.	negative *in vitro* *positive in vivo* (in patient)
				HD5	*DRB1*04:05/09:01*	positive	DR9 (*DRB1*09:01*)(L)	
				HD4	*DRB1*08:03/14:05*	negative		
				HD11	*DRB1*09:01/14:54*	positive	n.t.	
GPC3-LP4	386-412	SRRRELIQKLKSFISFYSALPGYICSH	27	HD3	*DRB1*08:02/15:02*	positive	DR15 (*DRB1*15:02*) (allo)	n.t.
				HD10	*DRB1*07:01/13:02*	positive	DR13 (*DRB1*13:02*) (L)	positive *in vitro*
				HD5	*DRB1*04:05/09:01*	negative		
GPC3-LP5	556-576	GNVHSPLKLLTSMAISVVCFF	21	HD3	*DRB1*08:02/15:02*	negative		
				HD10	*DRB1*07:01/13:02*	positive	DR13 (*DRB1*13:02*) (L)	positive *in vitro*
				HD5	*DRB1*04:05/09:01*	positive	DR9 (*DRB1*09:01*) (L)	n.t.

[a]We tested the immunogenicity of GPC3-derived long peptides in these healthy donors and [d]HLA-A2 transgenic mice with C57BL/6 background (Tgm); details of donors' *HLA alleles* are shown in Supplementary Table 1.

[b]An *in vitro* immune response of T helper (Th) cells to dendritic cells pulsed with GPC3 proteins; *in vivo*, a long peptide (LP)-specific Th cell response was observed in patients with hepatocellular carcinoma.

[c]Underlined and bold sequences are CTL epitopes;[9] GPC3-LP1,4,5 sequences were selected due to high-affinity binding to HLA class II molecules predicted by the algorithm; GPC3-LP2 and GPC3-LP3 were selected due to both the prediction of HLA class II binding and the proximity to the known CTL epitopes. a.a.: amino acid, negative: we could not obtain positive data and did not proceed further, n.t.: not tested, LP: long peptide, SP: short peptide, HD: healthy donor, L: restriction HLA class II molecules were confirmed using L-cell lines expressing single allelic product of HLA class II molecules, allo: confirmed by means of allogeneic peripheral blood mononuclear cells (PBMCs) in which at least one of the HLA class II alleles was shared with the donors.

