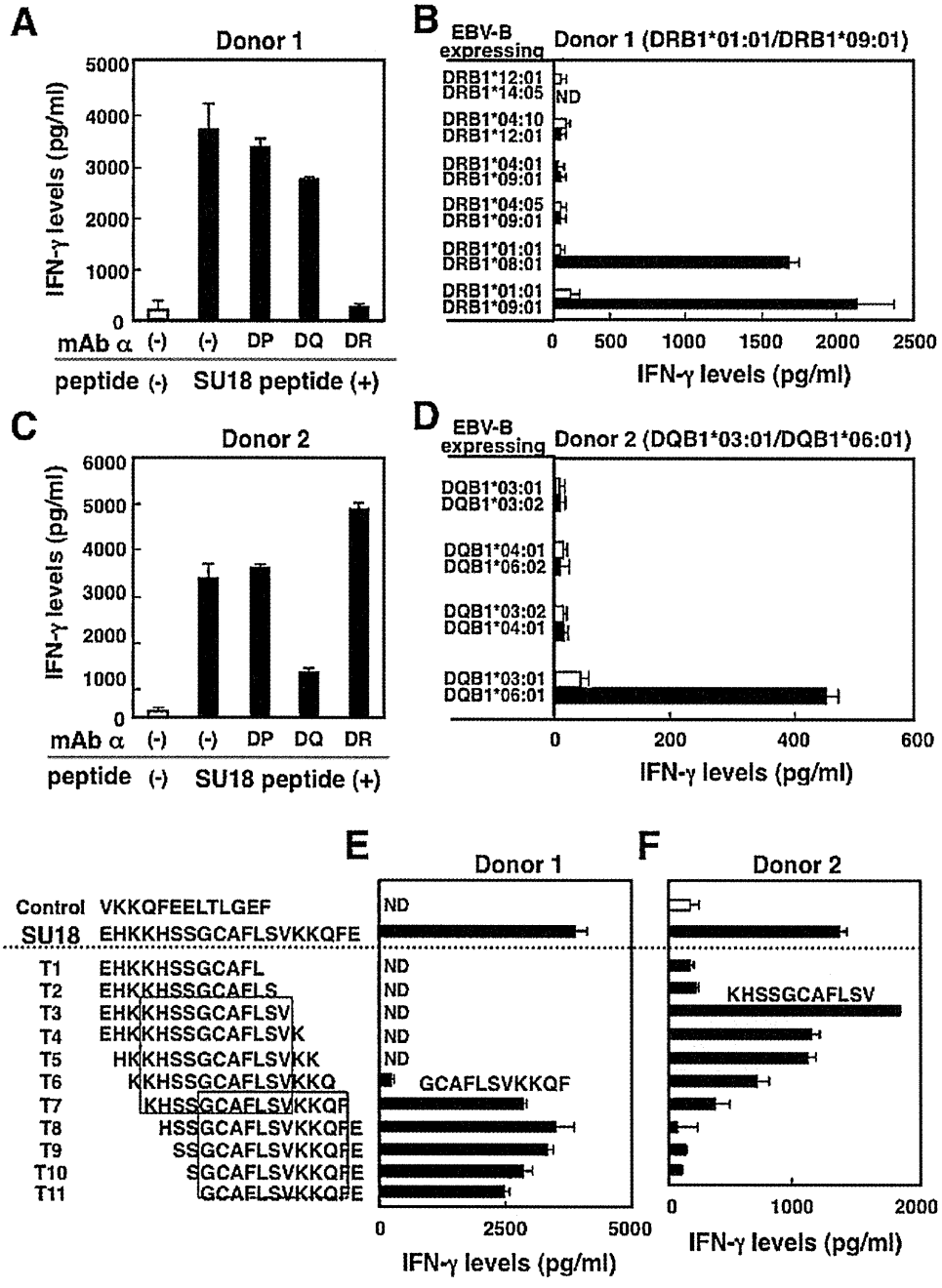

Fig. 1. Identification of novel Survivin-2B helper epitope peptide promiscuously bound to HLA-DRB1*01:01 and HLA-DQB1*06:01. Survivin-2B-specific CD4+ Th cells were induced from isolated CD4+ T cells by repetitive stimulation with MIX4 and donor 1- and donor 2-derived SU18 peptide (Survivin-$2B_{99-117}$)-reactive Th cells were used for this experiment. (A and C) Donor 1 or donor 2-derived SU18-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU18 peptide-pulsed autologous Mo-DC in the presence of mAbs against HLA-DP (DP), HLA-DQ (DQ), or HLA-DR (DR) (5 μg/ml). (B and D) SU18-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU18 peptide-pulsed autologous or allogenic EBV-B cells expressing indicated HLA-DR haplotypes. (E and F) SU18-specific CD4+ T cells were cultured with autologous Mo-DC in the presence of truncated 12–15-mer peptides of SU18 peptide. After culture for 20–24 h, IFN-γ levels in the culture supernatants were determined by ELISA. Means and SDs of the representative data are indicated. ND represents "not detected".

(5×10^4 cells), which were induced from autologous PBMC by stimulating with Survivin-H/K-HELP. To determine the antigen presentation capability of peptide-pulsed Mo-DC, IFN-γ levels in the culture supernatants were measured by ELISA kit (BD Biosciences, California, USA) 24 h after incubation. The change of APC function of Mo-Dc was determined by calculating the change of IFN-γ-production (%) as follows: Change of IFN-γ production (%) = (IFN-γ levels induced by co-cultured with Th1 cells and peptide-pulsed Mo-DC cultured for various hours – IFN-γ levels induced by co-cultured with Th1 cells and peptide-unpulsed Mo-DC cultured for various hours)/(IFN-γ levels induced by co-cultured with Th1 cells and peptide-pulsed Mo-DC cultured for 0 h – IFN-γ levels induced by co-culture with Th1 cells and peptide-unpulsed Mo-DC cultured for 0 h) × 100.

