Introduction

Tumor cells express various proteins different from those of normal somatic cells, or over-express proteins at higher levels than in normal cells. Thus, peptides derived from these proteins may be specifically recognized by T lymphocytes. Of the two tumor-specific T lymphocyte subsets, $CD8^+$ cytotoxic T lymphocytes (CTL) recognize tumor-associated antigen (TAA)-derived peptides in the context of MHC class I molecules (MHC-I), whereas $CD4^+$ helper T (Th) cells respond to peptide-MHC class II (MHC-II) complexes. Because of their ability to eradicate malignant cells directly, CTLs have long been defined as the critical effector cells in anti-tumor immunity, although Th cells can also induce robust anti-tumor immune responses [1-3]. It is well accepted that tumor-specific Th cells maintain the anti-tumor responses of CTLs by licensing dendritic cells (DC) to effectively prime CTLs [4,5] by generating effective memory CTL [6,7], or by direct stimulation of effector CTLs [8]. Thus, identification and vaccination of Th-cell epitopes that activate tumor-specific Th cells is might be a promising method to induce effective anti-tumor immunity in tumor-bearing hosts. Although it may be possible to identify such Th-cell epitopes using human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors or cancer patients, it requires frequent bleedings and assays using a large number of overlapping peptides, which is in many cases a very long and labor-intensive procedure.

Utilizing a computer algorithm for the prediction of peptides with high binding affinity to a specific HLA/MHC molecule might be a convenient alternative. For example, SYFPEITHI (http://www.syfpeithi.de/) and BIMAS (http://www-bimas.cit.nih.gov) are powerful tools to predict MHC class I binding and therefore potential CTL-epitopes. On the other hand, the confidence of predicting peptides that bind to a specific HLA/MHC-II molecule is still under discussion and the ability of those peptides to induce Th-cell responses must be evaluated. Therefore, we established transgenic mice expressing HLA-DR4 (*HLA-DRA/HLA-DRB1*04:05*). of which allele frequency is 13.6% in Japanese population, to evaluate HLA-DR4-restricted Th-cell responses to tumor-associated antigen (TAA)-derived peptides predicted to bind to HLA-DR4. Using those mice, immunization with peptides known to be recognized by HLA-DR4-restricted human Th cells successfully induced peptide-specific and HLA-DR4-restricted mouse Th cell responses, demonstrating the validity of the HLA-DR4 Tgm for the preliminary screening of novel TAA-derived and HLA-DR4-restricted Th epitopes, which could be clinically applicable for peptide vaccine-based cancer immunotherapy in the future.

Materials and Methods

Ethics Statement

This study was approved by the animal research committee of Kumamoto University (Permission Number: B25-115). The mice were maintained at the Center for Animal Resources and Development of Kumamoto University, and were handled in accordance with the animal care guidelines of Kumamoto University.

The Institutional Review Board of Kumamoto University approved the research protocol for collecting and using PBMCs from healthy donors performed in this study with written informed consent.