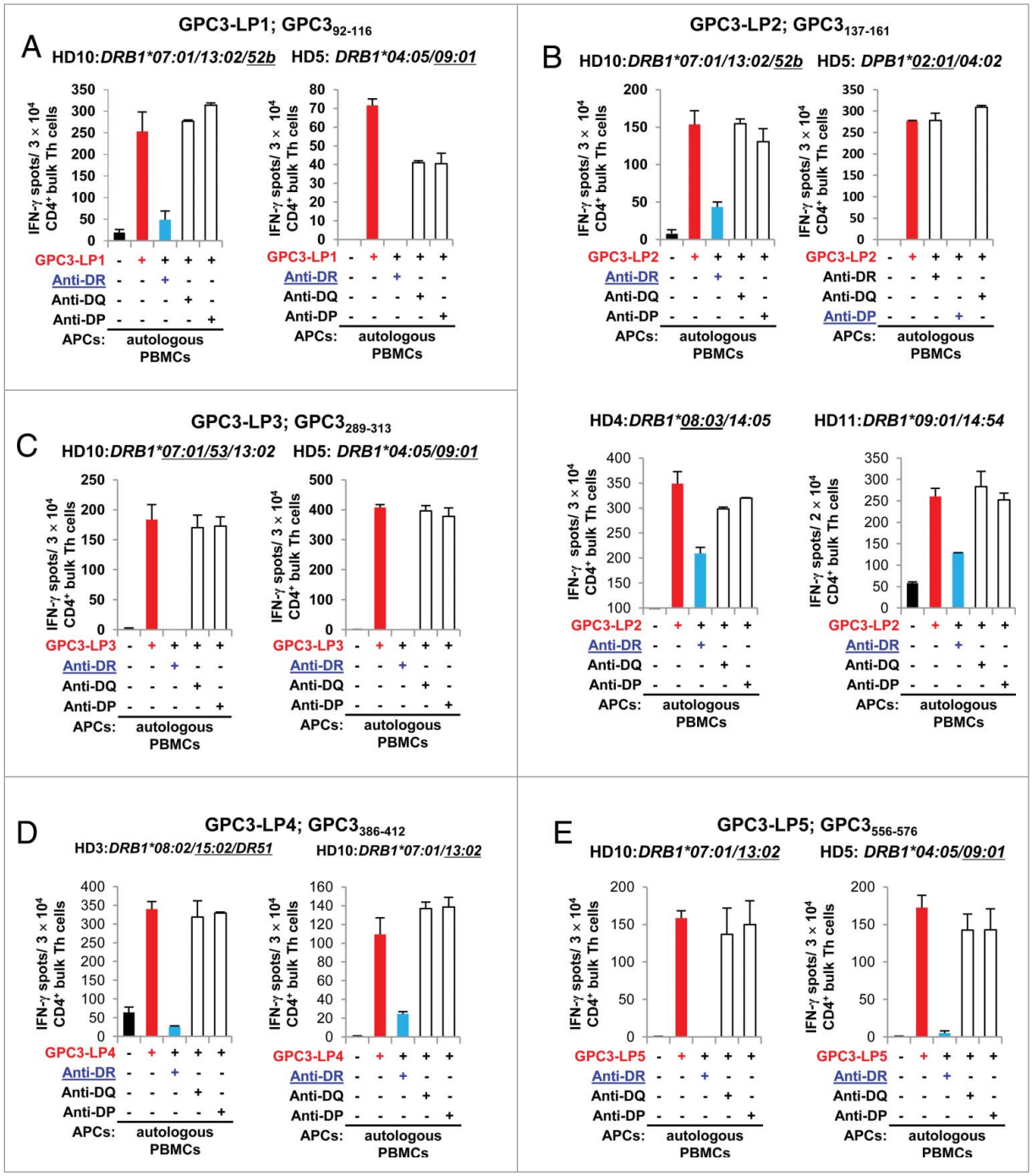

Figure 1. Induction of GPC3-LP-specific Th cells from healthy donors (HDs). (**A–E**) GPC3-specific Th cells were generated from healthy donors by stimulating isolated $CD4^+$ T cells with GPC3-LPs as indicated. The generated Th cells were restimulated with autologous PBMCs pulsed with GPC3-LPs. The number of IFNγ-producing Th cells was analyzed using ELISPOT. Representative data from at least three independent experiments (all yielded similar results) are shown. The HLA class II genotype of the donor is indicated at the top of the panels. The underlined HLA class II alleles encode an HLA class II molecule presenting the peptides to Th cells from **Figure 2**. The underlined mAb inhibited the Th cell response. (**A**) HLA-DR-restricted GPC3-LP1-specific Th cells were generated from PBMCs of an *HLA-DRB1*07:01/13:02*$^+$ healthy donor (HD10, left panel) and from PBMCs of an *HLA-DRB1*04:05/09:01*$^+$ healthy donor (HD5, right panel). (**B**) HLA-DR-restricted GPC3-LP2-specific Th cells were generated from PBMCs of HD10 (upper left panel), an *HLA-DRB1*08:03/14:05*$^+$ healthy donor (HD4, lower left panel), and an *HLA-DRB1*09:01/14:54*$^+$ healthy donor (HD11, lower right panel). HLA-DP-restricted GPC3-LP2-specific Th cells were generated from PBMCs of an *HLA-DPB1*02:01/04:02*$^+$ healthy donor (HD5, upper right panel). (**C**) HLA-DR-restricted GPC3-LP3-specific Th cells were generated from PBMCs of HD10 (left panel) and HD5 (right panel). (**D**) HLA-DR-restricted GPC3-LP4-specific Th cells were generated from PBMCs of an *HLA-DRB1*08:02/15:02*$^+$ healthy donor (HD3, left panel) and HD10 (right panel). (**E**) HLA-DR-restricted GPC3-LP5-specific Th cells were generated from HD10 (left panel) and HD5 (right panel).

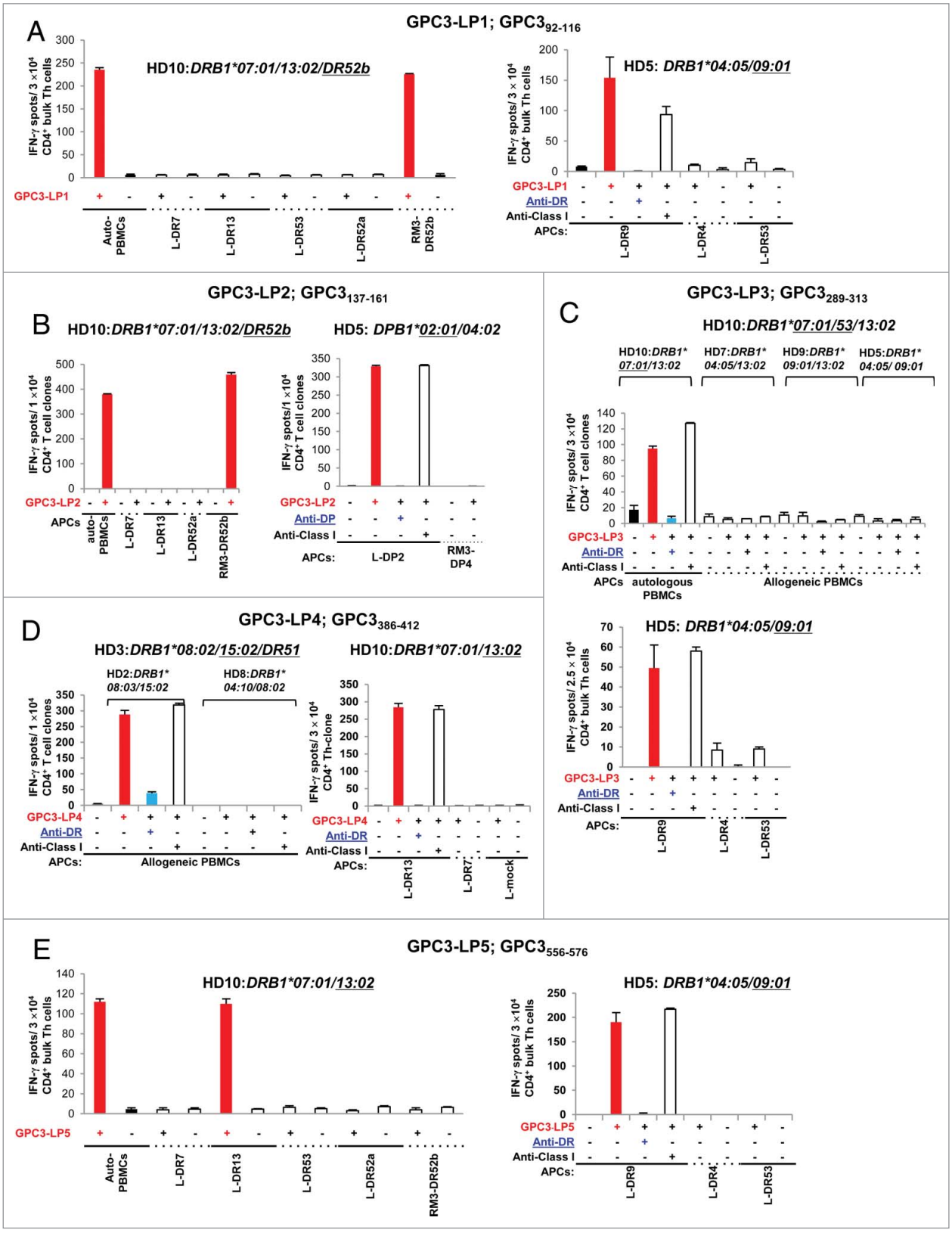

Figure 2. For figure legend see page no. 6.

RM3-DR52b cells (**Fig. 2A**) pulsed with GPC3-LP1 but not GPC3-LP1-pulsed L-DR7, L-DR13, L-DR53, and L-DR52a. The other bulk GPC3-LP1-specific Th cells derived from healthy donor HD5 (*DRB1*04:05/09:01/DR53*) specifically recognized L-DR9 cells pulsed with GPC3-LP1 but not respond to GPC3-LP1-pulsed L-DR4 or L-DR53 (**Fig. 2A**). These results indicated that GPC3-LP1 was presented at least by HLA-DR52b and HLA-DR9.

To identify the restriction HLA class II molecule of the bulk GPC3-LP2-specific T cells that were derived from healthy donor HD10 (*DRB1*07:0/13:02/DR53/DR52*), we generated a Th cell clone (Th-clone). Th-clone specifically recognized the GPC3-LP2-pulsed RM3-DR52b *(HLA-DRB3*02:02)* and allogeneic PBMCs from two *HLA-DR13*$^{+}$*DR7*$^{-}$ healthy donors but did not respond to L-DR7, L-DR13 and L-DR52a (**Fig. 2B** and **S2B**). These results suggested that GPC3-LP2 could be presented by HLA-DR52b.

The Th-clone from GPC3-LP2-specific T cells that were generated from healthy donor HD5 (*DPB1*02:01/04:02*) specifically recognized L-DP2 cells and allogeneic PBMCs carrying a shared HLA-DP2 molecule (pulsed with GPC3-LP2) but not RM3-DP4 cells or allogeneic PBMCs negative for HLA-DP2 (**Fig. 2B and S2C**). We confirmed that GPC3-LP2 induced HLA-DP2-restricted Th cells. GPC3-LP2 generated HLA-DR8 (*DRB1*08:03*)-restricted Th cells; this result was assessed using both allogeneic PBMCs and L-cell transfectant serving as APCs (**Fig. S2D and S2E**). Thus, GPC3-LP2 appeared to be a promiscuous Th cell epitope presented by several frequently occurring HLA class II molecules[24,25] including HLA-DR52b, HLA-DP2, HLADR8, HLA-DR9/14, and HLA-DR8/15 (**Table 1**).