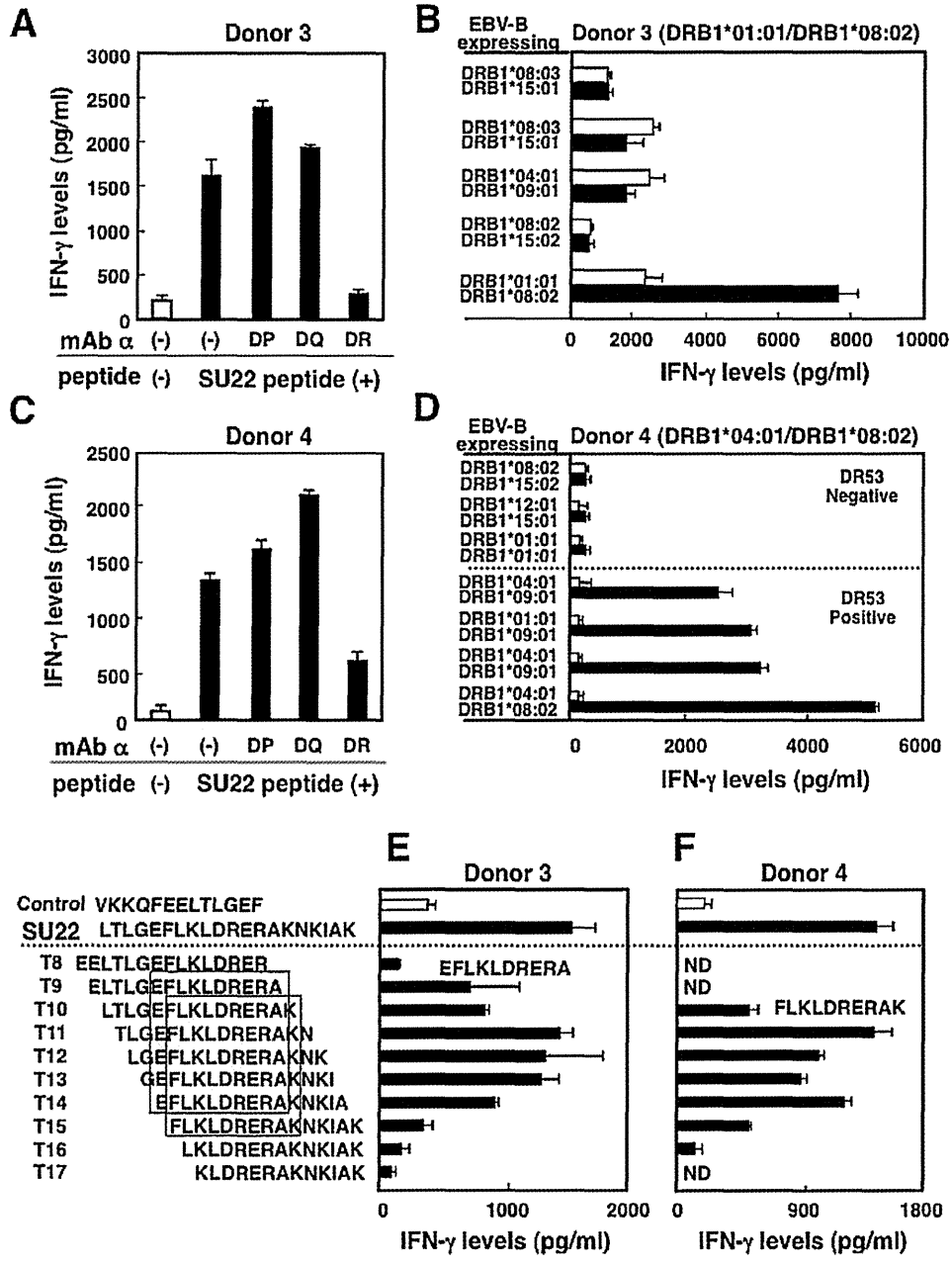

Fig. 2. Identification of HLA-DRB1*01:01- or HLA-DR53-restricted Survivin-2B helper epitope. (A and C) Donor 3 or donor 4-derived SU22-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU22 peptide-pulsed autologous Mo-DC in the presence of mAbs against HLA-DP (DP), HLA-DQ (DQ), or HLA-DR (DR) (5 μg/ml). (B and D) SU22-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU22 peptide-pulsed autologous or allogenic EBV-B cells expressing indicated HLA-DR haplotypes. (E and F) SU22-specific CD4+ T cells were cultured with autologous Mo-DC in the presence of truncated 12–15-mer peptides of SU22 peptide. After culture for 20–24 h, IFN-γ levels in the culture supernatants were determined by ELISA. Means and SDs of the representative data are indicated. ND represents "not detected".

2.7. Evaluation of HLA class II-restricted antigen-specific cytokine production of T cells

T cells (3×10^4 cells) expanded with Survivin-2B peptides were cultured with MMC-treated autologous PBMC (3×10^4 cells) or class II-expressing EBV-B cells (2×10^4 cells) in the presence of relevant peptide antigen for 20 h. After culture, the antigen-triggered IFN-γ levels in the culture supernatants were measured by ELISA kit (BD Biosciences, California, USA). HLA class II restriction was determined by the blocking experiment against peptide-specific T cell response (IFN-γ production) by adding 5 μg/ml anti-HLA-DR mAb (L243; BD Biosciences, California, USA), anti-HLA-DP mAb (BRAFB6; Serotech, Oxford, UK), or anti-HLA-DQ mAb (SPV-L3; Serotech, Oxford, UK). The detail HLA class II haplotype was determined by examining the APC function of EBV-B cells selected from our prepared EBV-B cell bank, which included various cells expressing various HLA class II haplotypes.

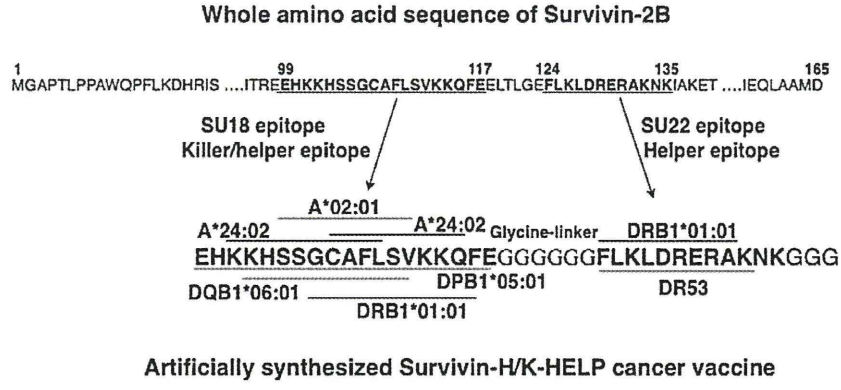

Fig. 3. Artificially synthesized cancer vaccine, Survivin-H/K-HELP containing helper and killer epitope peptides. Survivin-H/K-HELP was artificially synthesized by conjugating SU18 ($Survivin_{99-117}$) killer/helper epitope peptide (19 amino acid sequence indicated by boldface on the left) with SU22 ($Survivin_{124-135}$) helper epitope peptide (12 amino acid sequence indicated by boldface on the right) with a glycine-linker. The under lines indicated the sequence of helper and killer epitopes and HLA-restriction haplotypes. The figure illustrated Amino acid sequence and HLA-restriction portion of artificially synthesized.