Gene Constructs of HLA-DRA/I-$E^d\alpha$ and HLA-DRB1*0405/I-$E^d\beta$

To avoid possible problems caused by inter-species interactions between mouse CD4 and HLA-DR, a chimeric HLA-DR4/I-E^d molecule, of which translated α1 and β1 domains were derived from HLA-DR4 and the other domains were derived from respective I-E^d α and β molecules, was constructed [9]. The genomic fragments of exon 2 of *I-$E^d\alpha$* and β *genes* were replaced with those of *HLA-DRA*01:01* and *HLA*DRB1*04:05 genes*, respectively. The 8.5 kb HindIII and 16 kb KpnI fragments were used for the chimeric *HLA-DRA/I-$E^d\alpha$* and *HLA-DRB1*0405/I-$E^d\beta$* transgenes, which contain endogenous I-$E^d\alpha$ and I-$E^d\beta$ promoter regions spanning 3.2 kb and 5.2 kb of 5'-untranslated regions, respectively (Figure 1). The *HLA-DR4/I-E^d* transgenes were co-injected into C57BL/6 fertilized eggs [10] and were transferred into the oviduct of pseudopregnant ICR mice. A total of 25 pups were obtained and at 4-5 weeks after birth, their PBMCs were subjected to flow cytometric analyses using anti-HLA-DRα-chain monoclonal antibody (mAb) L243 and anti-HLA-DR4β-chain mAb TAL15.1. In addition, genomic PCR analyses was used using the following primer sets: 5'-CACCCAGACACTGTTTCTTC-3' and 5'-CAAAGCTGGCAAATCGTC-3' for *HLA-DRA/I-$E^d\alpha$* and 5'-CCCGTTAGTTGTGGTGACCT-3' and 5'-GCACTGTGAAGCTCTCACCA-3' for *HLA-DRB1*04:05/I-$E^d\beta$*, respectively after preparation of genomic DNA using DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). The presence and expression of *HLA-DR4/I-E^d* transgenes were confirmed by PCR and flow cytometry and positive offspring were crossed with wild-type (WT) C57BL/6 mice.

Cell lines

L-DR4, genetically engineered mouse fibroblast L cells that express HLA-DR4 (*HLA-DRA*01:01/DRB1*04:05*), were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 50 U/ml penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BM-DCs) were generated as described by Inaba et al [11]. Briefly, bone marrow cells obtained from mouse thighbones and hind limbs were cultured at 1×10^7 cells per well in 10 ml of RPMI 1640 supplemented with mouse GM-CSF 10 ng/ml, 10% FCS, 50 μM 2-mercaptoethanol (2ME) and 50 U/ml penicillin/streptomycin. On day 14, the cells were recovered for antigen presentation.

Synthetic peptides

Peptides were synthesized by Biomatik (Cambridge, Ontario, Canada). The peptides used in this study were: CMV-derived