Because the GPC3-LP3-specific bulk Th cells derived from healthy donor HD10 (*DRB1*07:0/13:02/DR53/DR52*) did not recognize allogeneic GPC3-LP3-pulsed PBMCs isolated from 2 *HLA-DR13*$^{+}$ healthy donors (HD7 and HD9) and *HLA-DR52* is linked with *HLA-DR13* allele, we concluded that GPC3-LP3 generated HLA-DR7- or DR53-restricted Th cells in HD10 (**Fig. 2C**). The GPC3-LP3-specific bulk Th cells from healthy donor HD5 (*DRB1*04:05/09:01/DR53*) were specifically activated by L-DR9 cells pulsed with GPC3-LP3 in an HLA-DR-dependent manner but not GPC3-LP3-pulsed L-DR4 or L-DR53 cells (**Fig. 2C**), indicating that GPC3-LP3 was presented by HLA-DR9.

We established a GPC3-LP4-reactive Th-clone from bulk Th cells generated from healthy donor HD3 (*DRB1*08:02/15:02*). We then used allogeneic PBMCs as APCs to determine restriction by shared HLA-DR molecules. We assessed that GPC3-LP4 generated HLA-DR15- or DR51-restricted Th-clone (**Fig. 2D**). We also found that GPC3-LP4 induced HLA-DR13-restricted Th cells (**Fig. 2D**) from healthy donor HD10 (*DRB1*07:01/13:02/DR53* /DR52) because this Th-clone specifically recognized L-DR13 but not L-DR7 pulsed with GPC3-LP4.

GPC3-LP5-reactive Th-clone from healthy donor HD10 (*DRB1*07:01/13:02/DR53* /DR52) could recognize L-DR13 (**Fig. 2E**) but not L-DR7, L-DR53, L-DR52a, or RM3-DR52b cells pulsed with GPC3-LP5. Another GPC3-LP5-reactive Th-clone from healthy donor HD5 (*DRB1*04:05/09:01/DR53*) could recognize L-DR9 but not L-DR4 or L-DR53 cells pulsed with GPC3-LP5. Thus, we suggest that GPC3-LP5 generated HLA-DR13- and HLA-DR9-restricted Th cells (**Fig. 2E**).

GPC3-LPs-induced Th cells secrete Th1 type cytokines in response to stimulation with the cognate peptide

For the characterization of the Th cells reactive to the GPC3-LPs, we measured various cytokines secreted by the Th cells in response to stimulation with the cognate peptide-pulsed autologous PBMCs. GPC3-LP1-, GPC3-LP2- and, GPC3-LP4-specific Th cell clones that were derived from healthy donor HD10 produced a large amount of IFNγ, TNF-α, IL-2, GM-CSF, and MIP1β after re-stimulation with cognate peptides; this finding suggested that GPC3-LPs have the ability to induce Th cells with Th1 polarization characteristics (**Fig. S3**).

Possible natural processing and presentation of GPC3-LPs by DCs

We proceeded to test whether DCs take up and process the GPC3 protein to stimulate GPC3-LP-specific Th cells that were generated by stimulation with synthetic LPs. DCs that were loaded with the recombinant GPC3 protein were used as APCs in IFNγ ELISPOT.[26,27] Four GPC3-LPs (GPC3-LP1, −2, −4, and −5)-reactive Th cell clones derived from healthy donor HD10 (*DRB1*07:01/13:02/DR53/DR52*) efficiently recognized DCs loaded with the GPC3 protein but did not recognize control protein-loaded DCs, indicating that these epitopes could be naturally processed from the GPC3 protein (**Fig. 3**).

Figure 2 (See previous page). Exact identification of restriction HLA class II molecules of GPC3-specific Th cells. (**A–E**) GPC3-specific Th cells were generated from healthy donors (HDs) by stimulating magnetic bead-isolated CD4^{+} T cells with GPC3-LPs as shown in **Figure 1**. The generated Th cells from healthy donors were then re-stimulated with autologous PBMCs, allogeneic PBMCs, or HLA class II-expressing L cells pulsed with individual GPC3 LPs. The number of IFNγ-producing cells was analyzed using an ELISPOT. Representative data from at least two independent experiments (yielded similar results) are shown. The HLA class II genotype of the donor is indicated at the top of the panels. The underlined HLA class II alleles encode HLA class II molecules presenting the peptides to Th cells. The underlined mAb inhibited the Th cell response. (**A**) HLA-DR52b- and DR9-restricted GPC3-LP1-specific Th cells were generated from PBMCs of healthy donors HD10 (left panel) and HD5 (right panel). (**B**) HLA-DR52b- and DP2-restricted GPC3-LP2-specific Th cells were generated from PBMCs of HD10 (left panel) and HD5 (right panel). (**C**) HLA-DR7/53- and DR9-restricted GPC3-LP3-specific Th cells were generated from PBMCs of HD10 (upper panel) and HD5 (lower panel). (**D**) HLA-DR15/51- and DR13-restricted GPC3-LP4-specific Th cells were generated from PBMCs of healthy donors HD3 (left panel) and HD10 (right panel). (**E**) HLA-DR13- and DR9-restricted GPC3-LP5-specific Th cells were generated from PBMCs of HD10 (left panel) and HD5 (right panel).

In vitro cross-presentation of SPs by human DCs loaded with GPC3-LP2

We evaluated the ability of GPC3-LP2 to stimulate A2-GPC3-SP-specific CTLs by means of IFNγ ELISPOTs as described in *Materials and Methods*. As shown in **Figure 4A**, A2-GPC3-SP-specific bulk CTLs that were derived from an HLA-A2$^+$ donor specifically produced IFNγ in response to stimulation with DCs loaded with GPC3-LP2 encapsulated in liposomes but not DCs loaded with liposomes plus GPC3-LP or control LP encapsulated in liposomes. The specific IFNγ production was markedly inhibited by the anti-HLA class I mAb but not by the anti-HLA-DR mAb, suggesting that the A2-GPC3-SP-reactive CTLs were stimulated through the cross-presentation of GPC3-LP2 by DCs *in vitro*.