2.8. Detection of antigen-specific expression of cytokine and cytotoxic molecules in T cells by intracellular staining

The induced T cells (5×10^4 cells) were stimulated with peptide-pulsed autologous Mo-DC (5×10^3 cells) for 2 h and further incubated in the presence of Brefeldin-A (Sigma–Aldrich Japan, Tokyo, Japan) for 20 h. Thereafter, the cells were first stained with anti-CD4 and anti-CD8 mAbs and then treated with Cytofix/Cytoperm (BD Biosciences, California, USA). The fixed cells were stained with anti-IFN-γ and anti-IL-5 mAbs to detect intracellular Th1 and Th2 cytokine production. Anti-Perforin, and anti-Granzyme B mAbs (BD Biosciences, California, USA) were used for detection of intracellular expression of cytotoxic molecules in $CD8^+$ T cells. The expression levels were determined by flow cytometry (FACSCanto II, BD Biosciences, California, USA).

2.9. A phase I clinical study on Survivin-H/K-HELP cancer vaccine therapy for a breast cancer patient

A phase I clinical trial of the Survivin-H/K-HELP vaccine was designed to evaluate the safety, immune response and tumor response. This clinical study was approved by all of institutional review boards in light of the Declaration of Helsinki. Written informed consent was obtained from patient before enrolling in the study. The consent for publication was also obtained from a patient by the written informed consent. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (UMIN000003489) on April 19, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

In December 2008, a 53-year-old woman was diagnosed with locally advanced breast cancer with axillar lymph node metastasis. Four courses of an anthracycline-based regimen (THP-adriamycin, cyclophosphamide; AC) were administered as pre-operative chemotherapy. However, the tumor remained same size (no response: NR). In April 30, 2009, left mastectomy with axillary lymph node resection was performed. Microscopically, the tumor diagnosed as invasive ductal carcinoma with a negative estrogen receptor, a negative progesterone receptor, and a negative human epidermal growth factor receptor 2 score (so-called "triple negative" tumor). Adjuvant treatment of weekly taxol (80 mg/m^2) was followed 12 times during May and August 2009. In September 2009, the patient presented a clinically asymptomatic supraclavicular lymph node recurrence. As this region seemed to be resistant to the standard chemotherapy, surgical resection of supraclavicular nodes was performed in September 8, 2009 and another chemotherapy, CPT-11 (80 mg/m^2) was initiated for 12 times during October and January, 2010. After a 4-month free-of relapse period, a local recurrence was detected again in left spinal accessory node. The radiotherapy in supraclavicular and chest wall in total 50 Gy/25 times was initiated from February 1, 2010. As the CT scan showed no response after 50 Gy irradiation, 10 Gy was boosted (total 60 Gy) in the same area. As this lymph node recurrence was considered to be chemo-resistant and radio-resistant, we conducted a vaccine immunotherapy. The cancer vaccine therapy using Survivin-H/K-HELP was initiated from April 14, 2010. Survivin-H/K-HELP vaccine was subcutaneously administered biweekly 4 times. Two weeks after the last administration, the safety, immune response and tumor response were evaluated as described previously [15].

3. Results

3.1. Identification of a novel Survivin-2B helper epitope

To identify Survivin-2B helper epitopes, purified $CD4^+$ T cells from four healthy donors were stimulated with IFN-γ-treated autologous Mo-DC with Survivin-2B peptide mixture (MIX1 to MIX5), which included 5 overlapping peptides derived from Survivin-2B (Supplementary Table S1). After repetitive stimulation with the cognate mixture, we obtained MIX4-reactive Th cells from all donors. Using these Th cells, the reactive peptides in the MIX4 were determined as SU18 peptide ($Survivin\text{-}2B_{99-117}$), SU21 peptide ($Survivin\text{-}2B_{112-131}$), and SU22 peptide ($Survivin\text{-}2B_{119-138}$), respectively. In the case of SU18 peptide, two types of Th cells were generated. The IFN-γ-production of donor 1-derived SU18-reactive Th cells was greatly inhibited by anti-HLA-DR mAb (Fig. 1A) and the Th cells recognized only HLA-DRB1*01:01-expressing EBV-B cells. These evidence suggested that the SU18 peptide was presented by HLA-DRB1*01:01 (Fig. 1B). In contrast, the response of donor 2-derived SU18-reactive Th cells was inhibited by anti-HLA-DQ mAb (Fig. 1C) and it was restricted to HLA-DQB1*06:01 (Fig. 1D). Therefore, SU18 peptide is a promiscuous peptide. To identify minimal epitopes recognized by these two Th cells, a series of 15-mer overlapping truncated peptides derived from the SU18 peptide was prepared and tested for their ability to stimulate the Th cells. The HLA-DRB1*01:01-restricted Th cells recognized EBV-B cells in the presence of T7 to T11 truncated peptides, suggesting that their recognizing minimal region was $Survivin\text{-}2B_{106-116}$ (GCAFLSVKKQF) (Fig. 1E). The minimal region of HLA-DQB1*06:01-restricted Th cells was determined as $Survivin\text{-}2B_{102-112}$ (KHSSGCAFLSV) (Fig. 1F). The same analysis was also performed using SU21 and SU22 peptides and demonstrated that donor 3-derived SU21-reactive Th cells were restricted to HLA-DRB1*15:01/15:02 and

its minimal region was Survivin-2B$_{117-128}$ (EELTLGEFLKLD) (data not shown). In the case of SU22 peptide, two types of Th cells were generated from donor 3 and 4. Donor 3-derived SU22-reactive Th cells were restricted to HLA-DRB1*01:01 (Fig. 2A and B), and donor 4-derived cells were restricted to HLA-DR53 (Fig. 2C and D), suggesting that SU22 peptide was also a promiscuous peptide. Each of minimal regions was Survivin-2B$_{123-132}$ (EFLKLDRERA) and Survivin-2B$_{124-133}$ (FLKLDRERAK), respectively (Fig. 2E and F).