Figure 1

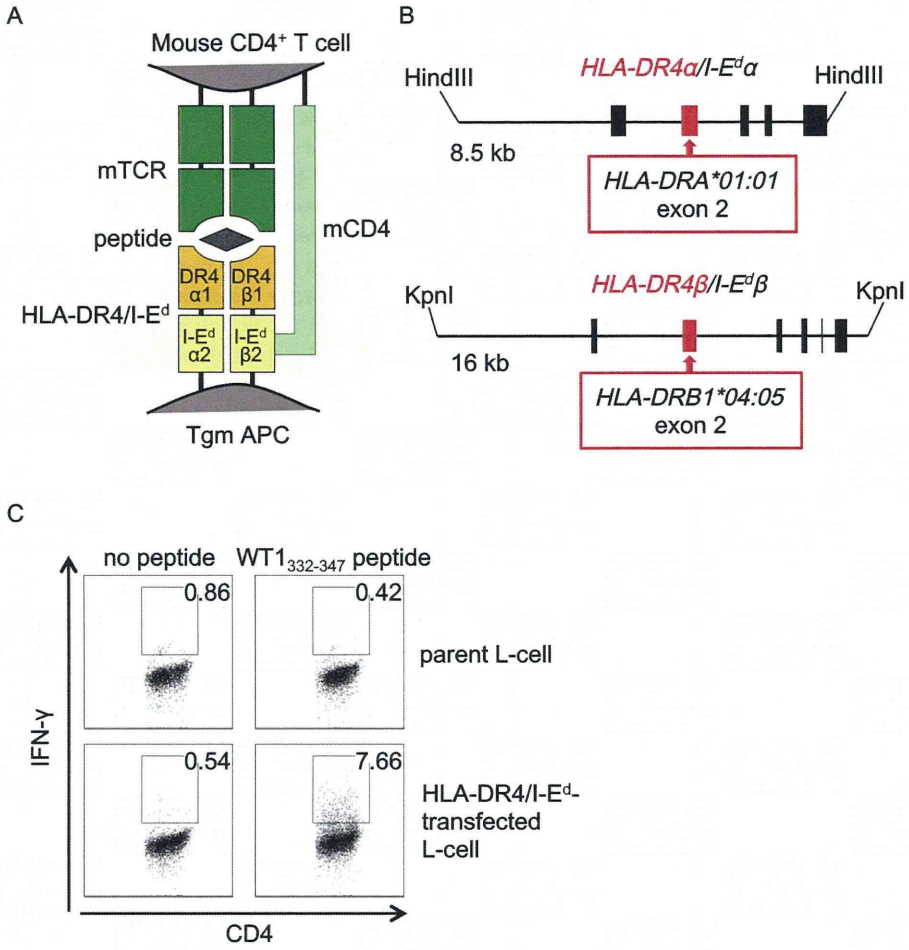

Figure 1. Transgenes encoding chimeric HLA-DR4/I-E^d molecule. (A) Schematic diagram of mouse T cell receptor (mTCR, green), mouse CD4 (mCD4, pale green) and HLA-DR4/I-E^d chimeric molecules (orange and yellow). (B) To avoid inter-species interactions between mCD4 and HLA-DR4, the second exons of *I-E^{d}α* and *I-E^{d}β* genes encoding α1 and β1 domains were substituted with those of HLA-DRA and *HLA-DR4B* genes (red boxes), respectively. The transgenes contain the endogenous I-E^{d}α and I-E^{d}β promoter regions spanning 3.2 kb and 5.2 kb of 5'-untranslated regions, respectively. (C) $WT1_{332-347}$ peptide-pulsed L-cells expressing HLA-DR4/I-E^d stimulated IFN-γ production by the $WT1_{332-347}$ peptide-specific and HLA-DR4-restricted human Th clone (gated on CD4).

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CMV-$egH_{290-302}$ peptide (SYLKDSDFLDAAL) [12], WT1-derived $WT1_{332-347}$ peptide (KRYFKLSHLQMHSRKH) [13], CDCA1-derived $CDCA1_{55-78}$ peptide (IVYGIRLEHFYMMPVNSEVMYPHL) [14], KIF20A -derived $KIF20A_{494-517}$ peptide (TLHVAKFSAIASQLVHAPPMQLGF) [15], DEPDC1-derived $DEPDC1_{191-213}$ peptide (RYVILIYLQTILGVPSLEEVINP) and a negative control $DEPDC1_{60-85}$ peptide (NSNFGPEVTRQQTIQLLRKFLKNHVI). The purity of the peptides was routinely >95%. Lyophilized peptides were dissolved in DMSO and stored at −20°C.

Flow Cytometry

After hemolysis, PBMCs were stained with various PE- or FITC-conjugated mAbs. The cells were analyzed by FACScan (BD Biosciences, Franklin Lakes, NJ, USA) using Cell Quest software. PE-conjugated anti-HLA-DR-α-chain mAb L243 was from BD and FITC-conjugated anti-HLA-DR-β-chain mAb TAL15.1, PE-conjugated anti-I-A^b mAb, PE-conjugated anti-mouse MHC-II mAb, FITC-conjugated anti-B220 mAb, PE- or FITC-conjugated anti-mouse CD4 mAb were from eBioscience (San Diego, CA, USA).

Intracellular IFN-γ staining

Parental L-cells and L cells expressing HLA-DR4/I-E^d were incubated with or without 10 μg/ml of $WT1_{332-347}$ peptide for 3 h, washed extensively, and then used as a stimulator. L cells were co-incubated with HLA-DR4-restricted $WT1_{332-347}$ peptide-specific $CD4^+$ T cell clones [13] in the presence of 2 μg/ml CD28/CD49d Costimulatory Reagent (BD Biosciences) and 10 μg/ml Brefeldin A (Sigma-Aldrich, St Louis, MO, USA) for 5 h. Intracellular staining of IFN-γ was performed using BD Cytofix/ Cytoperm Buffer (BD Biosciences) according to the manufacturer's procedures after surface staining of CD4 molecules. PE-conjugated anti-IFN-γ mAb and FITC-conjugated anti-CD4 mAb were from BD Biosciences. The cells were analyzed with FACSAria (BD Biosciences). The data were analyzed with FlowJo software.