An *in vivo* cross-priming assay using HLA-A2 Tgm

The ability of GPC3-LP2 to prime A2-GPC3-SP-specific CTLs was examined using *HLA-A2* Tgm. The CD8$^+$ T cells from *HLA-A2* Tgm that were vaccinated with GPC3-LP2 produced IFNγ specifically in response to *ex vivo* stimulation with bone marrow DCs pulsed with the A2-GPC3-SP (**Fig. 4B**). These results suggest that after uptake of GPC3-LP2, APCs can cross-prime A2-GPC3-SP-specific CTLs *in vivo* in *HLA-A2* Tgm. In the isolated CD8$^+$ cells, the number of A2-GPC3-SP-specific CTLs as estimated by an IFNγ ELISPOT was increased in the mice immunized with GPC3-LP2 compared to mice immunized with A2-GPC3-SP (**Fig. 4B**).

The CD4$^+$ T cells that were isolated from *HLA-A2* Tgm immunized with GPC3-LP2 produced IFNγ specifically in response to stimulation with murine BMDCs pulsed with the GPC3-LP2 (**Fig. 4C**) but not with the control (GPC3-LP5). These results suggested that GPC3-LP2 could also prime GPC3-LP2-specific and probably I-A^b-restricted Th cells *in vivo* in HLA-A2 Tgm.

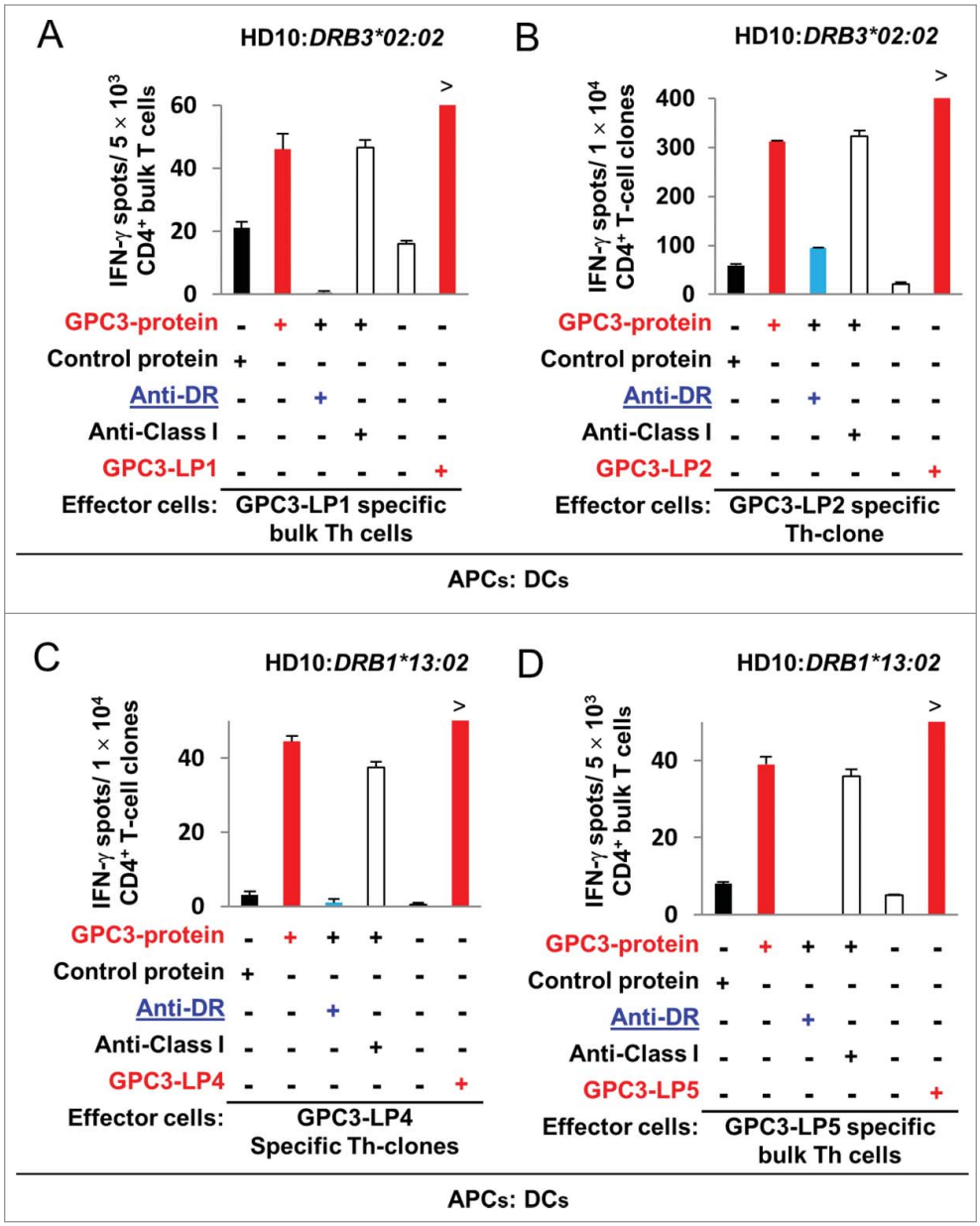

Figure 3. Natural processing and presentation of GPC3-LPs by DCs loaded with the recombinant human GPC3 protein. (**A**) An HLA-DR52b (*HLA-DRB3*02:02*)-restricted and GPC3-LP1-specific Th clone that was derived from healthy donor HD10 recognized autologous DCs loaded with the recombinant human GPC3 protein. Representative data from two independent experiments that were performed in duplicate (yielded similar results) are shown. (**B**) An HLA-DR52b-restricted GPC3-LP2-specific Th clone that was derived from HD10 recognized autologous DCs loaded with the recombinant human GPC3 protein. (**C**) An HLA-DR13-restricted and GPC3-LP4-specific Th clone that was derived from HD10 recognized autologous DCs loaded with the recombinant human GPC3 protein. Representative data from three independent experiments that were performed in duplicate (all yielded similar results) are shown. (**D**) An HLA-DR13-restricted and GPC3-LP5-specific Th cell clone that was derived from healthy donor HD10 recognized autologous DCs loaded with the recombinant human GPC3 protein.

The number of GPC3-LP-specifc CD4$^+$ T cells is increased in HCC patients vaccinated with GPC3-SPs

Cancer patients who are vaccinated with restricted epitopes often mount a T-cell response to epitopes not present in the vaccine.[15] To assess the induction of GPC3-LPs-specific Th cell