3.2. Development of a superior long peptide, Survivin-H/K-HELP consisting of SU18 and SU22 peptides

From the database searching, it was clarified that SU18 included a novel promiscuous helper epitope with three other killer epitopes and SU22 contained a widely applicable promiscuous helper peptide. Therefore, we decided to synthesize artificial Survivin-H/K-HELP by conjugating SU18 and SU22 Survivin-derived peptides using glycine-linker. As illustrated in Fig. 3A, Survivin-H/K-HELP consisted of two helper epitopes (presented to HLA-DRB1*01:01, DR53, DQB1*06:01, and DPB1*05:01) and three killer epitopes (presented to HLA-A*02:01 and A*24:02). The detail information concerning helper and killer epitopes presented to HLA molecules were indicated by underlines in Fig. 3A.

3.3. Survivin-H/K-HELP was superior to its component short peptides (SU18 and SU 22) for inducing Survivin-specific Th1 and Tc1 cells

CD4$^+$ T cells, isolated from 9 healthy donors were cocultured with IFN-γ-treated Mo-DC or OK-432-treated Mo-DC in the presence of Survivin-H/K-HELP. The CD4$^+$ T cells were expanded by weekly stimulation with Survivin-H/K-HELP 3 times. After 3 weeks-culture, the expanded T cells were restimulated with Survivin-peptides and their IFN-γ or IL-5 producing ability was determined by intracellular staining assay to evaluate the frequency of Th1 or Th2 cell induction. The representative FACS profile was shown in Fig. 4A. Survivin-H/K-HELP induced IFN-γ-producing Th1 cells but not IL-5-producing Th2 cells. We also demonstrated that IL-4-producing Th2 cells were not significantly induced by stimulation with short peptides (0.48%) or surviving-H/K-HELP (0%). The expanded numbers of CD4$^+$ T cells were also measured and indicated as the accumulated data of T cell growth rate of 9 donors (Fig. 4B). These results indicated that OK-432-treated Mo-DC were superior to IFN-γ-treated Mo-DC for inducing Survivin-specific IFN-γ-producing Th1 cells. Thus, we used OK-432-treated Mo-DC as antigen-presenting cells (APC) to induce Th1 cells using cancer-associated antigen peptides. Using this culture method, we assessed whether Survivin-H/K-HELP was superior to its component short peptide (SU18 and SU22) for inducing Survivin-specific Th1 and Tc1 cells. PBMC were cultured with Survivin-H/K-HELP or mixture of its component peptides (mixture of SU18 and SU22 short peptides) in the presence of OK-432-treated Mo-DC for 3 weeks. After culture, the frequency of Survivin-specific Th1 or Tc1 cells was determined by intracellular staining using flow cytometry. As shown in Fig. 5, the induction of Th1 and Tc1 cells was greatly enhanced by culture with Survivin-H/K-HELP but not with the mixture of SU18 and SU22 short peptides in 3 distinct healthy donors though SU18 (19 amino-acids) peptide contained both helper and killer epitopes as described above (Fig. 3A). The CD4$^+$ T cells and CD8$^+$ T cells, which were expanded by stimulation with Survivin-H/K-HELP recognized its component SU18 (Donor A and B) and SU22 (Donor C) short peptides on APC and produced IFN-γ (Fig. 5). The expanded CD8$^+$ T cells expressed cytotoxic molecules such as perforin and granzyme B (supplementary Fig. S1). Thus, Survivin-H/K-HELP is superior to its component short peptides

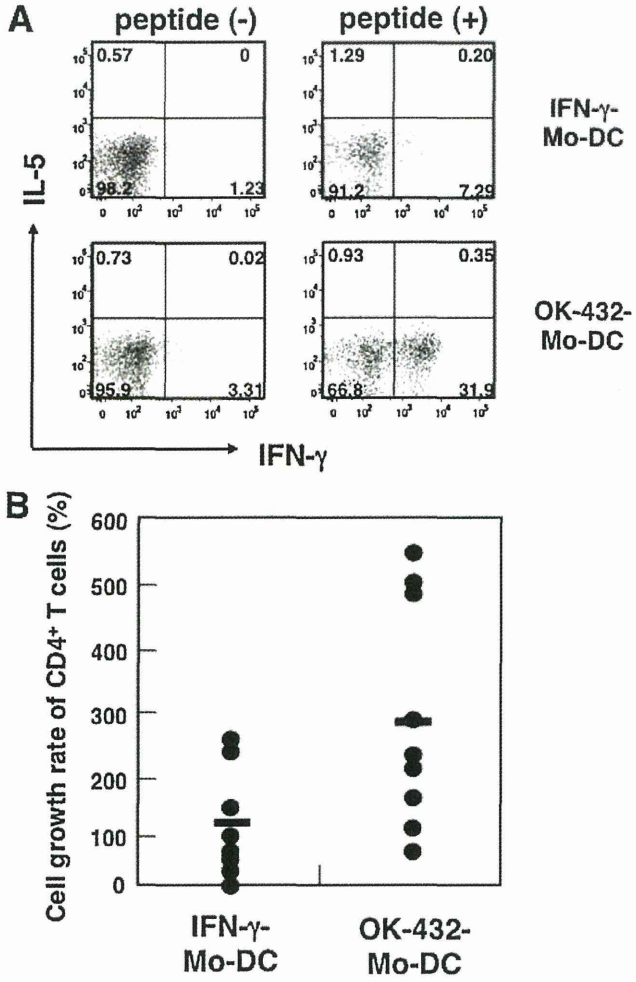

Fig. 4. OK-432-activated Mo-DC were superior to IFN-γ-treated Mo-DC for an efficient induction of Survivin-2B-specific CD4$^+$ Th1 cells by pulsing with Survivin-H/K-HELP. CD4$^+$ T cells were weekly stimulated with IFN-γ- or OK-432-treated Mo-DC pulsed with Survivin-H/K-HELP for 3 weeks. (A) IFN-γ and IL-5 production by CD4$^+$ T cells were evaluated by intracellular staining by flow cytometry after stimulation with (+) or without (−) Survivin-H/K-HELP. (B) Numbers of CD4$^+$ T cells in the culture were counted at day 21 and indicated as Cell growth rate of CD4$^+$ T cells (%), which was calculated as follows; cell growth rate of CD4$^+$ T cells (%) = numbers of activated T cells after culture/numbers of T cells before culture × 100. Means of triplicate data are presented.