Immunization of mice

Mice were primed either in the tail base with 50 μl of peptides in PBS (1 μg/μl) emulsified with 50 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich) or by intravenous injection with peptide-pulsed BM-DCs (5×10^5). Seven days after the first immunization, mice were boosted with 50 μl of peptide in PBS (1 μg/μl) emulsified with 50 μl of Incomplete Freund's Adjuvant (IFA, Sigma-Aldrich) or CFA. On day 14, the inguinal lymph nodes or spleen cells were collected and cultured with the peptides and IL-2 (20 U/ml) for 7 days. Th cells were isolated by MACS beads (Miltenyi Biotec, Bergish-Gladbach, Germany) according to the manufacturer's instruction and assayed as described below. In some experiments, the harvested lymph nodes cells on day 14 were subjected to *ex vivo* proliferation assay.

T cell proliferation assay and blocking experiment

Purified Th cells were co-cultured with peptide-pulsed L-DR4 cells for 48 h in RPMI 1640 medium containing 8% FCS, 50 U/ml penicillin/streptomycin and 2ME, then pulsed with 1 μCi [^{3}H-] thymidine and cultured for another 17 h. The cells were harvested and incorporated radioactivity was counted using a scintillation counter (I450 Microbeta, Trilux, PerkinElmer). To confirm the HLA-DR4 restriction, single cell-suspension of inguinal LN cells was cultured with or without peptide in the presence or absence of anti-HLA-DR blocking mAb L243 for 48 h. Then, incorporated radioactivity was counted as described above.

IFN-γ ELISPOT assay

The ELISPOT assay was performed as described previously [14]. Briefly, Th cells were incubated in triplicate in ELISPOT plates (BD Biosciences) under the presence of the indicated peptides (10 μg/ml) and L-DR4 as antigen presenting cells. According to the manufacturer's instructions, the plates were incubated for 18 h at 37°C and IFN-γ-positive spots were quantified using Eli photo (Minerva Tech, Tokyo, Japan).

Generation of antigen-specific human $CD4^+$ T cells

We obtained PBMCs from HLA-DR4-positive healthy donors (genotyped by the HLA Laboratory, Kyoto, Japan). Induction of antigen-specific $CD4^+$ T cells was performed as follows:. We isolated the PBMCs from the heparinized blood of Japanese healthy donors by means of Ficoll-Conray density gradient centrifugation. $CD4^+$ T cells and $CD14^+$ cells were purified from PBMCs. Monocyte-derived DCs were generated from $CD14^+$ cells and used as antigen-presenting cells (APCs) to induce antigen-specific $CD4^+$ T cells. $CD14^+$ cells co-cultured with human GM-CSF (100 ng/ml) and human IL-4 (10 ng/ml) in a 10 cm tissue culture dish for 7 days. On day 5, OK-432 (0.1 KE/ml) were added into the dish. On day 7, DCs (1×10^4 /well) were pulsed with 10 μg/ml peptide for 3 h, irradiated (45 Gy), and subsequently mixed with $CD4^+$ T cells (3×10^4 /well) in 200 μL AIM-V (Life Technologies) supplemented with 5% human decomplemented plasma in each well of a 96-well, flat-bottomed culture plate. After 7 days, half of the medium was removed from each culture, and fresh medium (100 μl/well) containing irradiated (50 Gy) autologous PBMCs (1×10^5) pulsed with peptide (10 μg/ml) and 5 ng/ml recombinant human IL-7 (rhIL-7) was added. Two days after the second stimulation with peptide, rhIL-2 was added to each well (10 IU/ml). A week later, the stimulated $CD4^+$ T cells in each well were analyzed for specificity in IFN-γ ELISPOT assays. The T cells showing a specific response to the cognate peptide were transferred to 24-well plates and re-stimulated at weekly intervals with irradiated autologous PBMCs (1×10^6 /well) pulsed with the peptide in medium supplemented with rhIL-2 (20 IU/ml) and rhIL-7 (5 ng/ml) [14].

