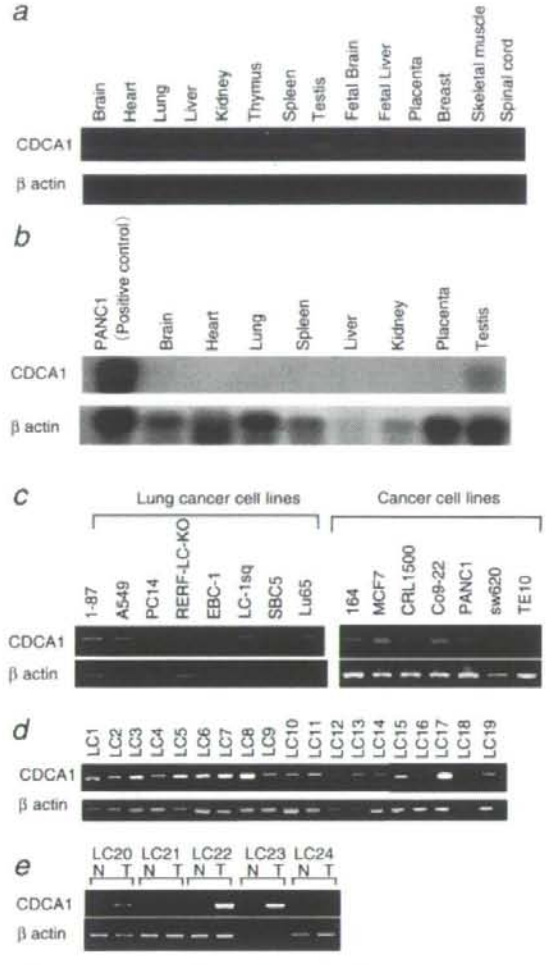

FIGURE 2 – Expression of *CDCA1* mRNA in human normal tissues detected by RT-PCR and Northern blot analysis. Expression of the *CDCA1* mRNA in normal tissues was also analyzed using RT-PCR (*a*) and Northern blot analysis (*b*). *CDCA1* mRNA was strongly expressed in the normal testis and very weakly expressed in thymus, but not in brain, heart, lung, spleen, liver, kidney and placenta. *CDCA1* gene expression detected by RT-PCR in cancer cell lines (*c*) and cancer tissues of NSCLC patients (*d*). (*c*) *CDCA1* mRNA was expressed in all 8 lung cancer cell lines tested and other cancer cell lines, including 1-87, A549, PC14, RERF-LC-KO (ADC), EBC-1, LC-1sq (SqCC), SBC5, LU65 (SCLC), 164 (melanoma), MCF7 (breast cancer), CRL1500 (breast cancer), Co9-22 (oral cancer), PANC1 (pancreas cancer), sw620 (colon cancer) and TE10 (esophageal cancer). (*d*) In 18 cases out of 19 NSCLC tissues, *CDCA1* gene was expressed in cancer tissue. (e) *CDCA1* mRNA was strongly expressed in lung cancer tissues compared with noncancerous lung tissues of NSCLC patients.

carcinoma: SqCC), SBC5 and Lu65 (small cell LC: SCLC) strongly expressed *CDCA1* mRNA (Fig. 2*c*). The other cancer cell lines including 164 (melanoma), MCF7 (breast cancer), CRL1500 (breast cancer), Co9-22 (oral cancer), PANC1 (pancreas cancer), sw620 (colon cancer) and TE10 (esophageal cancer) revealed high CDCA1 expression at mRNA level (Fig. 2*c*). In 18 of 19 NSCLC patients, *CDCA1* mRNA was highly expressed in cancer tissues (Fig. 2*d*). We also performed RT-PCR analysis of CDCA1 mRNA expression in both cancerous and normal lung tissues isolated from 5 LC patients in Figure 2*e*. These results indicated the expression of CDCA1 to be higher in LC tissues in comparison to that of normal lung tissues in all the 5 patients investigated.

Furthermore, we analyzed the expression level of *CDCA1* mRNA in other kind of cancer tissues by using microarray analysis[21,28–30] (Table I). *CDCA1* mRNA was overexpressed in all cases of SCLC ($n = 15$), cholangiocellular cancer ($n = 12$), bladder cancer ($n = 28$) and renal cell cancer ($n = 7$) in comparison to their adjacent normal counterparts.

Expression of CDCA1 protein in testis and cancer cell lines

To investigate the expression of CDCA1 protein, we then examined many paraffin-embedded normal tissues and 12 cases of paraffin-embedded NSCLC tissues. CDCA1 did not stained in the normal brain, liver, lung, kidney and spleen (Figs. 3*a*–3*e*), except for normal testis (Fig. 3*f*). In this study, we investigated 8 samples of lung ADC and 4 samples of lung SqCC, and strong CDCA1 staining was observed in 9 cases, and no staining was observed in any of their adjacent normal lung tissues (Figs. 3*g*–3*i*).

Identification of HLA-A2-restricted mouse CTL epitopes of human CDCA1 by using HLA-A2.1 (HHD) Tgm

As the candidates of HLA-A2-restricted and human CDCA1-derived CTL epitopes, we selected 40 kinds of peptides having high predicted binding scores to HLA-A2 (A*0201) calculated by using the BIMAS software program (Table II). We tried to identify HLA-A2-restricted CTL epitopes by using HLA-A2.1 HHD Tgm. CD4 negative spleen cells isolated from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of 4 peptides selected from these 40 CDCA1 peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide (Fig. 4*a*). As a result, we found that CD4-negative spleen cells stimulated *in vitro* with the $CDCA1_{65-73}$ and $CDCA1_{351-359}$ peptides produced a significant amount of IFN-γ in a peptide-specific manner in ELISPOT assays (Figs. 4*b* and 4*c*). These CD4 negative spleen cells (2×10^4/well) showed 106 ± 13.1 spot counts/well in response to the BM-DCs pulsed with the $CDCA1_{65-73}$ peptide, whereas they showed 42.0 ± 9.64 spot counts/well in the presence of the BM-DCs without peptide loading ($p < 0.05$) (Fig. 4*b*). These assays were done 3 times with similar results. Secondly, the CD4-negative spleen cells stimulated with BM-DC pulsed with $CDCA1_{351-359}$ peptides showed 42.3 ± 4.02 spot counts/well, whereas they showed 24.6 ± 7.19 spot counts/well in the presence of BM-DCs without peptide loading ($p < 0.05$) (Fig. 4*c*).

As for other peptides, no significant peptide-specific response was observed (data not shown). These results suggest that the $CDCA1_{65-73}$ and $CDCA1_{351-359}$ peptides could be HLA-A2-restricted CTL epitope peptides in HLA-A2.1 (HHD) Tgm, and we also expected these peptides to be epitopes for human CTLs.

Induction of CDCA1-reactive CTLs from PBMCs of HLA-A2-positive healthy donors and a NSCLC patient

We evaluated the CDCA1-specific immune responses of CTLs that were generated by the stimulation with the $CDCA1_{65-73}$ or $CDCA1_{351-359}$ peptide of PBMCs isolated from healthy donors and NSCLC patient. The PBMCs were isolated from these donors positive for HLA-A2 (A*0201), and the $CD8^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide. This stimulation of $CD8^+$ T cells was repeated 3 times in every week (Fig. 5*a*).

CTLs induced from donors were cocultured with target cells, and ELISPOT assay and ^{51}Cr release assay were done by using $CDCA1_{65-73}$ or $CDCA1_{351-359}$ peptide-pulsed T2 cell as a target cell. In the cancer patient 1, the IFNγ production of CTLs stimulated with $CDCA1_{65-73}$ peptide-pulsed T2 (HLA-A2+) was significantly greater than that of stimulated with T2 cells pulsed with the HLA-A2-binding irrelevant HIV-derived peptide (Fig. 5*b*).

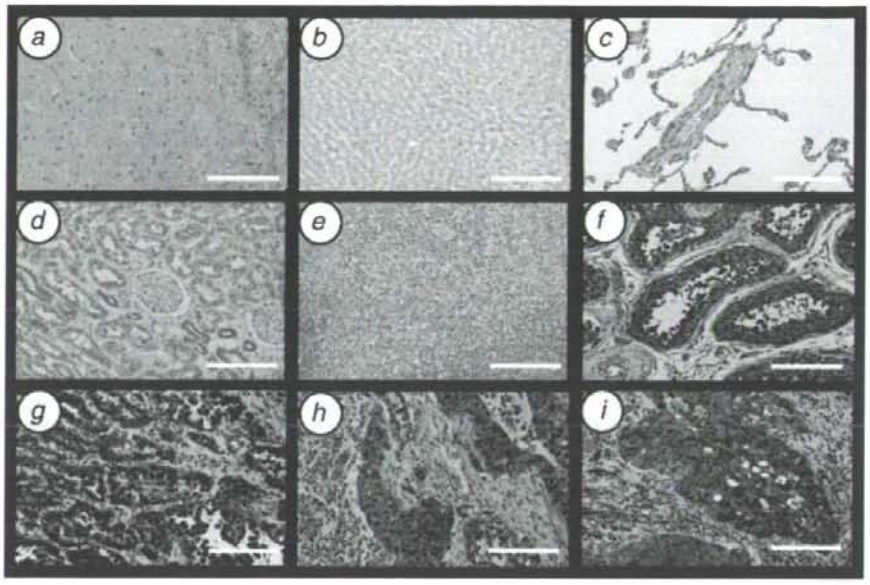

FIGURE 3 – Immunohistochemical staining of CDCA1 protein. Normal brain (*a*), normal liver (*b*), normal lung (*c*), normal kidney (*d*), normal spleen (*e*), normal testis (*f*) and NSCLC (*g*, *h*: ADC; *i*: SqCC) tissues were analyzed. The scale bars represent 100 μm.

The CTLs induced from the healthy donor 1 produced a considerable amount of IFNγ in response to the T2 cells pulsed with the $CDCA1_{351-359}$ peptide (more than 300 spot counts/well), but not to T2 cells without peptide loading (64.3 ± 12.9 spot count/well) by ELISPOT assay (Fig. 5*c*). The CTLs induced from PBMCs of the cancer patient 1 also showed cytotoxic activity to T2 cells pulsed with the $CDCA1_{65-73}$ peptide, but not to T2 cells pulsed with the HLA-A2-binding irrelevant HIV-derived peptide or peptide unpulsed T2 cells in ^{51}Cr release assay, and similar responses were observed in the healthy donor 1 (Fig. 5*d*). These results indicated that these CTLs had peptide-specific cytotoxic activity. In Figure 5*e*, CDCA1-peptide-induced CTLs were stimulated with T2 cells pulsed with various concentrations of CDCA1 peptide. We found the CTLs respond to CDCA1 peptide-pulsed T2 cells in a peptide dose-dependent manner, and that CTLs produced a significant amount of IFN-γ in response to the T2 cells pulsed with more than 0.2 μg/ml of the peptide in comparison to the responses observed in the presence of T2 cells unpulsed with the peptide or T2 cells pulsed with the HLA-A2-binding irrelevant HIV-derived peptide.

Furthermore, we used CDCA1-transfected COLO201 (COLO201/CDCA1, CDCA1+, HLA-A2+; Fig. 6*a*) as target cells and examined whether we could find a CDCA1-specific immune response of CTLs. As shown in Figure 6*b*, the IFNγ production of CTLs generated from the healthy donor 1 by using the $CDCA1_{65-73}$ peptide in response to COLO201/CDCA1 was significantly larger than that stimulated with mock transfected tumor cell line negative for CDCA1 gene expression, COLO201/Mock. The CTLs induced from PBMCs of healthy donor 2 by stimulation with the $CDCA1_{351-359}$ peptide also showed specific immune response against COLO201/CDCA1, but not against COLO201/Mock, using ELISPOT assay (Fig. 6*c*). In addition, the CTLs generated from the healthy donor 1 showed immune response to PANC1 (CDCA1+, HLA-A2+) but not to A549 (CDCA1+, HLA-2−) (Fig. 6*d*).