(SU18 and SU22) for an efficient induction of IFN-γ-producing CD4$^+$ Th1 and CD8$^+$ Tc1 cells in the presence of OK-432-treated Mo-DC.

3.4. Superior and sustained antigen presentation of OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP

We compared the antigen presentation capability of OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP or its component SU18 and SU22 short peptides in the activation of the antigen-specific Th1 cells. The change of the APC function of OK432-activated Mo-DC was determined by measuring the change of Th1-dependent IFN-γ production levels in the culture. Although the antigen presentation activity of OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP or its component short peptides was gradually decreased during incubation, the Mo-DC pulsed with Survivin-H/K-HELP exhibited stronger stimulatory activity to induce higher IFN-γ production from Survivin-reactive Th cells compared with those pulsed with the mixture of SU18 and SU22

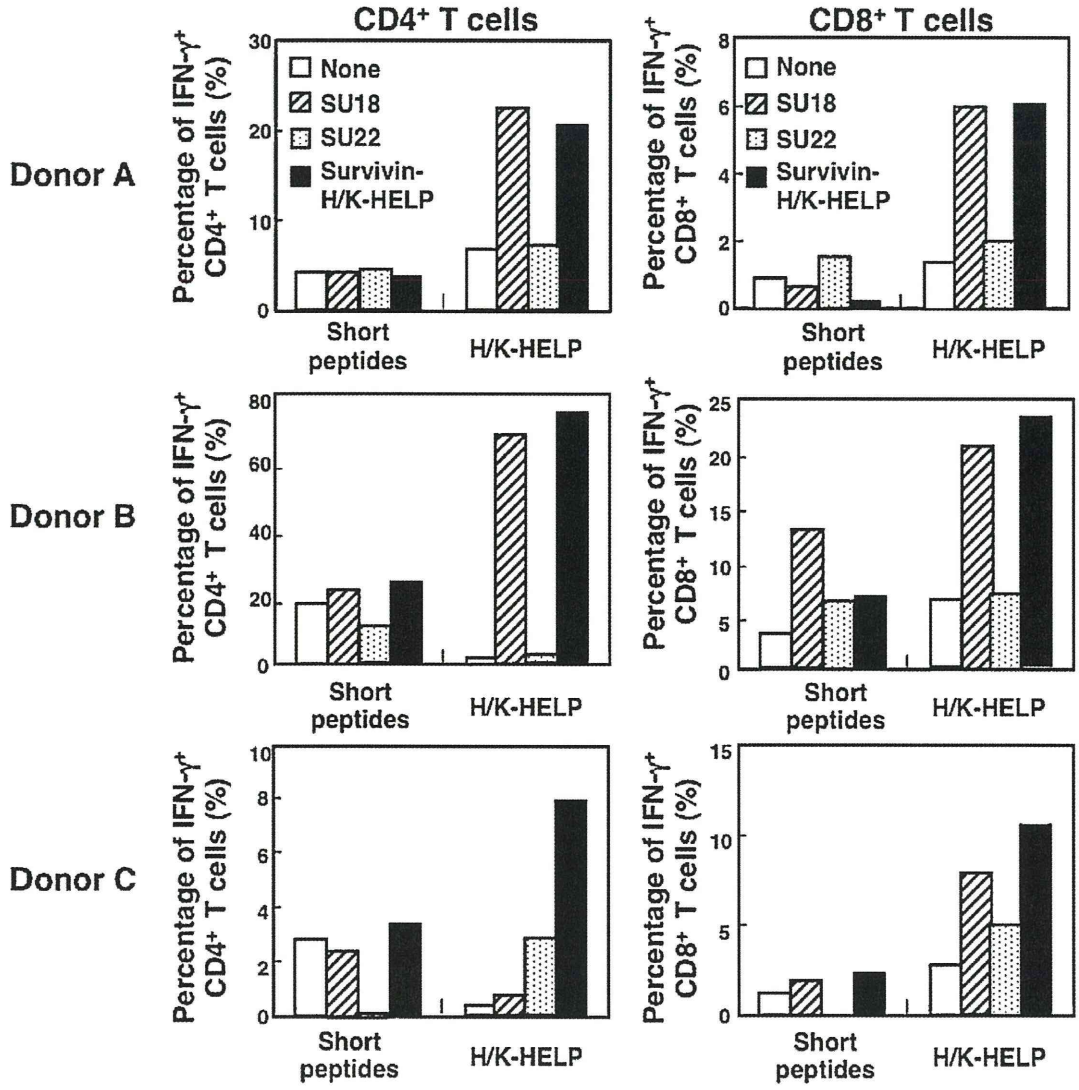

Fig. 5. Survivin-H/K-HELP induced an efficient induction of Survivin peptide-specific Th1 and Tc1 cells *in vitro*. PBMC of three donors were weekly stimulated with Mo-DC pulsed with Survivin-H/K-HELP or mixture of its component short peptides (SU18 and SU22) for 3 weeks. The induced T cells were restimulated with none (open bars), SU18 (slanted bars), SU22 (dotted bars), or Survivin-H/K-HELP (filled bars) for 20 h. IFN-γ production by stimulated $CD4^+$ T or $CD8^+$ T cells was evaluated by intracellular staining by flow cytometry. Representative data of three independent experiments are shown.

peptides at any point (24, 48, 72, 96 h) after culture (Fig. 6). Moreover, OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP exhibited sustained APC function to stimulate Th1-dependent IFN-γ production. Even 96 h after the initiation of culture, OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP possessed antigen-presenting capability of inducing IFN-γ from Th1 cells. While APC function of OK-432-activated Mo-DC pulsed with SU18 and SU22 short peptides almost completely disappeared 48 h after the initiation of culture.