Results

Expression and characterization of the chimeric HLA-DR4/I-E^d molecule *in vitro*

The *HLA-DRA/I-E^d*α and *HLA-DRB1*0405/I-$E^{d\beta}$* chimeric transgenes were first co-transfected into mouse fibroblast L cells and cell surface expression of HLA-DR4/I-E^d molecules were confirmed by positive staining with FITC-conjugated anti-HLA-DR antibody (TAL15.1) by flow cytometry (data not shown). HLA-DR4-restricted and $WT1_{332-347}$ peptide-specific T cell clones [13] produced IFN-γ in an HLA-DR4-restricted and a peptide-specific manner when T cells were co-cultured with the TAL15.1-positive L-cells pulsed with $WT1_{332-347}$ peptide (Figure 1C). These results suggested that the chimeric HLA-DR4/I-E^d molecules were expressed and could stimulate HLA-DR4-restricted T-cell responses by transduction of the *HLA-DRA/I-E^d*α and *HLA-DRB1*0405/I-E^d*β chimeric transgenes into the mice.

Characterization of HLA-DR4 Transgenic Mice

To select HLA-DR-positive F_0 mice, their PBMCs were collected and analyzed by cell surface staining with anti-HLA-DR antibodies (anti-HLA-DRα mAb L243 and anti-HLA-DRβ mAb TAL15.1) by FACScan. Genomic DNA prepared from the cells was then subjected to PCR analysis 2 mice were positive for the expression of both HLA-DRα and -DRβ molecules (Figure 2A) and for the presence of *HLA-DRA/I-E^d*α and *HLA-DRB1*0405/I-E^d*β transgenes (Figure 2B), and they were designated as #5 and #7 founder mice, respectively. These two mice were maintained by crossing with WT C57BL/6 mice, and

Figure 2

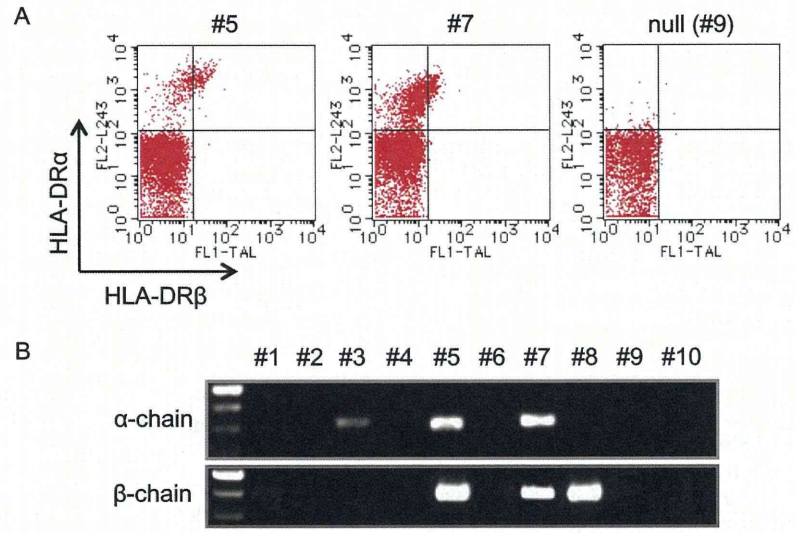

Figure 2. Selection of transgene-positive founder mice (F_0). Twenty five F_0 mice were arbitrarily numbered #1~#25. PBMCs were analyzed for HLA-DR4/I-E^d expression by flow-cytometric analyses (gated on lymphocytes) using anti-HLA-DRα and β mAbs (A) and genomic PCR analyses (B). A typical dot blot of PBMCs from other mice negative for transgenes is shown (null (#9) in A) and results from representative 10 mice from 25 F_0 mice are shown in B.