When we think about the application of CDCA1-derived peptides to cancer immunotherapy, the most important point is that these CDCA1 peptide-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing CDCA1. We thus investigated whether these CTLs could kill human cancer cell lines expressing endogenous CDCA1. As shown in Figure 6*e*, we could generate CDCA1-reactive CTLs by the stimulation of PBMC with the $CDCA1_{63-75}$ peptide and these CTLs exhibited cytotoxic activity to PANC1 (CDCA1+, HLA-A2+), but not to A549 (CDCA1+, HLA-2-) or COLO201 (CDCA1-, HLA-A2+) in the cancer patient 1 (Fig. 6*e*). Similarly, we could generate CDCA1-reactive CTLs by the stimulation of PBMCs of healthy donor 1 with the $CDCA1_{351-359}$ peptide and these CTLs exhibited cytotoxic activity to PANC1, but not to A549 (Fig. 6*f*). These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 can be expressed on the cell surface of cancer cells to be recognized by the CTLs. We have produced a HLA-A2-CDCA1 351–359 peptide tetramer to detect HLA-A2-restricted and CDCA1-specific CTLs. By using this tetramer, we observed a strong correlation between the frequency of the CDCA1 351–359 peptide-reactive CTLs and the frequency of the tetramer-positive CTLs observed in a given $CD8^+$ T cell population (Fig. 6*g*). These observations strongly proved the presence of HLA-A2-restricted and CDCA1 peptide-specific CTLs in $CD8^+$ T cells used in this study.

Discussion

The identification of TAA-derived peptides naturally processed in and presented on tumor cells is important for the establishment of peptide-based cancer immunotherapy. We identified a novel cancer-testis antigen, CDCA1, using a cDNA microarray analysis of NSCLC and normal tissues. CDCA1 was strongly expressed in NSCLC and in normal testis, but not in other normal tissues tested at both the mRNA and protein levels. Because the testis is an immune-privileged site, we think that CTLs reactive to CDCA1 peptides can attack only NSCLC cells without injuring normal tissues in the case of immunotherapy targeted on CDCA1. Thus, we chose CDCA1 as a candidate of TAA for the immunotherapy of patients with NSCLC.

We wanted to identify a TAA, which is indispensable for proliferation and survival of NSCLC, as a target for immunotherapy, because the use of such TAAs may minimize the well-described

TABLE II – HUMAN CDCA1-DERIVED PEPTIDES PREDICTED TO BE BOUND TO HLA-A2 AND USED FOR VACCINATION OF MICE[1]

No.	Position	Subsequence residue listing	Binding score
1	65	YMMPVNSEV	855
2	120	FLSGIINFI	607
3	222	RLNELKLLV	285
4	351	KLATAQFKI	211
5	182	QLSDGIQEL	201
6	141	FLWQYKSSA	190
7	3	TLSFPRYNV	69.6
8	285	CLPSCQLEV	69.6
9	228	LLVVSLKEI	40.8
10	386	AVYERVTTI	27.5
11	372	TVIEDCNKV	25.0
12	243	KIVDSPEKL	20.7
13	257	KMKDTVQKL	17.8
14	88	LVTHLDSFL	17.5
15	447	KIDEKTAEL	16.9
16	358	KINKKHEDV	16.4
17	416	KLKSQEIFL	14.4
18	82	FLPFSNLVT	14.1
19	344	LMIVKKEKL	12.9
20	44	VLHMIYMRA	12.7
21	227	KLLVVSLKEI	311
22	222	RLNEIKLLVV	269
23	294	QLYQKKIQDL	157
24	87	NLVTHLDSFL	117
25	181	KQLSDGIQEL	64.5
26	47	MIYMRALQIV	49.1
27	402	KLGIQQLKDA	40.0
28	343	RLMIVKKEKL	38.7
29	309	KLASILKESL	36.6
30	22	ILTGADGKNL	36.3
31	193	SLNQDFHQKT	28.3
32	52	ALQIVYGIRL	21.4
33	44	VLHMIYMRAL	16.7
34	35	DLYPNPKPEV	16.7
35	165	KLERIDSVPV	15.6
36	65	YMMPVNSEVM	12.3
37	154	QLNAAHQEAL	10.5
38	60	RLEHFYMMPV	10.2
39	344	LMIVKKEKLA	6.1
40	453	AELKRKMFKM	4.8

[1]To identify HLA-A2-restricted CTL epitopes of human CDCA1 by using HLA-A2.1 (HHD) transgenic mouse (Tgm), we selected 40 kinds of peptides having amino acid sequences with high predicted binding scores to HLA-A2 (A*0201).

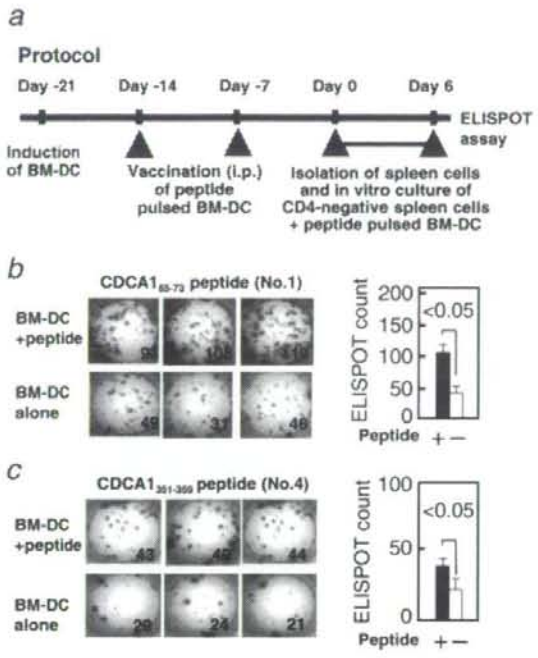

FIGURE 4 – The identification of HLA-A2-restricted mouse CTL epitopes of human CDCA1 by using HLA-A2.1 (HHD) Tgm and ELISPOT assay. (*a*) Protocol for identification of human CDCA1-derived and HLA-A2-restricted CTL epitopes. We immunized the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5/well) pulsed with the mixture of CDCA1-derived peptides carrying HLA-A2 (A*0201) binding motif into the peritoneal cavity once a week for 2 weeks. Seven days after the last DC vaccination, spleen cells (5×10^6/well) were stimulated with syngenic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured CD4-negative spleen cells as responder cells in ELISPOT assay to evaluate CDCA1-specific response of CTLs. (*b* and *c*) Two candidate peptides were selected from 40 kinds of CDCA1 peptides by using IFNγ ELISPOT assay. (*b*) CD4-negative spleen cells showed 102 ± 10.1 spot counts/well, in response to the BM-DC pulsed with the $CDCA1_{65-73}$ peptide (upper), whereas they showed 42.0 ± 9.64 spot counts/well in the presence of BM-DC without peptide loading (lower) ($p < 0.05$). (*c*) CD4-negative spleen cells showed 42.3 ± 4.02 spot counts/well, in response to the BM-DC pulsed with the CDCA1 351–359 peptide (upper), whereas they showed 24.6 ± 7.19 spot counts/well in the presence of BM-DC without peptide loading (lower) ($p < 0.05$).

risk of immune escape of cancer cells attributable to deletion, mutation or downregulation of TAAs, as a consequence of therapeutically driven immune selection.[12] One of the function of CDCA1 is reportedly to couple kinetochores to spindle microtubules and it is critical for retaining the cell cycles.[14] Furthermore, CDCA1 is one of the component of nuclear division cycle complex, which is an essential kinetochore component, highly conserved across species with a crucial role in proper chromosome segregation during mitosis.[38] CDCA1 is required for stable kinetochore localization of centromere-associated protein E (CENP-E) in Hela cells, and depletion by RNAi of CDCA1 caused aberrant chromosome segregation resulting in a prolonged mitotic blockade followed by cell death.[15] This aberrant exit from mitosis has characteristics of both apoptosis and catastrophe.[14] Consequently, CDCA1 is essential for normal cellular function, and it also plays an important role in proliferation and survival of cancer cells.

In addition, CDCA1 and kinetochore associated 2 (KNTC2) have been reported to be members of the evolutionarily conserved centromere protein complex.[13] Their elevated expressions were associated with poorer prognosis of NSCLC patients by using immunohistochemical analysis, and the growth of NSCLC was inhibited by the dominant negative peptides of CDCA1.[13] Therefore, the expression levels of CDCA1 in NSCLC tissue may be a useful marker for the prediction of the prognoses of the patients after surgical treatment. Furthermore, the results suggest a possible involvement of CDCA1 in the progression of NSCLC. Thus, immunotherapy targeting at CDCA1 may be effective for such NSCLC patients with a poor prognosis.

In this study, we identified 2 HLA-A2-restricted CDCA1 epitope peptides that can stimulate generation of HLA-A2-restricted mouse CTL by vaccination in HLA-A2.1 (HHD) Tgm without expression of endogenous mouse H-2^b-encoded class I molecules. In addition, we found that the CDCA1-reactive human CTLs could be generated from PBMCs stimulated with these peptides in healthy donors and a cancer patient. We demonstrated that these CTL lines specific to peptides derived from CDCA1 killed tumor cells expressing CDCA1 in a HLA-A2-restricted manner (Fig. 4). HLA-A2.1(HHD) Tgm has been reported to be a versatile animal model for the preclinical evaluation of peptide-based immunotherapy.[18,19,32] We could also identify its usefulness for the identification of HLA-A2-restricted CTL epitopes in this study.

In this study, we selected CDCA1-derived peptides which were predicted to have high binding affinity to HLA-A0201-encoded