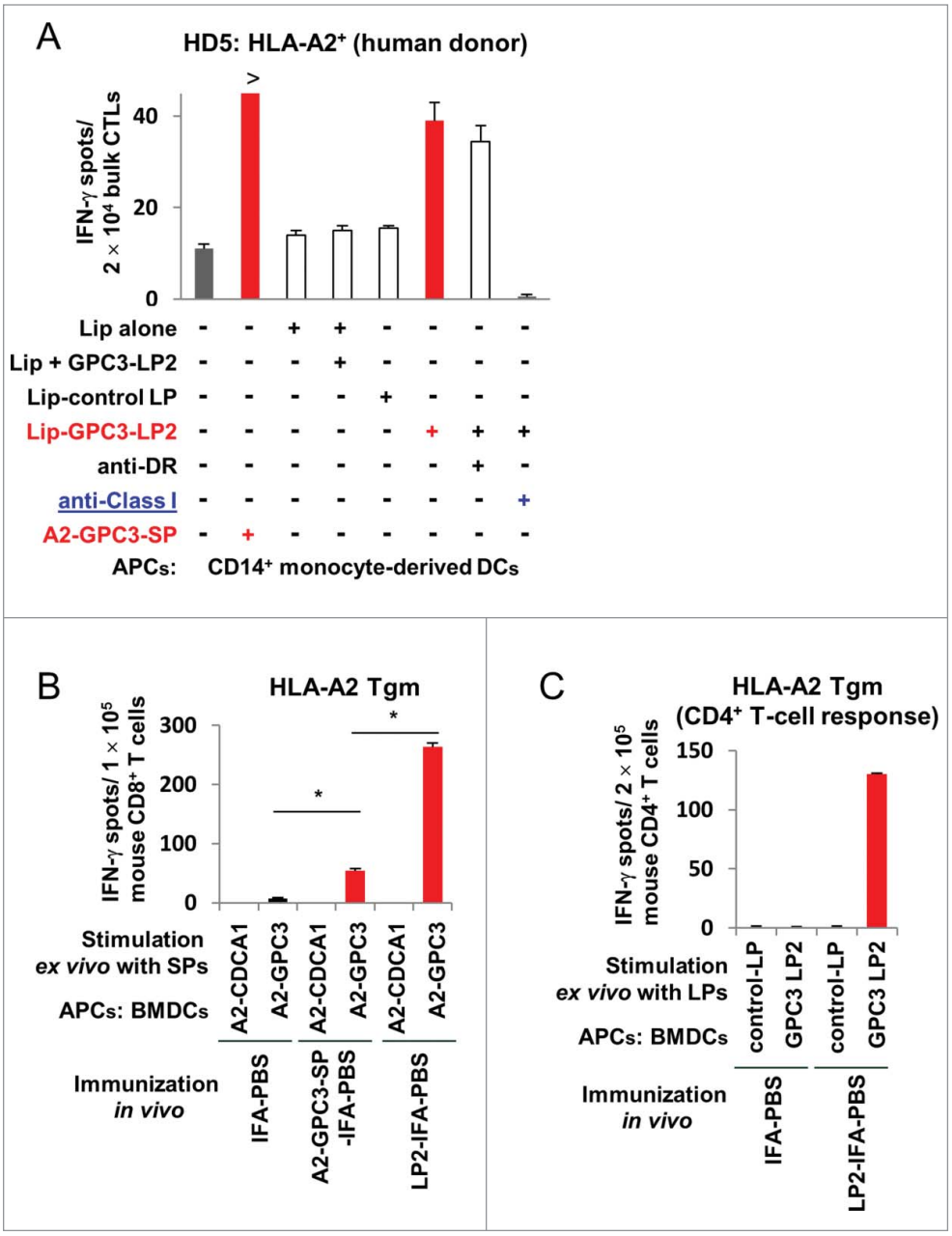

Figure 4. DCs induced efficient cross-presentation of GPC3-LP2 to A2-GPC3$_{144–152}$-SP-specific and HLA-A2-restricted CTLs *in vitro* and cross-priming in HLA-A2 Tgm *in vivo*. (**A**) A2-GPC3$_{144–152}$-SP-specific CTLs that were derived from healthy donor HD5 (*HLA-A2*$^+$ and *HLA-DP2*$^+$) were stimulated *in vitro* with autologous DCs pulsed with GPC3-LP2 encapsulated in liposomes (Lip-GPC3-LP2), IMP3$_{507–527}$-LP encapsulated in liposomes (Lip-control LP), liposomes plus soluble GPC3-LP2 (Lip + GPC3-LP2), or liposomes alone (Lip). Representative data of three independent experiments (all yielded similar results) are shown. (**B–C**) HLA-A2 Tgm was immunized with A2-GPC3$_{144–152}$-SP (A2-GPC3-SP-IFA-PBS), GPC3-LP2 (LP2-IFA-PBS), or PBS emulsified in incomplete Freund's adjuvant (IFA; IFA-PBS). Seven days after the second immunization, murine CD4$^+$/CD8$^+$ T cells were isolated from the pooled inguinal lymph nodes and were stimulated *ex vivo* with BMDCs pulsed with GPC3-LP2 or GPC3-LP5 (control LP) and A2-GPC3$_{144–152}$-SP, A2-CDCA1-SP, or A2-HIV-SP. The numbers of IFNγ-producing murine CD4$^+$/CD8$^+$ T cells were assessed using an *ex vivo* ELISPOT. Representative data from 2∼4 independent experiments (2–3 mice in each group) that were performed in duplicate or triplicate (all yielded similar results) are shown. (**B**) GPC3-LP2 immunization induced an enhanced SP-specific CTL response in comparison with GPC3-A2-SP immunization *in vivo* when an equimolar dose of the peptide was used. (**C**) An immune response of GPC3-LP2-specific CD4$^+$ Th cells isolated from the same pooled inguinal lymph nodes.

response in cancer patients, we stimulated PBMCs isolated from HCC patients vaccinated with A2-GPC3-SP or A24-GPC3-SP. The patients' characteristics are summarized in **Table S3**. After seven-day *in vitro* stimulation of PBMCs with GPC3-LPs, the frequency of individual GPC3-LPs-specifc T cells was measured (**Figs. 5A–E**). The responses were considered positive when the number of IFNγ-secreting cells was increased at least two-fold above the negative control. GPC3-LP-specific immune responses were observed in 14 of the 20 vaccinated patients (only representative four data was shown in **Figs. 5B–E**, rest of the data was shown in **Fig. S5** and **Table S3**). GPC3-LP-specific IFNγ production by T cells was significantly inhibited by addition of an anti-HLA-class II mAb (**Fig.5** and data not shown) but not an anti-HLA-class I mAb. These results demonstrated that IFNγ was produced by GPC3-LP-specific CD4$^+$ T cells. We compared these responses in patients' PBMCs collected just before the first vaccination, the seventh vaccination and the tenth vaccination, and found that LP-specific CD4$^+$ T-cell responses were induced, maintained or enhanced in 8 of 13 vaccinated patients after repeated vaccinations (**Figs. 5F–G**, **Fig. S5 and Table S4**). Th cell response was observed in 11 of 16 patients who enrolled in a phase II clinical trial. OS was significantly prolonged in patients with specific Th cell response compared to patients with negative Th response (**Fig. 5H** left and **Table S5**). We also compared OS between GPC3-LPs-specific Th cell response-positive and -negative patients who didn't mount vaccine specific CTL response, and found that OS was significantly