3.5. A case study in phase I clinical trial of Survivin-H/K-HELP cancer vaccine therapy

We documented a prominent case study of breast cancer patient treated with Survivin-H/K-HEKP cancer vaccine. As shown in Section 2, a patient suffered with postoperative chemo-resistant, radio-resistant lateral deep cervical node recurrence of breast cancer was enrolled in a phase I clinical trial of Survivin-H/K-HELP cancer vaccine therapy. After confirmation of Survivin expression in cancer tissue, the patient was intradermally treated with Survivin-H/K-HELP vaccine and montanide biweekly four times (1st cycle) (Fig. 7A). Compared with the pretherapy, the size of metastatic node significantly decreased and completely disappeared 4 weeks after first cycle of Survivin-H/K-HEKP cancer vaccine therapy (71 days after initiation of vaccination) (Fig. 7B and C). In parallel with the anticancer effect of Survivin-H/K-HELP cancer vaccine, it rapidly induced Survivin-peptide-specific IFN-γ-producing Th1 cells and the elevation of Survivin-peptide specific antibodies though the generation of Tc1 cells reached peak at late time (Fig. 7D–F). It was notable that Th1-dependently complement-fixing IgG1 and IgG3 antibodies against Survivin-peptide became major subtypes at the late stage of Survivin-H/K-HELP vaccination (Fig. 7F and G).

4. Discussion

Survivin-2B is an attractive target molecule for cancer vaccine therapy because it is highly expressed in almost all cancer cells examined thus far by PCR and immunohistochemistry [16–24].

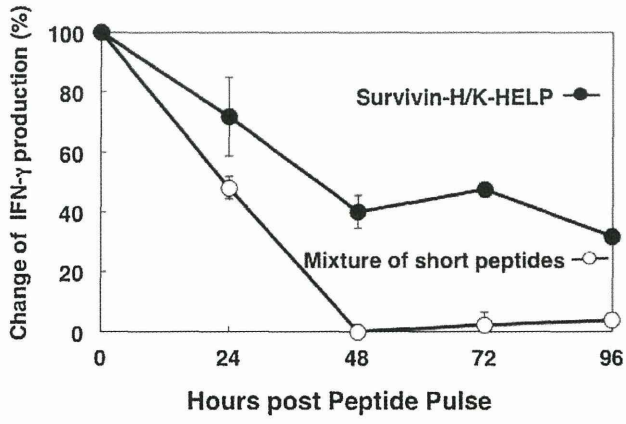

Fig. 6. Superior and sustained antigen presentation of Mo-DC pulsed with Survivin-H/K-HELP. OK-432-activated Mo-DC were pulsed with 5 μM Survivin-H/K-HELP (filled circle) or its component short peptides *SU18* and *SU22* (open circle) for 2 h at 37 °C. After washing twice, peptide-pulsed Mo-DC were cultured alone in AIM-V medium for various times (0, 24, 48, 72, 96 h). The peptide-pulsed-Mo-Dc were harvested from the culture 0, 24, 48, 72, or 96 h after incubation and cocultured with Survivin-peptide-reactive Th cells for 24 h to assess their antigen presentation capability. The antigen presenting ability of peptide-pulsed Mo-DC was determined by measuring Th cell-derived IFN-γ levels in the culture supernatants by ELISA. Change of IFN-γ production (%) was calculated as described in Section 2.6. Each data represents the mean of triplicate samples from representative experiment. The similar results were obtained in three separate experiments.

Here, we identify minimal peptide sequences of helper epitopes in Survivin cancer antigen. Among SU18 (Survivin-$2B_{99-117}$), SU21 (Survivin-$2B_{112-131}$), and SU22 peptides (Survivin-$2B_{119-138}$), the SU18 promiscuous helper peptide contains HLA-A*02:01- and HLA-A*24:02-restricted killer epitopes and the SU22 peptide contains a helper epitope restricted by DR53, which is a popular HLA class II phenotype in both Japanese (>60%) [25] and Caucasians (>50%) as determined by the Allele*Frequency Database. We also develop an artificially synthesized 40 amino-acid long peptide, Survivin-H/K-HELP by conjugating *SU18* helper epitope and *SU22* killer epitope peptides with a glycine-linker. SU18 includes a novel promiscuous helper epitope with three other killer epitopes and SU22 contains a widely applicable promiscuous helper peptide. Thus, Survivin-H/K-HELP, a long peptide including two helper and three killer epitopes, can activate both Th1 and Tc1 cells and it is applicable to the treatment of cancer patients worldwide judged from its binding HLA-haplotypes. We also demonstrate that Survivin-H/K-HELP is a superior cancer vaccine compared with its component short peptides (SU18 and SU22) to induce cancer antigen-specific Th1-dependent immunity *in vitro*.

During the past decades, many investigators have attempted to induce cancer-specific CTL in cancer patients by vaccination with class I-binding short (8–10 amino acids) peptides [26–28]. However, the overall results of cancer vaccine therapy have not been impressive [8,29–31]. This may be because of the existence of strong immunosuppression, tumor escape mechanisms and/or the lack of helper T cell activation [5,32–35].

We previously demonstrated a critical role for Th1 and Th2 immunity in tumor-bearing hosts and proposed that the introduction of Th1-dominant immunity is essential for inducing fully activated CTL and immunological memory [5,36,37]. Moreover, we demonstrated that Th1-dominant immunity could suppress the accumulation of immunosuppressive Treg cells into the tumor local site in an IFN-γ-dependent manner [38]. Therefore, the introduction of Th1 immunity in cancer patients might be a rational strategy to activate tumor-specific immunity and to inhibit immunosuppressive Treg functions. Indeed, recently, it was demonstrated that a mixture of various SLP containing both helper and killer epitopes corresponding to sequences of viral or tumor-associated antigens were superior to short peptides for inducing antitumor immunity. Melief and van der Burg et al. [11] reported that an HPV-16-derived 35 amino-acid long peptide eradicated an established HPV-16-expressing mouse tumor. SLP but not short peptides derived from the naturally occurring sequences of HPV-16 oncoproteins induced complete responses or partial responses in vulvar intraepithelial neoplasia [39]. Thus, long peptide vaccines containing both helper and killer epitopes might be a rational strategy to activate Th1-dependent antitumor immunity [5]. However, the first clinical trial using a synthetic 14 amino-acid peptide vaccine containing a naturally-occurring combination of helper and killer epitopes exhibited no significant therapeutic efficacy on the tumor [40]. We also demonstrate in this paper that a 19 amino-acid SU18 peptide, an SLP containing a naturally-occurring combination of helper and killer epitopes does not show stronger T cell stimulating activity *in vitro* compared with a 40 amino-acid Survivin-H/K-HELP containing SU18 and SU22 peptide. Therefore, long peptide with more than 23 amino acids might be suitable to induce an efficient T cell stimulation as described by Melief's group [11].