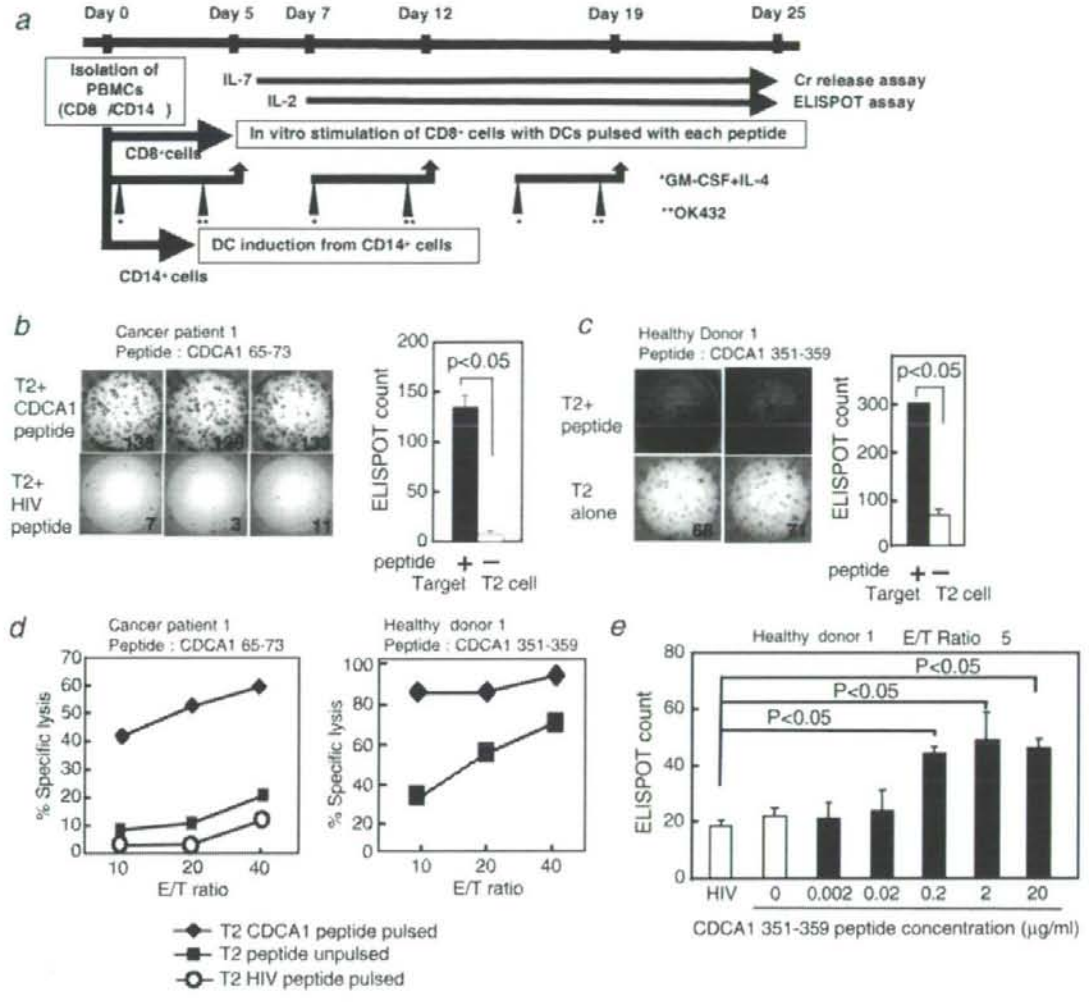

FIGURE 5 – CDCA1-specific immune response of CTLs induced from a healthy donor and a NSCLC patient. (*a*) Protocol for induction of CDCA1-specific CTLs from PBMC. We isolated PBMCs from donors and $CD8^+$ T cells and $CD14^+$ cells were isolated using each microbeads from the PBMC of the same donors. Thereafter, peptide-reactive $CD8^+$ CTLs were generated. We generated DC from $CD14^+$ cells, by culturing in the presence of GM-CSF and IL-4 for 5 days. DC were pulsed with HLA-A2-binding peptides in the presence of β2-microglobulin for 4 hr at 37°C. These peptide-pulsed DC were then irradiated and mixed at 1:20 ratio with autologous $CD8^+$ T cells. Cells were cultured with IL-7 in AIM-V with 2% auto serum. After 3 days, these cultures were supplemented with IL-2. On days 12 and 19, the T cells were further restimulated with the autologous peptide-pulsed DC. The DCs were prepared each time in the same way as described earlier. IFNγ ELISPOT assay and Cr release assay were performed after 5 or 6 days from the third round of peptide stimulation. CTL induced from a donor was cocultured with target cells and ELISPOT assay was done by using CDCA1 65–73 peptide (No. 1) (*b*) or CDCA1 351–359 peptide (No. 4) (*c*). The IFNγ production stimulated with peptide-pulsed T2 cells was significantly greater than that stimulated with nonpulsed T2 cells or HIV peptide-pulsed T2 cells. (*d*) The CTLs induced from PBMCs of the cancer patient 1 and the healthy donor 1 also showed cytotoxic activity to T2 cells pulsed with the CDCA1 peptide. (*e*) Peptide dose-dependent response of CDCA1 351–359 peptide-induced CTLs was investigated in the healthy donor 1. CTLs produced a significant amount of IFNγ in response to the T2 cells pulsed with more than 0.2 μg/ml of the peptide at E/T ratio 5.

molecules by the BIMAS software program; however, some of their amino acid sequences are not conserved between human and mouse CDCA1. There are 2 amino acid replacements between human and mouse $CDCA1_{65-73}$ peptide (human YMMPVNSEV/mouse YMMPMNIEV) and 1 amino acid replacement in $CDCA1_{351-359}$ peptide (human KLATAQFKI/mouse KLATARFKI). Hence, we worried that the mouse CTLs induced by stimulation with these nonself human CDCA1 peptides would stimulate strong responses in Tgm, but not in human, whereas we could induce CTLs responding to these epitope peptides from healthy donors and a cancer patient. Because it may be possible that human CTLs can recognize CDCA1-derived peptide in the context of HLA-A2, but that mouse CTL cannot recognize those peptides, we are planning to evaluate antigenicity of 38 other CDCA1 peptides by stimulating human PBMCs in a future study.

We found that it is possible to induce CDCA1-reactive CTLs by stimulation of PBMCs from healthy donors and a cancer patient with the CDCA1 peptides *in vitro*. The CTLs induced by the peptide-

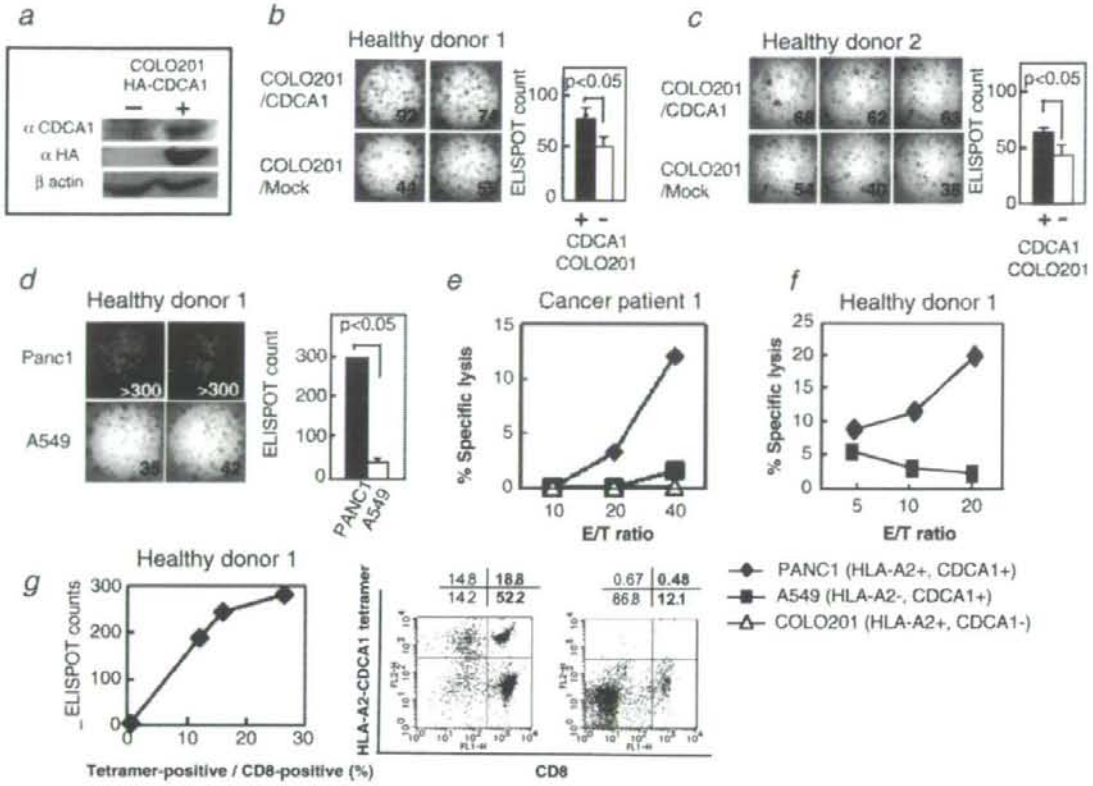

FIGURE 6 – The CDCA1-specific and CDCA1-positive tumor cell-directed cytotoxic activity of CTLs induced from healthy donors and a NSCLC patient. (*a*) The introduction and expression of CDCA1-gene expression vector in COLO201 cells. Lenti-viruses harboring EF-1a promoter and CMV promoter-driven CDCA1-HA expression vector were infected 3 times to cancer cell line, COLO201, which expresses HLA-A2 but not CDCA1. The whole cell lysate was subjected to the Western blot analyses using anti-HA antibody (middle) or anti-CDCA1 antibody (upper). (*b–d*) The IFNγ production by CTLs stimulated with COLO201-CDCA1 was significantly larger than that stimulated with mock transfected tumor cell line, COLO201. Furthermore, the IFNγ production by CTLs stimulated with PANC1 which endogenously expresses both CDCA1 and HLA-A2 was significantly greater than that stimulated with A549 which endogenously expresses CDCA1 but not HLA-A2. (*e, f*) The CTLs were cocultured with target cells and ^{51}Cr release assay was done. These CTLs exhibited cytotoxic activity to PANC1 (CDCA1$^+$, HLA-A2$^+$) but not to A549 (CDCA1$^+$, HLA-A2$^-$) nor COLO201 (CDCA1$^-$, HLA-A2$^+$). (*g*) Correlation between the frequency of CDCA1 peptide-reactive CTLs and the frequency of HLA-A2-CDCA1 tetramer-positive CTLs in CD8-positive T cells. Left: In ELISPOT assay, the target cells were peptide-pulsed T2 cells and E/T ratio was 5. Right: In FACS analyses, cells analyzed were CTLs generated from the healthy donor 1 by stimulation of PBMCs 3 times with peptide-pulsed DC (left) and freshly isolated naive CD8-positive cells separated from PBMC of the donor (right).

loaded DC exerted a significant cytotoxic activity against CDCA1-expressing cancer cells in a HLA-A2-restricted manner. Propagation of the CDCA1 peptide-specific and HLA-A2 (*A0201)-restricted CTLs from the donor's PBMCs was also confirmed by the specific HLA-peptide tetramer assay. Although the background CTL responses were high and/or the antigen-specific CTL responses were small in several experiments, these responses were highly reproducible in experiments repeated several times. Therefore, we are convinced that these CTL responses were specific to the CDCA1 peptides. These relatively weak CTL responses to PANC1 tumor cell line may be due to poor processing of the CTL epitope from CDCA1 protein in PANC1 cells or relatively CTL-resistant nature of PANC1 cells used as the target cells. Whereas, these CTLs killed very well the T2 cells pulsed with the CDCA1 peptide. These problems await solving in our future experiment.

The induction of CDCA1-specific CTLs from healthy donors and a cancer patient has important implications for ongoing efforts to search additional TAAs. In addition, we are now also trying to induce the CDCA1-reactive CTLs from PBMCs isolated from patients with NSCLC, SCLC, cholangiocellular cancer, bladder cancer and renal cell cancer. There are several methods for cell-mediated cancer immunotherapy, including the vaccination of peptide or protein,[39] immunization with dendritic cells pulsed with a peptide, protein or tumor lysate,[40,41] the immunization with dendritic cell/tumor cell hybrids[42] and adaptive transfer of tumor-specific CTL lines propagated *ex vivo*.[43] Our CDCA1 peptides may well be applicable in some of these immunotherapeutic approaches.

In summary, we have found a novel cancer testis antigen, CDCA1, expressed in NSCLC, SCLC, cholangiocellular cancer, bladder cancer and renal cell cancer. We can induce tumor-reactive CTLs from PBMCs, which were stimulated with the specific peptide. The CDCA1 epitope peptides identified in this study may well provide a new cancer immune therapy for NSCLC.