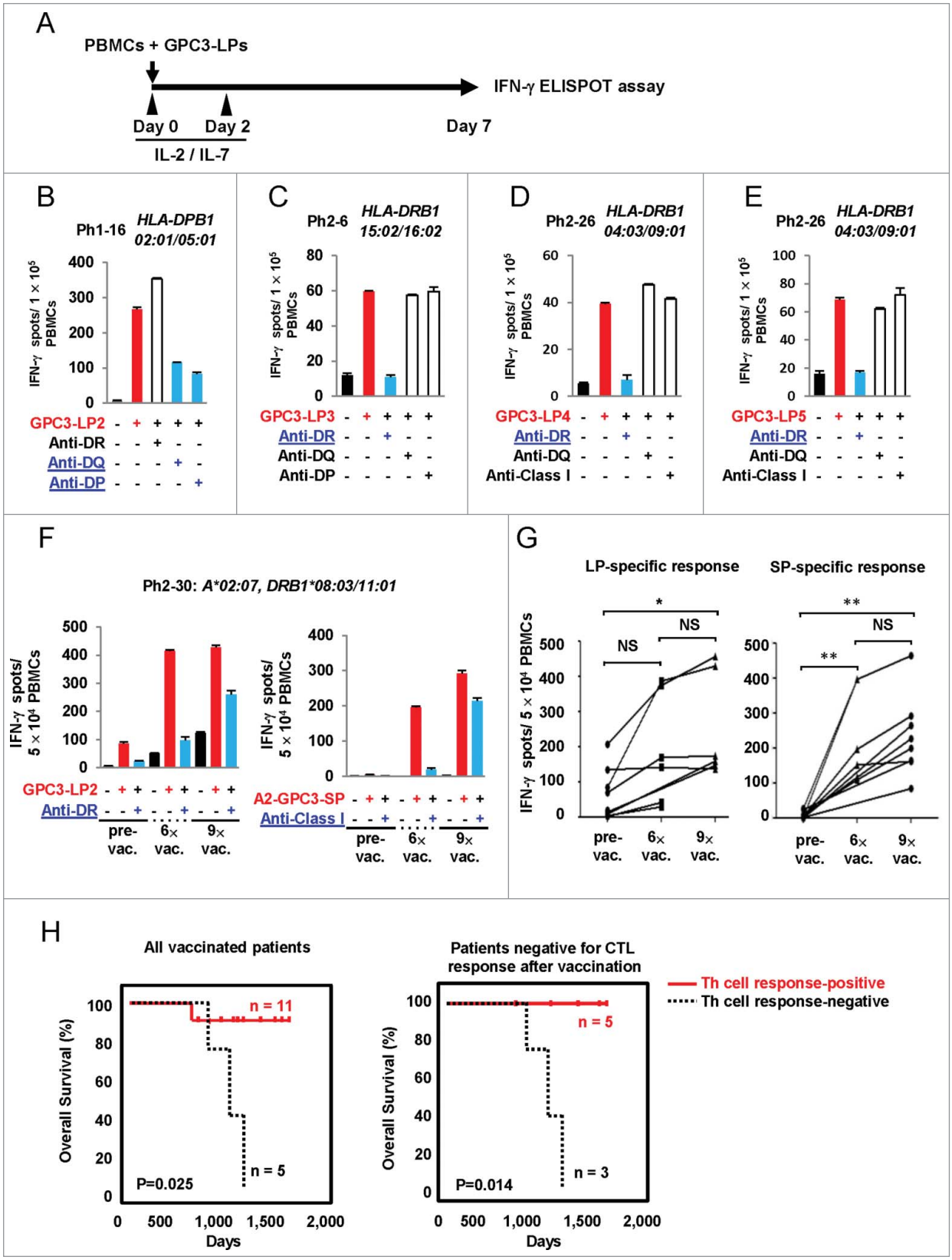

Figure 5. For figure legend see page no. 10.

prolonged in Th response-positive group (**Fig. 5H** right and **Table S5**).

Discussion

In our previous studies, although a patient showed strong specific CTL responses along with tumor lysis after vaccination with GPC3-SPs,[28] no complete response was observed when GPC3-SP was used as monotherapy for advanced HCC.[8] Maintenance of the memory CTLs induced by GPC3-SPs can be improved by the help of tumor-specific $CD4^+$ Th cells.[18] Therefore in this study, we sought to identify GPC3-derived LPs capable of inducing both CTL and Th1 cells.

Our key findings are as follows, (1) Five GPC3-LPs capable of eliciting promiscuous HLA class II-restricted Th1 cell responses were identified and four of them are likely to be naturally processed from the GPC3 protein by DCs *in vitro*. (2) GPC3-LP2, which bears a natural HLA-A2-restricted CTL epitope, was cross-presented effectively when encapsulated in newly developed liposomes. GPC3-LP2 alone was also cross-presented to prime specific CTLs *in vivo* in HLA-A2 Tgm. (3) Immunization of *HLA-A2* Tgm with GPC3-LP2 encompassing A2-GPC3-SP was more effective in eliciting SP-specific CTLs in comparison with immunization with A2-GPC3-SP alone. (4) Th cell responses were also observed in HCC patients repeatedly vaccinated with GPC3-SP and they strongly correlated with the prolonged OS of the patients.

$CD4^+$ T cell help was needed to mount an effective secondary $CD8^+$ T cell response.[29] This help may be mediated through costimulatory signals induced by CD40/CD40L interaction necessary to activate APC or through signals induced by cytokines including Il-2, IL-7 and IL-15 for the maintenance of memory T cells.[18] In our study, we found increased number of SP-specific CTLs in mice that were immunized with GPC3-LP2 compared to mice immunized with SP alone. A part of this augmented CTL response may be attributed to the help of the $CD4^+$ T cell response, because GPC3-LP2 stimulated a specific murine $CD4^+$ T cell response.

The use of LPs increases a possibility that any DC taking up LPs stimulate both $CD4^+$ T cells and $CD8^+$ T cells.[21,30–34] LPs can be taken up, processed into SPs and presented to $CD8^+$ T cells by DC rather than nonprofessional APC, and this can evoke a stronger and more effective CTL response. Such DC-focused antigen presentation may be another mechanism that caused increased CTL response in mice immunized with GPC3-LP2.

In cancer patients treated with immunotherapy, growth arrest and cancer regression correlated with the presence of tumor-specific, IFNγ producing $CD4^+$ cells.[35] One of the plausible mechanisms of Th1 cells mediated tumor regression could be cellular senescence, because IFNγ and TNF produced by Th1 cells can induce senescence in numerous murine and human cancer cells.[36] As most of the Th cells induced by GPC3-LPs were found to secrete a large amount of Th1 type cytokines including IFNγ and TNF-α (**Fig. S3**), we speculate that the use of GPC3-LPs will be beneficial for immunotherapy of HCC.