Previously [41], we demonstrated that culture of $CD4^+$ T cells with IFN-γ-treated autologous Mo-DC in the presence of helper peptides was a superior method to induce MAGE-A4-specific Th1 cells. Moreover we developed an artificially synthesized 40 amino acid long peptide, which conjugated MAGE-A4 class I-binding epitope and a defined helper epitope and demonstrated the safety and immunological effect of artificially synthesized MAGE-A4-H/K-HELP [15]. In contrast to short (14 amino-acid) hybrid peptides including helper and killer epitopes [13], administration of 40 amino acid H/K-HELP successfully induce cancer-specific Th1, Tc1, and complement-fixing antibodies (IgG1 and IgG3) [15,42]. This discrepancy may be explained as an artificially synthesized 40 amino acid long peptide, but not its component short peptides, has a beneficial structure favorable for DC presentation and subsequent activation of Th1 and Tc1. In the present paper, we develop Survivin-H/K-HELP including two helper and three killer epitopes by conjugating SU18 and SU22 short peptides. Survivin-H/K-HELP is superior to its component short peptides (*SU18* and *SU22*) to induce Survivin peptide-specific IFN-γ-producing Th1 and Tc1 cells. Furthermore, a 19 amino acid SU18 peptide, similar to SLP containing a naturally-occurring combination of helper and killer epitopes, does not show stronger T cell stimulating activity *in vitro* compared with the 40 amino-acid Survivin-H/K-HELP containing SU18 and SU22 peptides. We do not show any cytotoxicity data of $CD8^+$ T cells induced by culture with Survivin-H/K-HELP-pulsed Mo-DC. However, we have demonstrated that Survivin-H/K-HELP can induce higher numbers of $CD8^+$ T cells expressing perforin and granzyme B compared with those stimulated with short peptides. Therefore, it is possible to speculate that IFN-γ-producing $CD8^+$ T cells induced by Survivin-H/K-HELP act as cytotoxic T cells. Moreover, in one breast cancer patient of phase I clinical study, it is demonstrated that Survivin-H/K-HELP cancer vaccine successfully induce a complete response in parallel with the induction of Th1-dependent cellular and humoral responses. It is greatly interesting that Survivin-H/K-HELP vaccination allows the elevation of complement-fixing IgG1 and IgG3 Th1-dependent antibodies in patient's serum. Thus, it is suggested that 40 amino acid long peptide, Survivin-H/K-HELP is a superior cancer vaccine *in vivo* though this is still a result of one patient. Thus, we believe that a 40 amino-acid long peptide, Survivin-H/K-HELP is a superior cancer vaccine, which may act both *in vitro* and *in vivo*.

Melief et al. [43] reported that SLP of extended class I-binding long cancer peptides were efficiently processed by professional antigen presenting cells and subsequently exhibited sustained stimulating activity of DC to induce Th-dependent tumor-specific CTL. Consistent with their results, we also demonstrate that