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Identification of the H2-K^d-restricted cytotoxic T lymphocyte epitopes of a tumor-associated antigen, SPARC, which can stimulate antitumor immunity without causing autoimmune disease in mice

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We previously reported that the secreted protein acidic and rich in cystein (SPARC) was overexpressed in melanoma in humans, and the serum SPARC level was useful as a novel tumor marker for melanoma. SPARC was also reported to be overexpressed in various human cancers. In this study, we asked whether SPARC-specific cytotoxic T lymphocytes (CTL) could induce antitumor immunity to SPARC-expressing tumor in mice or not as a preclinical study of SPARC-directed anticancer immunotherapy. Because of similarities in the structural motifs of major histocompatibility complex-binding peptides between H2-K^d and HLA-A24 (*A*2402*), the most common human leukocyte antigen class I allele in the Japanese population, we attempted to identify the H2-K^d-restricted SPARC epitope for CTL in BALB/c mice and we found that the mouse SPARC$_{143-151}$ (DYIGPCKYI) and SPARC$_{225-234}$ (MYIFPVHWQF) peptides could induce peptide-reactive CTL in BALB/c mice without causing autoimmune diseases. The immunization of mice with SPARC$_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) inhibited the growth of s.c. inoculated mouse mammary cancer cell line, N2C, expressing SPARC and these mice lived longer than the mice immunized with peptide-unpulsed BMDC. In conclusion, our study indicated that SPARC peptide-based cancer immunotherapy was effective and safe at least in a mouse tumor prevention model. (*Cancer Sci* 2009; 100: 132–137)

Secreted protein acidic and rich in cystein (SPARC), also called osteonectin or BM-40, was identified in 1981 as a major non-collagenous constituent of bovine bone.[1] SPARC is a matricellular glycoprotein secreted by many cells types,[2] that modulates cellular interaction with extracellular matrix during tissue remodeling.[3] SPARC plays an important role in wound repair, cell proliferation, cell migration, morphogenesis, cellular differentiation and angiogenesis.[2–5] Targeted disruption of the *SPARC* gene in mice results in early cataractogenesis,[6,7] osteopenia[8] and curly tails.[2]

SPARC was reported to be overexpressed in various human cancers,[9–12] including primary and metastatic melanomas. The overexpression of SPARC by melanoma cells was associated with an invasive phenotype *in vivo*.[13,14] We previously reported that serum SPARC levels observed in melanoma patients were higher than those observed in healthy donors.[15] Increased level of serum SPARC was observed in 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of SPARC and glypican-3, which was reported by us as a novel tumor marker for melanoma,[16] enabled a 66.2% detection rate of melanoma patients at an early stage (0–II). Thus, SPARC is considered to be a useful tumor marker for melanoma. However, the usefulness of SPARC as a target for cancer immunotherapy has not been previously investigated.

One of the actual methods of the immunotherapy for cancer was vaccination of epitope peptides derived from tumor-associated antigen. Recently, several investigators have reported the effect of peptide vaccination on cancer.[17,18] However, the effect was partial, and more useful antigens were required. We previously identified several tumor-associated antigens, including glypican-3, heart shock protein 105, proliferation potential-related protein, KM-HN-1, cell division cycle associated 1 and cadherin-3/P-cadherin.[16,19–27] In addition, we identified several HLA-A2- or HLA-A24-restricted cytotoxic T lymphocytes (CTL) epitopes derived from these antigens. The immunization with these epitopes was effective in a mouse tumor model and some of these were applied to phase I clinical trials of cancer immunotherapy.

In this study, we identified the H2-K^d-restricted and SPARC-derived CTL epitopes useful for SPARC-directed immunotherapy, and the vaccination with these peptides elicited effective antitumor immunity with no evidence of autoimmune diseases in mice.

Materials and Methods

Cell lines. Mouse cancer cell lines, B16, B16F1, B16F10, EL4, MCA, NIH3T3, 3LL, BALB/3T3, Colon26, A20, RL male1 and MethA were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan). A mouse mammary cancer cell line, N2C, was provided by Dr Sangaletti Sabina of the National Institute of Tumors (Milan, Italy). T2K^d, a TAP-deficient T2 cell transfected with *K^d*-gene expression vector, was provided by Dr Paul M. Allen of Washington University School of Medicine (St Louis, MO, USA). These cells were maintained *in vitro* in RPMI-1640 or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Expression of H2-K^d was examined with flow cytometry analysis by using a fluorescein isothiocyanate (FITC)-conjugated antimouse H2-K^d-specific antibody (clone SF1-1.1, mIgG2ak; BD Biosciences Pharmingen, San Diego, CA, USA).

Mice. Seven-week-old female BALB/c mice (H-2^d), purchased from Charles River Laboratories Japan (Yokohama, Japan),

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were kept in the Center for Animal Resources and Development of Kumamoto University and handled in accordance with the animal care policy of Kumamoto University.

Identification of the CTL epitopes of SPARC in BALB/c mice. Mouse SPARC-derived peptides (purity, >90%), sharing the amino acid sequences with human SPARC and carrying binding motifs for both H2-K^d and HLA-A24 (*A*2402*), were searched for using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, USA), and we purchased four kinds of peptides (Table 1) from AnyGen (Gwangju, Korea). Identification of the CTL epitopes of SPARC was done using BALB/c mice as described.[19] In brief, the BALB/c mice were immunized i.p. with bone marrow-derived dendritic cells (BMDC) pulsed with the mixture of SPARC candidate peptides once a week for 2 weeks. Seven days after the last immunization, $CD4^-$ spleen cells collected from immunized BALB/c were stimulated with syngeneic BMDC pulsed with each peptide *in vitro*. Then, 6 days later, $CD4^-$ T cells were collected from the culture and the CTL-producing γ-interferon (IFN-γ) was detected by an enzyme-linked immunospot (ELISPOT) assay. Moreover, after 5 days culture *in vitro* under the same conditions, cytotoxic activities of these cells directed against target cells were tested by standard 6-h ^{51}Cr release assays as described previously.[19]

Table 1. SPARC-derived peptides conserved between human and mouse SPARC and predicted to bind to H2-K^d and HLA-A24

Designation	Position	Subsequence residue listing	Binding score	
			H2-K^d	HLA-A24
SPARC-1	143–151	DYIGPCKYI	4000	75
SPARC-2	123–131	HFFATKCTL	1382	20
SPARC-3	161–170	EFPLRMRDWL	960	30
SPARC-4	225–234	MYIFPVHWQF	120	210

These peptides were searched for using BIMAS (Bioinformatics and Molecular Sections, Center for Information Technology, NIH, Bethesda, MD, USA) software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

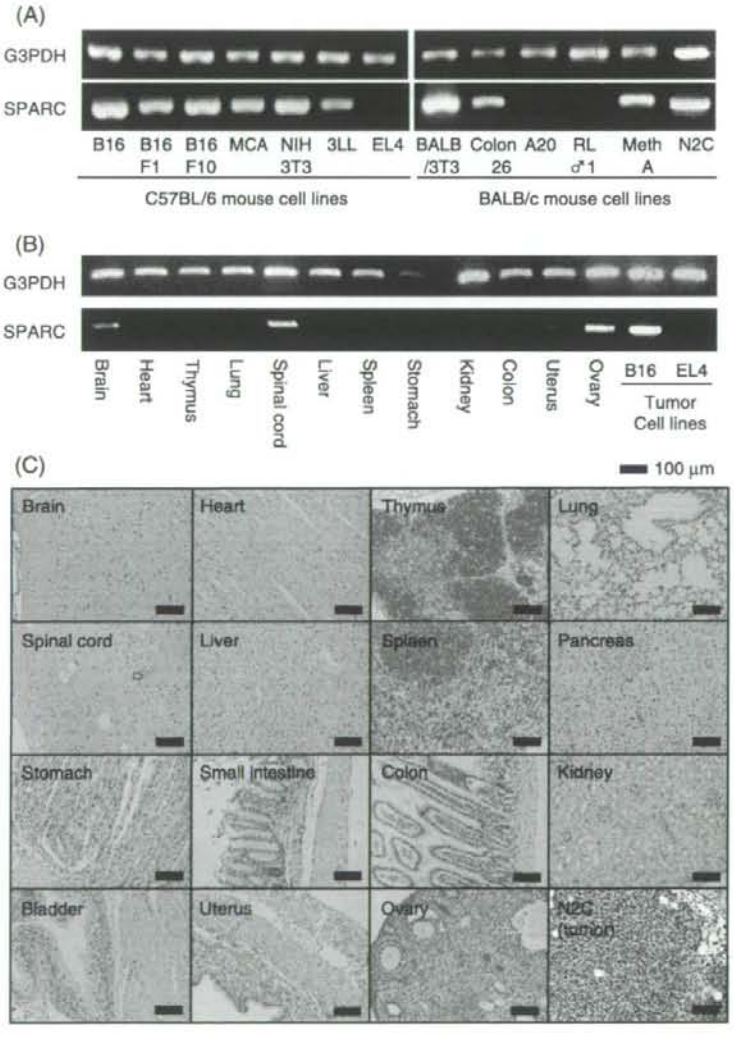

Fig. 1. The reverse transcription polymerase chain reaction (RT-PCR) analysis of *SPARC* mRNA expression in mouse cancer cell lines and normal tissues. (A) Various C57BL/6 and BALB/c mouse-derived cancer cell lines showed strong expression of *SPARC* except EL4, A20 and RL male 1. (B) Mouse *SPARC* gene was expressed in the normal tissues including ovary, spinal cord, brain and uterus by the RT-PCR analysis. (C) SPARC was not detected in the normal tissues by immunohistochemical staining.

Histological and immunohistochemical analysis. Immunohistochemical detections of SPARC was done as described previously.[21] We purchased Human, Normal Organs, and Cancers, Tissue Array, BC4 (SuperBioChips Laboratories, Seoul, Korea) for immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 was done as described previously.[28]

***In vivo* tumor prevention model.** BMDC (5×10^5 cells/body) loaded with or without SPARC-$4_{225-234}$ peptide or phosphate-buffered saline (PBS) were transferred i.p. into BALB/c mice ($n = 8$, each group) twice on days –14 and –7, and N2C cells (3×10^4/body) were challenged s.c. into the shaved back region on day 0. The tumor sizes were determined biweekly using a caliper square, and the tumor volume (mm^3) was calculated as long diameter × squared short diameter.

Statistical analysis. We analyzed all data with the StatView statistical program for Macintosh (SAS Institute, Cary, NC, USA) and evaluated the statistical significance with an unpaired Student's *t*-test. $P < 0.05$ was considered significant. The percentage of overall survival rate was calculated by the Kaplan–Meier method, and statistical significance was evaluated with the Wilcoxon rank sum test.

Results

Expression of *SPARC* mRNA in mouse cancer cell lines and normal tissues. We examined the expression level of *SPARC* mRNA using reverse transcription polymerase chain reaction (RT-PCR). The mouse fibroblast cell line NIH/3T3 and various mouse cancer cell lines, including melanoma (B16, B16F1 and B16F10), fibrosarcoma (MCA) and lung cancer (3LL) originated from C57BL/6 mice, and sarcoma (BALB/3T3 and MethA), colon cancer (colon26) and breast cancer (N2C) originated from BALB/c mice, showed strong expression of *SPARC* (Fig. 1A). C57BL/6 mouse leukemia/lymphoma cell line, EL4, and BALB/c mouse lymphoma cell line, A20 and RL male 1, did not express *SPARC*. Although *SPARC* mRNA was expressed in the ovary, spinal cord, brain and uterus, the expression levels observed in these tissues were lower than those observed in cancer cell lines (Fig. 1B). Moreover, normal tissues including ovary, spinal cord, brain and uterus did not express SPARC at protein level investigated by the immunohistochemical analysis (Fig. 1C).