Cancer patients vaccinated with a restricted epitope often mount a T-cell response to peptides that were not included in the vaccine, a phenomenon being called "epitope spreading."[37] We observed that GPC3-LPs-specific Th cell responses were induced, maintained or enhanced in HCC patients by repeated GPC3-SP vaccinations. Therefore, the GPC3-LPs-specific Th cell response might be induced through epitope spreading. So far no correlation was observed between responses to SPs and responses to LPs among the patients possibly due to the low number of patients investigated and the heterogeneity in magnitude of epitope spreading among patients. However, when OS of HCC patients was assessed, on the basis of GPC3-LPs-specific Th cell response we observed a strong correlation between prolonged OS and positive Th cell response.

MGlu-PG-modified liposomes are taken up efficiently by DCs and reportedly deliver entrapped ovalbumin molecules into the cytosol.[38] These liposomes are regarded as a promising system to deliver antigens. The pH-sensitive liposomes were prepared by surface modification of egg yolk phosphatidylcholine liposomes with pH-sensitive poly (glycidol) derivatives containing MGlu-PG. In our *in vitro* experiments we didn't observe cross presentation of soluble GPC3-LP2. We thought this was due to inefficient delivery of the LPs into HLA class I-mediated antigen processing pathway in APC. However, the use of LP encapsulated in our liposome resulted in efficient cross presentation probably through efficient delivery of LP to cytoplasm of DC. Although in our *in vivo* cross priming experiments we didn't use LP encapsulated in liposome, we observed sufficient cross priming.

One possible reason is that we used IFA as an adjuvant in *in vivo* settings. IFA causes accumulation of mature DC.[39] Mature DCs present the IFA-embedded peptide and cross prime CTLs *in vivo* in *HLA-A2* Tgm.

MHC class II proteins are highly polymorphic.[40] Thus, it is advisable to use a peptide or a cocktail of peptides vaccine that can be presented by multiple HLA class II molecules, so that the

Figure 5 (See previous page). The presence of GPC3-LP-specific Th cells in the PBMCs of patients with HCC who were vaccinated with a GPC3-SP. (**A–H**). Frozen PBMCs that were derived from HCC patients vaccinated with GPC3-SP (**Tables S3** and **Table S4**) were stimulated with a mixture of GPC3-LP1, −2, −3, −4, and −5 plus IL-2 and IL-7 *in vitro*. After 7 days, the frequency of individual GPC3-LP-specific T cells was assessed using an ELISPOT. Th cell responses specific to GPC3-LP2 (**B**), LP3 (**C**), LP4 (**D**), and LP5 (**E**) were observed in 14 of the 20 HCC patients tested. HLA class II restriction of the GPC3-LP-specific Th cells was determined by means of a blocking assay using monoclonal antibodies specific to HLA-DR, -DQ, or -DP. (**F**) The number of LP-specific $CD4^+$ T cells increased after repeated SP vaccinations. (**G**) Both GPC3-LP- and SP-specific responses were assessed in 13 HCC patients vaccinated with GPC3-SPs. The results represent specific IFNγ spots after subtraction of the background response. The PBMCs were isolated from the same patient at different time points as follows: just before the first vaccination with SPs (pre-vac), before the seventh vaccination (6×-vac), and before the tenth vaccination (9×-vac). (**H**) Kaplan–Meir curves for OS. Patients with GPC3-specific Th cell responses vs. patients with no-response (left panel) and patients with GPC3-specific Th cell responses vs. patients without response among patients negative for CTL induction (right panel).

vaccine can be applied to a large number of patients. However, several common HLA-DR types share largely overlapping peptide repertoires bound by HLA-DR[41] due to which broad coverage of population could be achieved with a relatively limited number of peptides in a vaccine. Although we checked immunogenicity in a small number of healthy donors, five GPC3-LPs used in the present study are expected to be applicable to the majority of the Japanese population (**Table S2**).[42]

Among the various clinical and histopathological markers, intratumoral T cell infiltration is one of the reliable predictive indicators for prolonged survival.[43] So far there is no correlation between $CD4^+$ infiltration and OS of the HCC patients vaccinated with GPC3-SP (data not shown). More detailed quantitative and qualitative analysis of the infiltrated GPC3-specific T cells is necessary to elucidate the role of immune infiltrate in the patients with a longer survival. The analysis of direct recognition of autologous tumor cells by patients' GPC3-LPs-specific Th cells is also necessary to evaluate their capacity of direct killing.

Taken together, we propose the use of vaccines consisting of GPC3-LPs bearing both $CD4^+$ and $CD8^+$ T-cell epitopes that possesses many promises to improve the GPC3 peptide-based cancer immunotherapy for HCC.

Materials and Methods

Clinical trials of GPC3-SPs vaccination in HCC patients

A phase I clinical trial was approved by the Ethics Committee of the National Cancer Center and was conducted from February 2007 to November 2009. The trial was registered in the University Hospital Medical Information Network Clinical Trial Registry (UMIN-CTR number, 000001395). Patients with advanced or metastatic HCC were enrolled after providing written informed consent as described previously.[8] A phase II trial (UMIN-CTR number 000002614) was conducted for patients with HCC who received curative surgical treatment from 2010 to 2013 (manuscript in preparation). Unlike the phase I trial, the phase II trial was a single-arm study without dose escalation of the GPC3 peptide, and 3 mg of the GPC3 peptide was used for each vaccination. In the phase II clinical trial, 10 times vaccinations were performed: the initial 6 times every 2 weeks and the last 4 times every 2 mo. The primary endpoints were 1- and 2-year recurrence rates. The secondary endpoints were safety and immunological responses.

Cell lines

Mouse fibroblast cell lines (L cells), genetically engineered to express DP5 (*DPA1*02:02/DPB1*05:01*, L-DP5) and DR4 (*DRB1*04:05*, L-DR4), DR8 (*DRB1*08:03*, L-DR8), DR13 (*DRB1*13:02*, L-DR13), or DR15 (*DRB1*15:02*, L-DR15) were used as APCs. These L cells were maintained *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. L cells expressing DR7 (*DRB1*07:01*, L-DR7), DR13 (*DRB1*13:01*, L-DR13), DR52a (*DRB3*01:01*, L-DR52a), DR52b (*DRB3*02:02*, RM3-DR52b), DR15 (*DRB1*15:01*, L-DR15), DP2 (*DPA1*01:03/DPB1*02:01*, L-DP2), and DP4 (*DPA1*01:03/DPB1*04:01*, RM3-DP4) were kindly provided by Dr. Alessandro Sette of La Jolla Institute for Allergy and Immunology, California, USA.[44]

Prediction and selection of possible promiscuous HLA class II-binding GPC3-LPs

To predict possible promiscuous HLA class II-binding human GPC3-derived peptides, the amino acid sequence of the human GPC3 protein was analyzed using a computer algorithm, http://tools.immuneepitope.org/mhcii/).[45] We defined the score of percentile rank less than 10 as indicating stronger binding affinity to HLA class II molecules and selected the regions which were predicted to have high binding affinity to multiple frequently observed HLA class II molecules (encoded by *DPB1*05:01, DRB1*07:01, DRB1*08:03, DRB1*09:01, DRB1*13:02*, or *DRB1*15:02* alleles. We also considered the regions that were proximal to known 9- or 10-mer CTL epitopes recognized by HLA-A2- or A24-restricted CTLs (**Fig. S1B**) and predicted to have strong binding affinity to HLA class II molecules. We avoid the regions that have potential glycosylation sites.