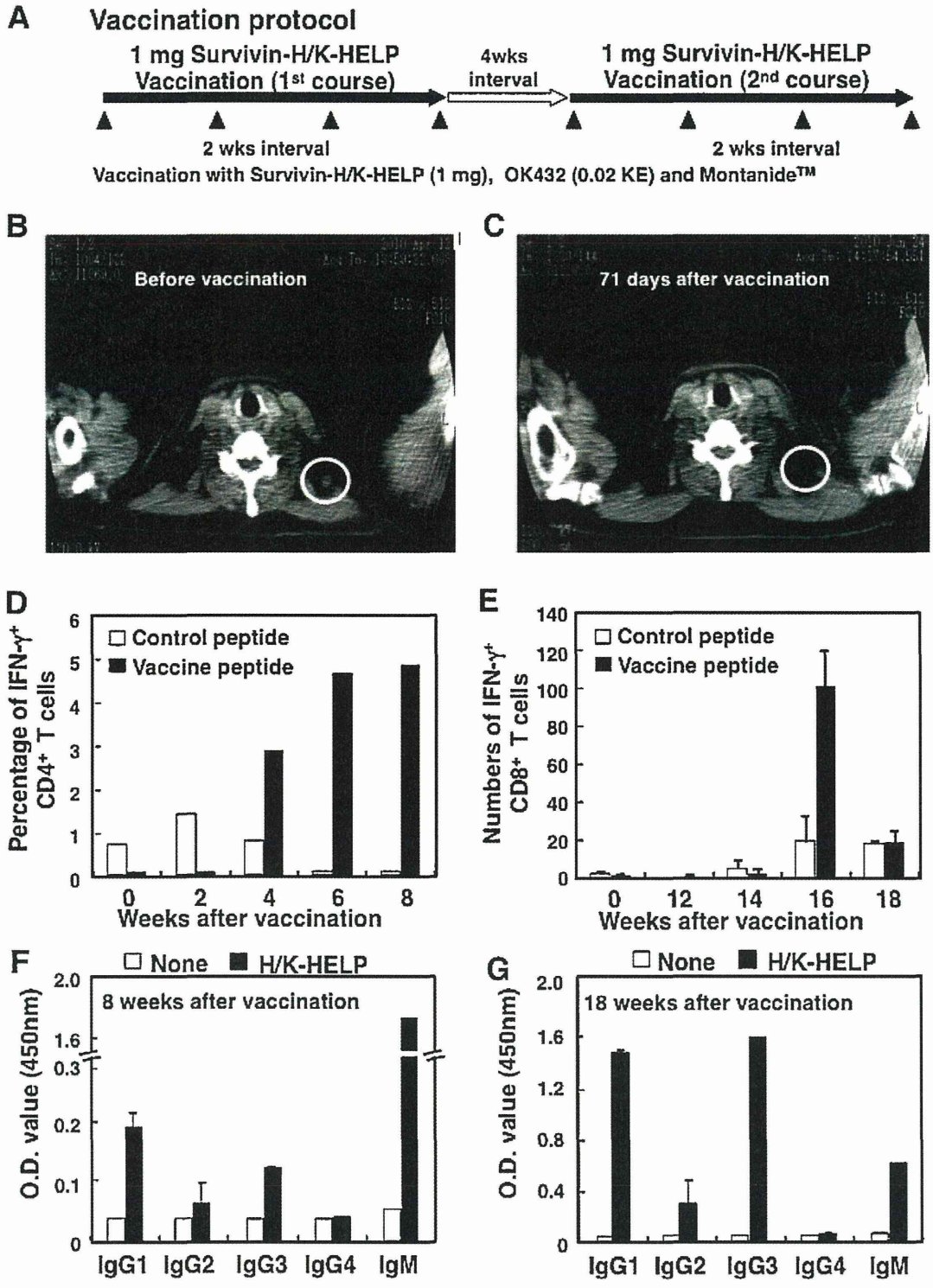

Fig. 7. Case study in phase I clinical trial of Survivin-H/K-HELP cancer vaccine therapy. A patient suffered with chemo-resistant, radio-resistant lateral deep cervical node recurrence of breast cancer was treated with Survivin-H/K-HELP vaccine therapy. (A) Vaccination protocol for the patient in phase I study. The patient was vaccinated with Survivin-H/K-HELP (1 mg) mixed with OK-432 (0.02 KE) and Montanide ISA-51 4 times (1st course) at 2 weeks interval and further 4 times (2nd course) after 4 weeks interval. (B and C) Growth inhibition of breast cancer by Survivin-H/K-HELP vaccine therapy. Tumor growth was monitored by computed tomograohy (CT) before or after the 1st cycle of Survivin-H/K-HELP vaccination. (D) Survivin-H/K-HELP-specific IFN-γ production by $CD4^+$ T cells was assessed by intracellular staining using flow cytometry various weeks after initiation of Survivin-H/K-HELP vaccination. (E) Survivin-H/K-HELP-specific IFN-γ production of $CD8^+$ T cells was determined by ELISPOT assay various weeks after initiation of Survivin-H/K-HELP vaccination. (F and G) The levels of IgG1, IgG2, IgG3, IgG4, or IgM antibody against none (□) or Survivin-H/K-HELP (■) peptide was determined using the patient's serum sample (400-fold diluted) 8 (F) or 18 (G) weeks after initiation of Survivin-H/K-HELP vaccination. Means and SDs were indicated in the figure.

OK-432-activated Mo-DC pulsed with 40 amino-acid Survivin-H/K-HELP but not its component short peptide (mixture of SU18 and SU22) allows superior antigen presentation capability to Mo-DC to stimulate IFN-γ-producing Th1 cells.

In summary, we identify novel helper epitopes and develop an innovative cancer vaccine, Survivin-H/K-HELP, which exhibits a superior capability of inducing Survivin-peptide-specific Th1 and Tc1 compared with its component short peptides (mixture of SU18 and SU22 peptides). We also demonstrate that Survivin-H/K-HELP compared with short peptides allows superior and sustained APC function to OK-432-activated Mo-DC, which is preferable for the induction of IFN-γ-producing Th1 cells. Thus, we believe that artificially synthesized Survivin-H/K-HELP of Survivin cancer-associated antigen will become an innovative cancer vaccine to induce Th1-dependent cellular and humoral immunity in cancer patients.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.04.010.

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Identification of a meiosis-specific protein, MEIOB, as a novel cancer/testis antigen and its augmented expression in demethylated cancer cells

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ABSTRACT

Cancer/testis (CT) antigens, which are expressed in various cancer cells but not in normal cells except germline cells of the testis, have been used as targets for cancer vaccine therapy. 5-Aza-2′-deoxycytidine (DAC), a potent inhibitor of genomic and promoter-specific DNA methylation, inhibits DNA methyltransferase activity and is reported to induce the expression of certain CT antigens by the demethylation of promoter CpG islands of the treated cells. Here, using DAC-treated cancer cells, we searched for novel attractive target molecules that would be useful for cancer immunotherapy and found a meiosis-specific protein, meiosis specific with OB domains (MEIOB), to be a novel CT antigen. Indeed, the MEIOB gene is expressed only in the testis and not in other normal tissues. The mRNA expression of MEIOB was greatly enhanced in several lung cancer cell lines after the treatment with DAC. Furthermore, we identified a variety of helper epitopes of the MEIOB antigen, which were recognized by MEIOB antigen-specific T cells in a HLA-restriction manner. Finally, we demonstrated that IFN-γ production of MEIOB peptide-specific helper T cells in response to HLA-matched cancer cells was greatly augmented by treatment with DAC and IFN-γ. Taken together, these findings show DAC to be a promising tool for finding novel CT antigens and for developing a future novel combination cancer vaccine chemotherapy.

1. Introduction

Cancer immunotherapy, including cancer vaccine, has been considered as an attractive therapy for patients with cancer because of the fewer side effects caused. Since the cancer/testis (CT) antigen was first found in a human melanoma [1], numerous CT antigens (such as MAGE and NY-ESO-1) have been identified as rational targets of various cancers. CT antigens are widely expressed in various types of human cancers, but their expression in normal cells is tightly restricted in germline cells of the testis [2–10]. It has been demonstrated that cancer vaccine therapy using the HLA class I-binding short peptide derived from the CT antigen caused an increase of tetramer-positive cytotoxic T lymphocytes (CTLs) in cancer patients and maintained the patients at long stable disease. However, we have proposed that Th1-dominant immunity in a tumor-bearing host was essential for the induction of fully activated CTLs and the subsequent generation of memory T cells [11–16].

Recently, it has been reported that the human papillomavirus (HPV)-16-derived long peptide, containing both helper and killer epitopes, remarkably eradicated established HPV-16-expressing tumors [17]. In a previous paper, we had identified the MAGE-A4-helper epitope [18] and applied it to a novel cancer vaccine immunotherapy, using the helper/killer-hybrid epitope long peptide (H/K-HELP). The H/K-HELP cancer vaccine induced superior antitumor responses, including T cell responses and antibody responses. In fact, in a phase I clinical study, we found that MAGE-A4-specific Th1 and Tc1 cells were effectively generated in patients with colon cancer after vaccination with MAGE-A4-H/K-HELP [19].

5-Aza-2′-deoxycytidine (DAC), an inhibitor of DNA methyltransferases (DNMTs), is approved by the U.S. FDA for the treatment of myelodysplastic syndromes and is currently under evaluation in phase I and II clinical trials for the treatment of solid tumors.

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