Identification of the SPARC-derived and H2-K^d-restricted CTL epitopes in BALB/c mice. Structural motifs of peptides bound to human HLA-A24 (*A*2402*) and mouse H2-K^d are similar. The amino acid sequences of human and mouse SPARC have a 92% homology.[4] Thereby, we searched for SPARC-derived and H2-K^d- or HLA-A24 (*A*2402*)-restricted peptides of which amino acid sequences were completely shared between human and mouse SPARC, and prepared four different synthetic peptides (Table 1). CD4$^-$ spleen cells isolated from BALB/c mice immunized twice with BMDC pulsed with mixture of these four peptides were stimulated *in vitro* with BMDC pulsed with each peptide for 5–6 days. Subsequently, we collected these CD4$^-$ T cells from the culture and we found that CD4$^-$ T cells stimulated with the SPARC-$1_{143-151}$ (DYIGPCKYI) or SPARC-$4_{225-234}$ (MYIFPVHWQF) peptides produced a large amount of IFN-γ in a peptide-specific manner in ELISPOT assays (Fig. 2A). Moreover, we tested cytotoxic activities of these cells directed against target cells by standard 6-h ^{51}Cr release assays. CTL induced by SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptides showed specific cytotoxicity against T2K^d (H2-K^d+, TAP negative) cells pulsed with each SPARC peptide but not against T2K^d cells unpulsed with SPARC peptide (Fig. 2B). In addition, those CTL had cytotoxic activities directed against MethA (SPARC$^+$, H-2^d) but not against RL male 1 (SPARC$^-$, H-2^d). These findings suggest that these SPARC-$1_{143-151}$ and SPARC-$4_{225-234}$ peptides had the capacity to induce the H2-K^d-restricted peptide-reactive CTL and that the CTL killed the tumor naturally expressing both SPARC and H2-K^d.

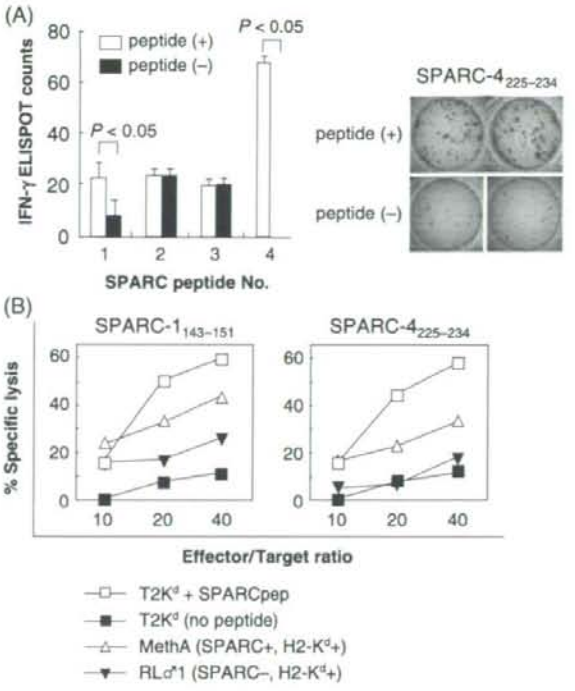

Fig. 2. Identification of the SPARC-derived and H2-K^d-restricted cytotoxic T lymphocyte (CTL) epitopes in BALB/c mice. We immunized the BALB/c mice with syngeneic bone marrow-derived dendritic cells (BMDC) (5×10^5/mouse) pulsed with the mixture of candidate peptides *in vivo* once a week for 2 weeks. Seven days after the last immunization, CD4$^-$ spleen cells isolated from immunized mice (2×10^6/well) were stimulated with BMDC (2×10^5/well) pulsed with each peptide *in vitro*. (A) Six days after the stimulation *in vitro*, the CTL-producing γ-interferon (IFN-γ) in response to the peptide-pulsed BMDC were detected by an enzyme-linked immunospot (ELISPOT) assay. (B) Five days after the stimulation *in vitro* under the same conditions, cytotoxic activities of these cells directed against indicated target cells was tested with standard ^{51}Cr release assays. We found that CD4$^-$ spleen cells stimulated with the SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptide produced a large amount of IFN-γ, and had cytotoxic activities directed against both H2-K^d and SPARC-expressing MethA tumor cell line or T2K^d pulsed with each peptide, but not to SPARC-negative RL male 1 and peptide-unpulsed T2K^d. These assays were done twice with similar results.

Immunization of SPARC-$4_{225-234}$ peptide did not induce the autoimmune diseases in BALB/c mice. To investigate whether the immunization of mice with the SPARC-derived H2-K^d-restricted peptide causes autoimmune diseases, the immunohistochemical staining of several important organs with anti-CD4 and anti-CD8 monoclonal antibody was performed in BALB/c mice immunized with BMDC pulsed with a mixture of four SPARC peptides once a week for 2 weeks. Tissue specimens of these mice were removed and analyzed 7 days after the second dendritic cell (DC) vaccination. As shown in Fig. 3, we could not find any pathological changes, such as lymphocyte infiltration or tissue destruction, in brain, heart, lung, liver, kidney, uterus, ovary and spinal cord of BALB/c mice. Although SPARC was expressed in spinal cord and brain by RT-PCR, the BALB/c mice immunized with BMDC pulsed with SPARC-$4_{225-234}$ peptide did not show any neurological disorders such as paralysis or abnormal behavior. No sign of autoimmune diseases

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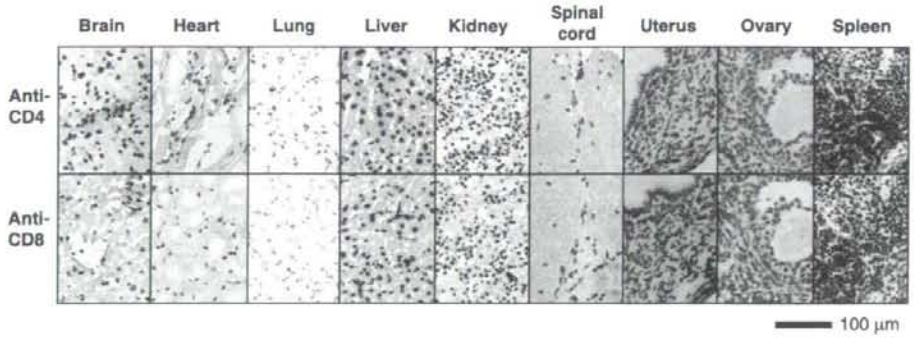

Fig. 3. Immunohistochemical staining with anti-CD4 or anti-CD8 monoclonal antibody in tissue specimens of BALB/c mice twice immunized with BMDC pulsed with the mixture of H2-K^d-restricted SPARC-derived peptides twice. These tissue specimens were removed and analyzed 7 days after the second dendritic cells (DC) vaccination (original magnification, ×200).

such as weight loss, diarrhea and skin abnormalities was observed (data not shown). These results indicate that the immunization of mice with SPARC-4$_{225-234}$ peptide does not induce autoimmune diseases.

Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-4$_{225-234}$ peptide-pulsed BMDC. We investigated whether the immunization of mice with the SPARC epitope peptide inhibit the growth of tumor expressing SPARC *in vivo* or not. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-4$_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or PBS ($n = 8$, each group). During the vaccination period, none of the treated mice showed any abnormalities. Subcutaneous inoculation of N2C cells (3×10^4) into the right flank was given 7 days after the last vaccination. Growth curves of N2C tumor mass are shown in Fig. 4(A,B). The N2C tumor appeared 25 days after the inoculation in the PBS-injected group. Measurement of tumor size was continued until 62 days after inoculation of the tumor cells when one mouse in the PBS-injected group died. Mean tumor size (1024.8 ± 1820.7 mm^3) on day 62 observed in the mouse group inoculated with SPARC-4$_{225-234}$ peptide-pulsed BMDC was significantly smaller (5343.6 ± 3117.2 mm^3, $P < 0.01$) than that observed in the mice inoculated with peptide-unpulsed BMDC and in those injected with PBS (6623.1 ± 3883.9 mm^3, $P < 0.01$). Complete tumor rejection was observed in four out of the eight mice in the SPARC-4$_{225-234}$ peptide-pulsed BMDC group. Although one out of the eight mice in the peptide-unpulsed BMDC group also completely rejected the tumor growth, there was no statistical significance in difference of tumor growth between the mice inoculated with peptide-unpulsed BMDC and those injected with PBS ($P = 0.48$). All mice injected with PBS died within 125 days after inoculation of the tumor cells. Mice inoculated with the SPARC-4$_{225-234}$ peptide-pulsed BMDC lived significantly longer than the mice of the other two groups (Fig. 4C). No significant abnormalities including neurological disorders were observed in four tumor-free mice in the SPARC-4$_{225-234}$ peptide-pulsed BMDC group for over 150 days after vaccination. This experiment was repeated twice with similar results. These findings indicate that vaccination of mice with BMDC pulsed with the SPARC epitopes resulted in significant inhibition of tumor growth and prolongation of overall survival *in vivo*.

Discussion

In this study, we demonstrated that: (i) *SPARC* was overexpressed in mouse cancer cell lines; (ii) the CTL induced by the SPARC-derived peptides were reactive to tumor overexpressing SPARC; and (iii) the immunization of BALB/c mice with SPARC peptide-pulsed BMDC protected mice from tumor growth and induced prolonged survival without causing autoimmune diseases.

Although immunohistochemical staining of these tissues with anti-SPARC antibody was negative (Fig. 1C), *SPARC* mRNA was expressed in several important normal tissues including brain and spinal cord using RT-PCR. Therefore, we needed to investigate whether induction of SPARC-specific CTL could induce antitumor immunity without causing autoimmune diseases in the mouse model system. One of the reasons for lack of autoimmune diseases might be that the H2-K^d-positive SPARC epitope peptide complexes are more densely expressed on tumor cells in comparison to those expressed on normal tissues including brain and spinal cord, if any. These possibilities must be evaluated in a future study.

The HLA-A24 is positive in 60% of the Japanese population (95% of whom are genotypically *A*2402*), 20% of Caucasians and 12% of Africans.[29] It is important especially for the Japanese to identify HLA-A24-restricted CTL epitope peptides. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H2-K^d are similar,[30–32] and the amino acid sequences of human and mouse SPARC protein exhibit a 92% homology.[33] SPARC-derived and H2-K^d-restricted CTL epitopes identified in BALB/c mice may well be applicable to induce human HLA-A24-restricted CTL. Therefore, in this study, we used BALB/c mice, and searched for SPARC-derived peptides having amino acid sequences shared between mouse and human SPARC. We could identify the SPARC-derived and H2-K^d-restricted CTL epitopes. According to these findings, we tried to induce the HLA-A24-restricted human CTL reactive to these peptides by stimulating peripheral blood mononuclear cells (PBMC) of healthy donors or various cancer patients *in vitro* with the peptides. In a preliminary study, these SPARC peptide-specific and HLA-A24-restricted human CTL were also generated *in vitro* (unpublished observation, 2008).