Synthetic peptides and recombinant proteins

Two human GPC3-derived SPs presented by HLA-A2 (A2-$GPC3_{144–152}$; A2-GPC3-SP) or HLA-A24 (A24-$GPC3_{298–306}$; A24-GPC3-SP), and five GPC3-LPs described above were synthesized by MBL (Nagoya, Japan; purity >95%; **Fig. S1B**). An HIV-related SP (A2-HIV) and a CDCA1-derived SP (A2-CDCA1) that binds to HLA-A2 were used as negative control SPs.[27,46] In some experiments, $IMP3_{507–527}$-LP served as a control LP. The recombinant full-length GPC3 protein was purchased from R&D Systems (Minneapolis, USA; purity >90%) and was reconstituted as a 1 mg/mL solution in PBS containing 0.2% fetal calf serum. The recombinant human full-length CDCA1 protein was used as a control.[46] The liposomes loaded with GPC3-LP2 and IMP-$3_{507–527}$-LP (as a control) were prepared as described previously.[47]

Generation of antigen-specific $CD4^+$ T cells from healthy donors

The protocols for isolation and use of peripheral blood mononuclear cells (PBMCs) from healthy donors were approved by the Institutional Review Board of Kumamoto University. We obtained PBMCs from 11 healthy donors with written informed consent. Genotyping of *HLA-A, DRB1*, and *DPB1* alleles was performed at the HLA Laboratory (Kyoto, Japan; **Table S1**). Induction of antigen-specific $CD4^+$ T cells was performed as described previously.[33] Briefly, $CD4^+$ T cells were purified from PBMCs via positive selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA).[48] Monocyte-derived DCs were generated from $CD14^+$ cells by means of *in vitro* cultivation,[26] and were used as APCs to induce antigen-specific $CD4^+$ T cells.[30] In some instances, T cells were cloned by limiting dilution for further analysis.[49]

Assessment of T-cell responses to peptides and proteins

The immune responses of Th cells to APCs pulsed with peptides (10 μg/mL) or proteins (10 μg/mL) were assessed using IFNγ enzyme-linked immunospot assays (ELISPOTs; BD Biosciences).[46] We analyzed the frequency of peptide-specific and IFNγ producing $CD4^+$ T cells per 3×10^4 of bulk $CD4^+$ T cells after stimulation with peptide-pulsed PBMCs (3×10^4) or 1×10^4 of bulk $CD4^+$ T cells after stimulation with peptide-pulsed and HLA-DR-expressing L cells (5×10^4/well) or RM3 (5×10^4/well).[33]

The *in vitro* cross-presentation assay

Yuba et al.[38] developed pH-sensitive 3-methylglutarylated poly (glycidol) (MGLu-PG)-modified liposomes containing the tumor antigen to enhance the efficiency of cross-presentation by DCs. To assess the cross-presentation of GPC3-LP2, we utilized DCs pulsed with GPC3-LPs encapsulated in liposomes. Briefly, a peptide (0.22 μmol) that was dissolved in N, N-dimethylformamide or deionized water (5 mg/mL) was added to a dry thin membrane of EYPC/CHexPG-PE (97/3, mol/mol; 6.25 μmol), and then the solvent was removed under vacuum for >3 h. The resulting lipid-peptide mixture was resuspended in PBS (500 μL) with 2-min sonication using a bath-type sonicator, yielding a suspension of liposomes that incorporated the peptide. The liposome suspension was further hydrated by freezing and thawing and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was centrifuged at 55,000 rpm for 1.5 h at 4°C twice to remove the free peptide from the liposomes. The lipid and peptide concentrations were measured by means of Phospholipids C (Wako) and Micro BCA Protein assays (Thermo Scientific), respectively.

Immature DCs were prepared from positively isolated $CD14^+$ cells. $CD14^+$ cells were cultured in the presence of IL-4 (10 ng/mL) and granulocyte macrophage colony-stimulating factor (GM-CSF, 100 ng/mL). Immature DCs were harvested on day 5 and pulsed with an LP encapsulated in liposomes (equivalent to an LP at 20 μg/mL) for 4 h. We counted the number of IFNγ-producing-A2-$GPC3_{144–152}$-SP-specific bulk CTLs in response to DCs loaded with GPC3-LP2 encapsulated in liposomes using an ELISPOT. Stimulation with SP-pulsed DCs was used as a positive control; un-pulsed DCs, DCs pulsed with liposomes alone, liposomes mixed with soluble GPC3-LP2, and DCs pulsed with $IMP3_{502–527}$-LP encapsulated in liposomes served as negative controls.

In vivo cross priming and induction of LP-specific murine $CD4^+$ T cells

HLA-A2 (HHD) Tgm were kindly provided by Dr. F.A. Lemonnier.[50] The mice were subcutaneously injected (at 7-day intervals) at the tail base with equimolar (50 μM,) solutions of GPC3-LP2 or A2-GPC3-SP emulsified in IFA.[30]

The cytokine assay

GPC3-LPs-specific bulk Th cells or Th cell clones (3×10^4/well) were cultured in the presence of cognate peptide-pulsed autologous PBMCs in 96-well plates. After 24 h, the culture supernatants were collected, and indicated cytokine concentrations were measured using the Bio-Plex System (Bio-Rad).

Assessment of GPC3-LP- or SP-specific $CD4^+$ or $CD8^+$ T-cell responses in HCC patients immunized with A2 or A24-GPC3-SP

After thawing the frozen PBMCs that were isolated from patients with HCC, we cultured the cells in the presence of a mixture of five or individual GPC3-LPs (10 μg/mL each) in a final volume of 2 mL of the AIM-V medium (supplemented with 5% human decomplemented plasma) at 37°C (2×10^6/well, 24-well plates). IL-2 and IL-7 were added on days 0 and 2. After 1 week of cell culture, the cells were collected, washed, and cultured in ELISPOT plates ($5–10 \times 10^4$/well) with the individual GPC3-LP (or GPC3-SP) or control LP (or SP) for 18 h. The numbers of GPC3-LP- and SP-specific $CD4^+$ or $CD8^+$ T cells were estimated as described previously.[31]

Statistical analysis

We compared the means using 1-way ANOVA (bar graphs and scatter dot graph). OS rates were analyzed by the Kaplan–Meir method. The significance of these parameters was estimated with the log-rank test. Differences with a p value < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

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Supplemental Material

Supplemental data for this article can be accessed on the pub lisher's website.

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