We observed the inhibitory effect of immunization of mice with BMDC pulsed with SPARC-derived epitope peptide on growth of the inoculated N2C tumor cell line in BALB/c mice. N2C expressing SPARC is a mouse breast cancer cell line originating from Her-2/neu transgenic BALB/c mice.[34] It was reported that SPARC was overexpressed in human breast cancers, and associated with poor prognosis or invasive phenotype type.[35–38] N2C tumors grew as solid nests forming lobules embedded in dense, well-vascularized, connective tissue and surrounded by the stromal septa.[34] The stromal cells in N2C tumors also expressed SPARC. N2C tumor grown in SPARC

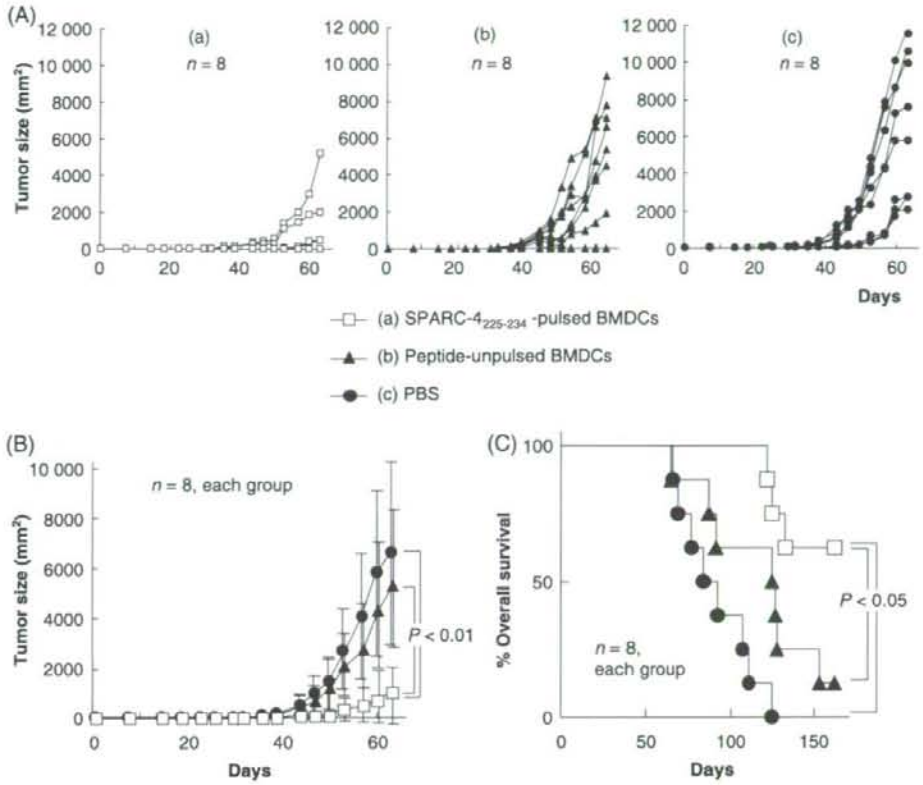

Fig. 4. Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-$4_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) *in vivo*. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-$4_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or phosphate-buffered saline (PBS) only. Subcutaneous inoculation of N2C cells (3×10^6/mouse) into the right flank was given 7 days after the last vaccination. (A) Growth curves of N2C tumor mass in individual mice in each group: (a) SPARC-$4_{225-234}$ peptide-pulsed BMDC inoculated group; (b) peptide-unpulsed BMDC inoculated group; (c) PBS injected group ($n = 8$, each group). (B) The mean tumor volumes ± standard deviation in three groups were compared in this panel. Statistical significance of the differences between each group were evaluated using the unpaired Student's *t*-test. (C) Survival rate of mice in each group. Mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group lived significantly longer than the mice in the other two groups. Statistical significance of the differences between each group were evaluated using the Wilcoxon rank sum test.

knockout mice were smaller and histologically characterized by undefined lobules, frequently presenting necrotic central areas. The lobules were not completely delineated by the stromal septa, which appeared generally thin and sometimes heavily infiltrated by leukocytes. Therefore, the destruction of tumor stromal cells by immunity directed against SPARC may well be a possible mechanism for inhibition of N2C tumor cell proliferation observed in this study. This possibility awaits evaluation in a future study.

Taken together, these findings indicate that the antitumor immunity stimulated with SPARC-derived peptide is effective and safe at least in a preclinical study using a mouse cancer-prevention model system.

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特集：特異抗原をターゲットとした **Immunotherapy**
総　説

新規癌胎児性抗原 Glypican-3 の肝細胞癌の診断と免疫療法への応用

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Usefulness of a novel oncofetal antigen, Glypican-3, for diagnosis and immunotherapy of hepatocellular carcinoma

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summary

We identified glypican-3 (GPC3), as a novel oncofetal antigen, overexpressed specifically in hepatocellular carcinoma (HCC) and melanoma in humans by utilizing genome-wide cDNA microarray analyses of HCC tissues and normal fetal and adult tissues. We also found that GPC3 is a novel tumor marker for HCC and melanoma, and that the pre-immunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse GPC3 298-306 (EYILSLEEL) peptide prevented the growth of tumor expressing mouse GPC3. Because of similarities in the binding peptide motifs between H-2K^d and HLA-A24 (*A*2402*), the H-2K^d-restricted GPC3 298-306 peptide thus seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. We investigated whether the GPC3 298-306 peptide could induce GPC3 reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A24 (*A*2402*)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice (Tgm) to identify the HLA-A2 (*A*0201*)-restricted GPC3 epitopes to expand the applications of GPC3 based immunotherapy to the HLA-A2$^+$ HCC patients. We found that the GPC3 144-152 (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) Tgm without inducing autoimmunity. In 5 out of 8 HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3 144-152 peptide-reactive CTLs were generated from PBMCs by in vitro stimulation with the peptide and the GPC3 298-306 peptide-reactive CTLs were also generated from PBMCs in 4 of 6 HLA-A24$^+$ GPC3$^+$ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into NOD/SCID mice. We have recently started a phase I clinical trial of GPC3 peptide vaccine-based immunotherapy of patients with advanced HCC.

We have also succeeded in inhibition of growth of tumors expressing mouse GPC3 by immunization of mice with dendritic cells differentiated in vitro from mouse embryonic stem cells and pulsed with the GPC3 peptides. Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of patients with HCC and melanoma.

Key words　肝細胞癌（HCC）；癌特異抗原；Glypican-3（GPC3）；癌免疫療法；ES-DC

抄　録

ヒトの肝細胞癌組織と正常組織における cDNA マイクロアレイ解析により，肝細胞癌に高発現する遺伝子として Glypican-3（GPC3）を同定した．GPI アンカー膜蛋白質である GPC3 は，肝細胞癌患者の約 40%の血清中に検出される新規癌胎児性抗原であり，αフェト蛋白，PIVKA-II につぐ肝細胞癌の第 3 の腫瘍マーカーとして有用であることを示した．また，マウスに GPC3 ペプチドを負荷した樹状細胞を投与した後に，マウス GPC3 を発現する癌細胞株を移植すると，自己免疫現象を伴うことなく著明な腫瘍の増殖抑制と生存期間の延長を誘導できた．さらに，HLA-A2 トランスジェニックマウスや，癌患者の血液検体を利用して，HLA-A2 あるいは A24 によりヒト・キラーT 細胞に提示される GPC3 ペプチドを同定した．これらのペプチドで癌患者のリンパ球を刺激することにより，GPC3 発現ヒト肝細胞癌細胞株を傷害するヒト・キラーT 細胞を誘導できた．これらの GPC3 ペプチドを用いた，肝細胞癌の免疫療法に関する臨床試験を開始した．また，我々はマウス胚性幹（ES）細胞から樹状細胞（ES-DC）を分化誘導する方法を開発し，マウス GPC3 を発現する ES-DC をマウスに免疫したところ，GPC3 発現マウス癌細胞株に対する in vivo 抗腫瘍効果の誘導が観察された．

熊本大学大学院医学薬学研究部・免疫識別学分野

はじめに

癌細胞にのみ発現する抗原を免疫することにより，癌細胞を攻撃して破壊するT細胞を誘導する免疫療法を確立するために，様々な癌抗原ワクチンの開発が試みられている．従来，正常組織に発現を認めず癌細胞に特異的に高発現する癌抗原を同定することは困難であったが，cDNAマイクロアレイ解析による癌組織と正常組織におけるゲノムワイドの遺伝子発現プロフィール解析により，癌特異抗原の同定が飛躍的に進んだ．我々は，この手法を用いて多数の癌特異抗原を同定し，これを用いた癌免疫療法の臨床試験を開始している．本稿では，肝細胞癌に高発現する新規癌胎児性抗原であるGlypican-3（GPC3）の発見と，その癌免疫療法への応用について紹介する．

さらに，筆者らは，細胞ワクチンとして用いる樹状細胞の供給源として胚性幹（ES）細胞に着目し，ES細胞由来の樹状細胞（ES-DC）を用いた免疫療法の開発に関する基礎研究を行っている．本稿では，マウスの腫瘍モデルを用いたES細胞から分化誘導したES-DCによる腫瘍免疫の誘導に関する研究成果，ならびに，最近開発したヒトES-DCの分化誘導法についても紹介する．

1. 肝細胞癌（HCC）に対する免疫療法の現況

肝細胞癌（HCC）の患者数は，欧米およびアジア諸国において依然として増加している．HCCは治療後も高頻度に再発を繰り返すため予後不良な癌であり，B型およびC型肝炎と，それに引き続いて発症する肝硬変から発生する．ごく初期の癌に対する早期治療法や，治療後の再発予防のために有効な補助療法の確立が望まれている．

慢性肝炎，肝硬変患者におけるHCCの発症予防や，HCC術後における術後化学療法は，いまだ開発途上にある．HCCに対する免疫療法についても，1990年代よりlymphokine-activated killer（LAK）cells, tumor-infiltrating lymphocytes（TIL），peripheral blood mononuclear cell（PBMC）を用いた養子免疫療法，DCワクチン療法，αフェト蛋白質由来のペプチドワクチン療法などが試みられている．またHCCにおいて高発現する癌特異的抗原も複数報告されており，各施設でその有用性が検討されている[1]．

II. 新規癌胎児性抗原Glypican-3（GPC3）の発見

1. cDNAマイクロアレイ解析によるHCC特異的な癌胎児性抗原の発見

我々は，東京大学医科学研究所ヒトゲノムセンターの中村祐輔博士らとの共同研究により，癌部と非癌部におけるcDNAマイクロアレイ解析データ[2]を用いて肝細胞癌（HCC）特異的に高発現する遺伝子としてGlypican-3（GPC3）を同定した[3]．図1Aに示すように，GPC3は正常な肝細胞と比較してHCCの約80%の症例のHCC組織において高発現しているが，成人の正常組織には，ほとんど発現していない．いっぽうGPC3は胎盤や胎児期の肝臓，肺あるいは腎臓に高発現しており，いわゆる癌胎児性抗原（Carcinoembryonic antigen あるいはOncofetal antigen）の範疇に入る蛋白質である．

2. GPC3の構造と機能

膜結合型糖蛋白質であるGlypicanファミリーは，現在までのところ6種類が報告されている[4]．GPC3は，580アミノ酸からなる60 kDのコア蛋白質にヘパラン硫酸糖鎖修飾が加わった膜蛋白質で，C末端がGPIアンカーにより形質膜に結合している．Piliaらは，X染色体（Xq26）連鎖疾患である巨人症の一つである，Simpson-Golabi-Behmel症候群において，GPC3の遺伝子変異を報告している．また，GPC3ノックアウトマウスでも，Simpson-Golabi-Behmel症候群と同様に体の巨大化などの表現型を示すことが報告されている．

GPC3は，ある種の腫瘍細胞では増殖を抑制したり，あるいはアポトーシスの誘導に関連があると報告されている[5]．近年，GPC3コア蛋白質が直接Wntと結合することにより，Wntシグナルを活性化し，肝細胞癌の増殖を促進することが報告されている[6]．

III. HCC癌組織におけるGPC3の発現と腫瘍マーカーとしての有用性

我々は，GPC3遺伝子の発現量の差が，その遺伝子産物である蛋白質量の差として反映されているか否かについてRT-PCR法，ならびに組織切片における免疫組織化学的解析を用いて確認した（図1B, C）．その結果，GPC3は蛋白質レベルにおいても，胎児期の肝臓組織に発現するが出生後発現しなくなり，HCCにおいて再び発現することを確認し

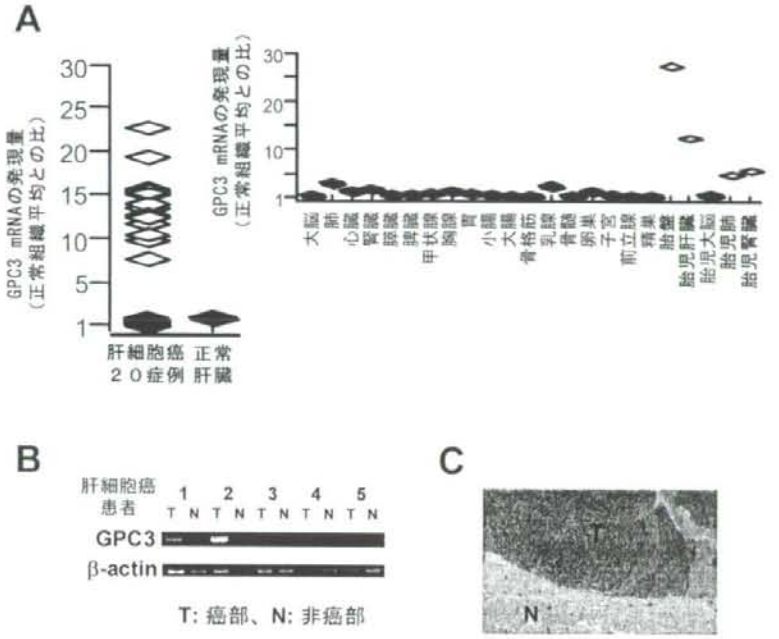

図１　HCC 20 症例の癌部，非癌部および多様な正常臓器における GPC3 遺伝子発現の cDNA マイクロアレイ解析データ[2]と HCC 組織における GPC3mRNA および蛋白質の発現

A：HCC 患者 20 症例の癌部と非癌部における 23,040 種類の遺伝子の発現を比較検討し，さらに胎生期の 4 臓器を含む 23 臓器の正常組織において，各遺伝子の発現プロフィールを解析した．GPC3 は，肝臓癌患者 20 症例中 16 症例で癌部/非癌部の発現の比が 5 以上（平均 396.2）で，胎盤や胎生期の肝臓および腎臓に発現する以外は，ほとんどの成人の正常臓器に発現を認めない，癌胎児性抗原をコードする遺伝子であった．B：HCC 組織の癌部（T）と非癌部（N）における GPC3 mRNA の発現の有無を RT-PCR 法にて検討したところ，癌部においてのみ GPC3 の発現を認めた．C：HCC 組織切片における GPC3 蛋白質の発現を，抗 GPC3 抗体を用いた免疫組織学的解析により確認した．

た．

さらに HCC 患者の約 40％の血清中に可溶性 GPC3 が検出されるが，健常人，慢性肝炎，その他の肝疾患では全く検出されず，HCC の血清腫瘍マーカーとして有用であることを発見した[3]．また HCC の外科的な治療後に，血清 GPC3 が消失あるいは減少することから，治療効果の判定などの臨床への応用が期待される．

IV．癌免疫療法のターゲットとしての GPC3 の有用性

1．マウスにおける抗腫瘍免疫の解析

発現の組織特異性が優れていることから，我々は癌胎児性抗原 GPC3 が，理想的な腫瘍拒絶抗原になり得るかどうかについてマウスを用いて検討した．日本人の約 60％が所有する HLA-A24 と，BALB/c マウスのクラス I 分子の K^d に結合するペプチドの構造モチーフは，非常に類似していることがわかっている．さらに，ヒトとマウスの GPC3 では，アミノ酸配列のレベルで 95％以上のホモロジーを認めることから，ヒトとマウスの GPC3 でアミノ酸配列が完全に一致し，HLA-A24 および K^d のいずれにも結合すると予測される GPC3 由来のペプチドを合成した．このペプチドを骨髄由来樹状細胞に負荷し，BALB/c マウスに免疫して解析することにより，K^d 分子に結合して細胞傷害性 T 細胞（CTL）に提示される K^d 拘束性 CTL エピトープペプチドを同定した[7]．

このエピトープペプチドを負荷した骨髄由来樹状細胞ワクチンを腹腔内に予防的に投与した BALB/c マウスでは，コントロール群に比べてマウス GPC3 遺伝子を強制発現させたマウス大腸癌細胞株の増殖は著明に抑制され，さらにマウスの生存期間の延長が確認された[7]．このエピトープペプチドは HLA-A24 によっても提示され，ヒトでも同様に CTL エピトープとなる可能性があると思われた．

2．HCC 患者における GPC3 特異的 CTL の誘導

日本人の HLA-クラス I 対立遺伝子のうち，HLA-A24（*A***2402*）は日本人の約 60％が所有し，

HLA-A2（*A*0201*）は約20%が所有する，ありふれた対立遺伝子である．そこでヒトとマウスのGPC3に保存されたアミノ酸配列をもつペプチドで，HLA-A2（*A*0201*）に結合すると推定されるGPC3由来の9～10個のアミノ酸からなるペプチドを9種類選択した．これらのペプチドをHLA-A2トランスジェニックマウス（HLA-A2 Tgm）に免疫した後に，ELISPOTアッセイにより最も強くGPC3特異的CTLを誘導出来るエピトープペプチドを探索することにより，ペプチドA2-3；$GPC3_{144-152}$を同定した．さらに，このGPC3 A2-3ペプチドを負荷したBM-DCにて2回免疫したHLA-A2 Tgmでは，重要臓器（脳，皮膚，心，肺，肝，腎）において自己免疫反応は生じておらず，その安全性が示唆された．

HLA-A2拘束性CTLエピトープペプチド$GPC3_{144-152}$と，H-$2K^d$（≒HLA-A24）拘束性CTLエピトープペプチド$GPC3_{298-306}$を用いて，HLA-A2またはHLA-A24陽性のHCC患者の末梢血単核細胞（PBMC）を刺激して，ペプチド特異的CTLの誘導を試みた．その結果，$GPC3_{144-152}$ペプチドを用いてHLA-A2陽性GPC3陽性HCC患者10名中5名のPBMCより，また，$GPC3_{298-306}$ペプチドを用いてHLA-A24陽性GPC3陽性HCC患者12名中6名のPBMCより，各CTLエピトープに特異的なCTLを誘導できた[8]（表1）．

さらに，重症混合型免疫不全をもつことより，ヒトの細胞を拒絶できないNOD/SCIDマウスにGPC3遺伝子を強制発現させたヒトHCC細胞株SK-Hep1/GPC3を皮下移植して生着させた．その後に，HLA-A2拘束性エピトープペプチド$GPC3_{144-152}$あるいはHLA-A24拘束性エピトープペプチド$GPC3_{298-306}$で刺激することにより，HCC患者のPBMCより誘導されたヒトGPC3特異的CTL株を静脈注射により養子免疫した．GPC3エピトープペプチドで誘導したCTL株を投与した

表1　HLA-A2あるいは-A24陽性HCC患者（それぞれPt-A2，Pt-A24）の約50%において，GPC3特異的なCTLが誘導された．

患　者	年　令	性　別	癌の進行度[1]	GPC3の発現[2]	HLAの発現[3]	CTLの誘導[4]
Pt-A2-1	80	F	IIIa	+	+	+
Pt-A2-2	72	M	II	+	+	+
Pt-A2-3	67	F	II	ND	ND	+
Pt-A2-4	54	M	I	+	+	+
Pt-A2-5	57	M	I	ND	ND	−
Pt-A2-6	66	M	I	−	−	−
Pt-A2-7	54	M	IIIa	+	+	−
Pt-A2-8	73	M	II	ND	ND	+
Pt-A2-9	68	F	IIIa	+	+	−
Pt-A2-10	54	M	II	+	+	−
患　者	年　令	性　別	癌の進行度[1]	GPC3の発現[2]	HLAの発現[3]	CTLの誘導[4]
Pt-A24-1	60	M	IVa	+	+	+
Pt-A24-2	57	M	IVa	+	+	−
Pt-A24-3	75	F	IIIa	+	+	+
Pt-A24-4	59	M	IIIa	ND	ND	+
Pt-A24-5	52	M	IVb	−	+	−
Pt-A24-6	65	M	I	ND	ND	+
Pt-A24-7	61	M	I	ND	ND	+
Pt-A24-8	74	M	II	ND	ND	−
Pt-A24-9	59	M	IVb	−	−	−
Pt-A24-10	69	M	IVa	+	+	−
Pt-A24-11	72	M	II	−	+	−
Pt-A24-12	61	M	IIIa	+	+	+

1）TNM分類を用いた．
2）免疫染色を用いて，肺癌周囲の正常組織と比較して発現を確認した．
3）免疫染色により膜が染色された場合に，発現ありと判断した．
4）GPC3発現HCC細胞株HepG2に対する細胞傷害活性が，E/T比20で20%以上観察された場合に，CTLを誘導できたと判断した．

NOD/SCID マウスでは，コントロールの T 細胞株あるいは生理食塩水のみを投与した群と比較して，有意差をもって腫瘍の増殖抑制が観察された[8]（図2）．

現在，国立癌センター東病院にて HLA-A2 あるいは -A24 陽性の HCC 患者を対象にして，これらのペプチドを用いた癌免疫療法の臨床第 1 相試験を展開中である．

V. 樹状細胞ワクチンを作製するための材料としての ES 細胞の有用性

現在，樹状細胞を用いた抗腫瘍免疫療法には，アフェレーシス（成分採血）により分離した末梢血白血球中の単球を GM-CSF 等のサイトカインを加えて培養し，分化誘導することにより作製された樹状細胞が用いられている．しかしながら，この方法には，アフェレーシス操作に伴う患者への負担，さらに，末梢血白血球から分離される単球の数や単球から樹状細胞への分化誘導効率に個人差があるため，樹状細胞の収量が不安定であることなど，医療技術として広く普及するにはいくつかの問題がある．

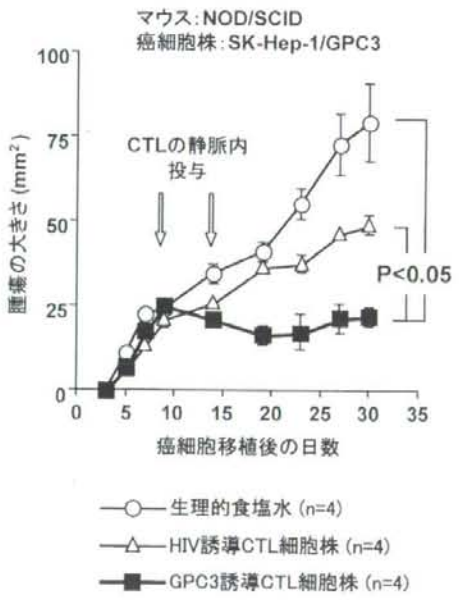

図2　免疫不全マウスに移植した GPC3 発現ヒト HCC 細胞株に対するヒト CTL 養子免疫療法の有効性
NOD/SCID マウスの背部の皮下に，ヒト HCC 細胞株 SK-Hep-1 に GPC3 遺伝子を強制発現させた SK-Hep-1/GPC3 を 1×10^7 個移植し，移植後 9 日目に 5×5 mm の大きさになった時点と，その 5 日後（移植後 14 日目）に CTL を 8×10^7 個，計 2 回静脈内に投与した．HCC 患者の PBMC を GPC3 エピトープペプチドで刺激して誘導した CTL 投与群（■）と，コントロールとして HIV エピトープペプチドで誘導した CTL 投与群（△），生理食塩水のみを投与した群（○）の間で比較すると，GPC3 特異的 CTL 投与群ではコントロール群に比べ，有意に腫瘍の増殖が抑制されていた．

筆者らは，ES 細胞を材料にして細胞ワクチンとして用いる樹状細胞を作製することを考え，数年前に研究を開始した．ES 細胞は，適切な条件の下で培養することにより，未分化な状態を保ったまま無限に増殖させることが可能である．したがって，樹状細胞の材料として ES 細胞を用いることが可能になれば，材料をいくらでも増やすことができることになり，細胞ドナーへ新たな負担をかけることなく大量の樹状細胞を作製できる．また，ES 細胞は，電気穿孔法あるいはリポフェクション等により，ウイルスベクターを使用することなく，遺伝子導入を行うことが可能であり，さらに，遺伝子導入細胞のクローンを作製することも可能である．そこで，ES 細胞の段階で遺伝的改変を行い，適切な遺伝子改変 ES 細胞クローンを選択し，これを樹状細胞に分化誘導すれば，樹状細胞の遺伝的改変を容易に行うことができる．これにより，抗原分子あるいは各種の免疫制御分子を人為的に発現させるなど，機能を様々に修飾した樹状細胞を作製することができるという利点もある．

VI. マウス ES 細胞からの樹状細胞の作製法

筆者らは，まず，マウスの ES 細胞から樹状細胞を分化誘導する方法の開発に取り組んだ．そして，OP9 細胞（正常な M-CSF 遺伝子を欠損した op/op マウスに由来する骨髄ストローマ細胞株）と共培養することにより，マウス ES 細胞から血液細胞への分化誘導を行う仲野らの方法[9]を参考にして，マウス ES 細胞から樹状細胞を作製する培養プロトコールを確立した[10]．

まず，ES 細胞の中胚葉性細胞への分化を促すために，ES 細胞を単層培養している OP9 細胞とともに 5-6 日間培養する．この結果，ほとんどの ES 細胞が，中胚葉系細胞へ分化する．次に，分化した細胞をトリプシン/EDTA を用いて培養プレートから回収し，新たに準備した OP9 細胞上で GM-CSF の存在下で 5-6 日間培養する．この結果誘導される ES 細胞由来のミエロイド系の細胞を細菌培養用のペトリディッシュに移し，さらに GM-CSF の存在下で培養を続けると 7-10 日目頃より不規則な樹状突起を有する浮遊性の細胞が出現する．この細胞は，マウスの骨髄細胞から GM-CSF を用いて分化

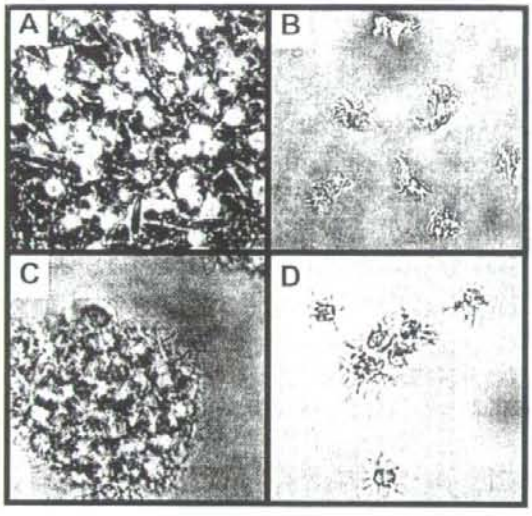

図3　マウスES-DCの分化にともなう細胞形態の変化
A：OP9細胞上でES細胞から分化した血球系細胞，B：分化細胞をペトリディッシュへ植え継いだ後に出現したES-DC，C：IL-4，TNF-α，抗CD40抗体を同時に加えて成熟したES-DC，D：IL-4，LPS，抗CD40抗体を同時に加えて成熟したES-DC．

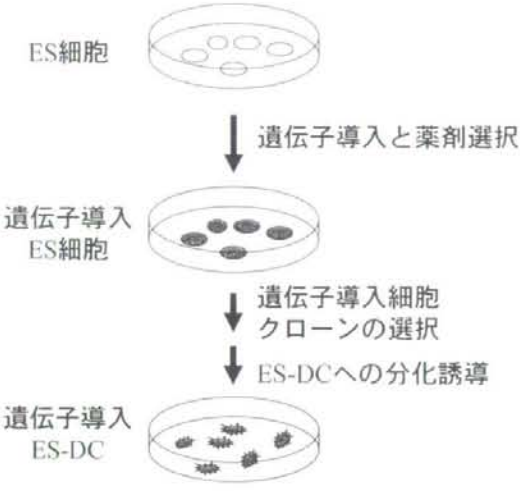

図4　遺伝子導入ES-DCの作製手順
まず，リポフェクションあるいは電気穿孔法により，未分化状態にあるES細胞へ，抗原あるいは免疫制御分子等の遺伝子発現ベクターを導入する．ベクターに付加している薬剤耐性遺伝子に合わせてG418あるいはピューロマイシン等の薬剤を加えて培養すると，ベクターが導入されたES細胞のクローンがコロニーとして出現する．複数のES細胞クローンを単離し，その中から適切な遺伝子改変ES細胞クローンを選択して増殖させる．樹立した遺伝子導入ES細胞クローンをES-DCに分化誘導することにより，遺伝子導入ES-DCをいくらでも作製することができる．

誘導した樹状細胞と同等の抗原提示機能とT細胞刺激活性を有しており，また，形態および表面マーカー等から，ミエロイド系樹状細胞に相当すると考えられる．これをさらにTNF-α，IL-4，抗CD40抗体，LPS等で刺激すると，著明な樹状突起を有し，より強力なT細胞刺激活性を有する成熟樹状細胞が誘導される．このES細胞由来の樹状細胞を，ES-DCと名付けた．図3にOP9細胞の上に出現したES細胞由来の浮遊細胞からES-DCへの分化に伴う細胞形態の変化を示す．

VII.　GPC3遺伝子を強制発現させたマウスES-DCによる抗腫瘍免疫の誘導

筆者らは，図4に示す遺伝子導入ES-DCを作製する手順により，モデル腫瘍抗原としてOVA（卵白アルブミン）抗原を発現するES-DCを作製した．このOVA発現ES-DCをマウス個体に移入することにより，OVA抗原に特異的な細胞傷害性T細胞を感作することができた．また，このES-DCをin vitroでマウスの脾臓由来のT細胞と共培養して刺激した場合も，OVA抗原特異的な細胞傷害性T細胞を活性化することができた．さらに，この樹状細胞を投与することによりOVA抗原に対して感作したマウスは，OVAを発現するマウス腫瘍細胞（MO4）を移植した場合に，これを拒絶することができた[11]．

ES-DCによる免疫療法では，モデル抗原であるOVAを用いた場合だけでなく，腫瘍細胞に自然に発現している腫瘍抗原を標的とした抗腫瘍免疫応答の誘導も可能である．Glypican-3は，筆者らがヒトの肝細胞がんおよびメラノーマに発現する新規癌胎児性抗原として同定したものである．前述の方法を用いてES-DCにGlypican-3を強制発現させたものをマウス個体に予防的に投与することにより，図5に示すようにマウスの皮下に移植された，Glypican-3を自然に発現するマウスメラノーマ細胞B16-F10の増殖を著明に抑制し，さらにマウスの生存期間を延長することが可能であった[12]．

VIII.　抗原とケモカインを同時に発現するマウスES-DCを用いた抗腫瘍免疫応答の誘導

筆者らは，ES-DCにT細胞の遊走を促すケモカインを発現させることにより，生体移入したES-DCがリンパ性臓器へ遊走できなくても，ES-DCが存在する場所へT細胞が集まり，その場所でES-DCからT細胞へ抗原刺激が伝えられ，抗原特異的なT細胞を活性化する効果を高めることがで

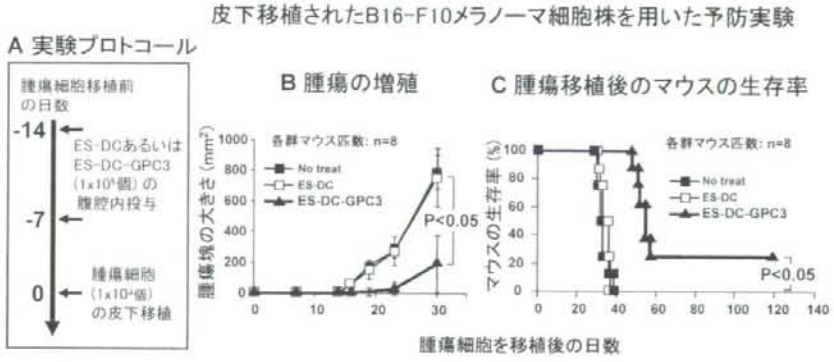

図5　肝細胞癌の新規癌胎児性抗原 Glypican-3 を発現する ES-DC による抗腫瘍効果の誘導
マウス ES 細胞にマウス GPC3 遺伝子を導入して発現させ，ES-DC（ES-DC-GPC3）を分化誘導した．A；遺伝子改変を加える前の ES-DC（□），あるいは ES-DC-GPC3（▲）をマウスの腹腔内に 7 日間隔で 2 回投与した．そして，2 回目の投与から 7 日後に，これらのマウスと未処置対照群マウス（■）の皮下に，GPC3 遺伝子を自然に発現するメラノーマ細胞（B16-F10）を移植し，B；その後の腫瘍の大きさの変化と，C；マウスの生存期間を観察した．

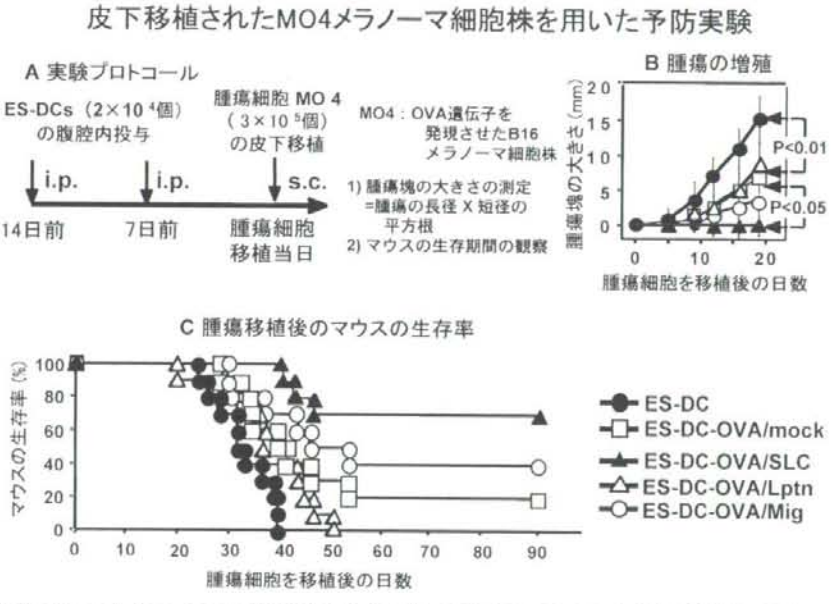

図6　導入遺伝子由来のケモカインとモデル腫瘍抗原 OVA を同時に発現する ES-DC による抗腫瘍効果の増強
マウス ES 細胞への遺伝子導入とクローンの選択を 2 回繰り返すことにより，モデル腫瘍抗原として OVA 抗原と，T 細胞の遊走を誘導するケモカイン（SLC，Lymphotactin，あるいは Mig）の遺伝子を同時に発現する ES-DC（それぞれ ES-DC-OVA/SLC ▲，ES-DC-OVA/Lptn △，ES-DC-OVA/Mig ○）を作製した．A；これらの遺伝的改変 ES-DC（▲，△，○），遺伝子改変を加える前の ES-DC（●），あるいは OVA のみを発現する ES-DC（ES-DC-OVA/mock □）をマウス腹腔内に 7 日間隔で 2 回投与した．そして，2 回目の投与から 7 日後に，OVA 遺伝子を強制発現させた腫瘍細胞（MO4）を皮下に移植し，B；その後の腫瘍の大きさの変化と，C；マウスの生存期間を観察した．

きるのではないかと考えた．

これを検証するために，前述した OVA 遺伝子を導入したマウス ES 細胞に，さらに，T 細胞に対する遊走活性を有するケモカインの遺伝子を導入し，この ES 細胞から OVA とケモカインを同時に発現する ES-DC を作製した．T 細胞に対する遊走活性を有するケモカインとして，生理的に存在する樹状細胞からは産生されない，SLC（CCL21），Mig（CXCL9），および Lymphotactin（XCL1）の遺伝子をそれぞれ導入し，各々の効果を比較した．その結果，この 3 種類のケモカインのいずれについても，OVA を単独で発現する ES-DC よりも，OVA とケモカインを同時に発現する ES-DC の方が，より効果的に T 細胞を活性化することがわかった[11]．さ