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the presence and expression of the transgenes were stably transmitted to their descendants (data not shown). Fluorescence in situ hybridization (FISH) analyses using fluorescent-labeled transgenes as probes revealed that the transgenes were inserted in the H2-H4 region of chromosome 3 in the line #5 Tgm (Figure 3A) and in the B-region of Y-chromosome in line #7 Tgm (Figure S1A), respectively.

To examine which subsets of cells expressed HLA-DR4/I-E^d molecules, PBMCs isolated from Tgm were analyzed by flow cytometry using fluorescent-labeled mAbs against HLA-DRα, mouse I-A^b and mouse B220. The majority of B220-positive cells were positive for HLA-DRα (Figure 3B, left panel), and most of the endogenous I-A^b-positive cells were positive for HLA-DRα (Figure 3B, right panel), indicating the expression profile of chimeric HLA-DR4/I-E^d was similar to the endogenous MHC-II, (I-A^b of C57BL/6 mice). Similar observation was also obtained in #7 Tgm (Figure S1B). We noticed that the expression level and positive ratio of HLA-DR4/I-E^d in I-A^b-positive cells were always slightly higher in the chromosome 3-linked line #5 Tgm compared with the Y chromosome-linked line #7 Tgm (Figure 3B and Figure S1B). Therefore, line #5 Tgm (hereafter called Tgm unless otherwise mentioned) were mainly used in the following immunological assays.

To examine the function of chimeric HLA-DR4/I-E^d molecules, Tgm that lacked expression of endogenous *MHC-II* gene were generated by crossing Tgm with *MHC-II* knock-out mice (B6.129S2-H2$^{dlAb1\text{-}Ea/J}$, Jackson Laboratory) and the presence of CD4$^+$ cells in PBMCs was examined. In PBMCs of *MHC-II* knock-out mice (*DR*$^{-/-}$*MHC-II*$^{-/-}$, Figure 4 left panels), both MHC-II positive cells (1.8%, lower panel) and CD4$^+$ cells (1.7%, upper panel) were almost absent. In PBMCs of Tgm (*DR*$^{+/-}$ *MHC-II*$^{+/+}$, Figure 4 center panels), both MHC-II positive (27.6%, lower panel) and HLA-DR4/I-E^d positive (16.4%, upper panel) cells were present and normal amount of CD4$^+$ cells were present (66.5%, upper panel). In PBMCs of *DR*$^{+/-}$*MHC-II*$^{-/-}$ mice (Figure 4 right panels), although MHC-II positive cells were absent (0.6%, lower panel), the expression of HLA-DR4/I-E^d (upper panel) in the mice restored the presence of CD4$^+$ cells (59.3%, upper panel). This indicated that expression of either HLA-DR/I-E^d or I-A^b in mice were indispensable for CD4$^+$ cell differentiation and since a comparable proportion of CD4$^+$ cells in PBMCs of *DR*$^{+/-}$*MHC-II*$^{-/-}$ mice with that of *DR*$^{+/-MHC\text{-}II+/+}$ mice was observed (59.3% vs 66.5%, Figure 4, upper center and right panels), the chimeric HLA-DR4/I-E^d molecules had an equivalent function to I-A^b and could induce normal differentiation of CD4$^+$ cells. Thus, the chimeric HLA-DR4/I-E^d molecules were functionally expressed in the Tgm.

Induction of HLA-DR4-restricted and non-self peptide-specific Th cells in Tgm

To check that Tgm could induce peptide-specific and HLA-DR4-restricted Th-cell responses, Tgm were immunized with a CMV-derived peptide, CMV-egH$_{290-302}$; SYLKDSDFLDAAL, that binds HLA-DR4 [12] using CFA and IFA as adjuvants. Th cells derived from immunized Tgm strongly proliferated when co-cultured with CMV-egH$_{290-302}$ peptide-pulsed L-DR4 cells, but not with unpulsed cells (Figure 5A). In addition, splenocytes isolated from immunized WT C57BL/6 mice did not respond to either peptide-pulsed or unpulsed L-DR4 cells. Therefore, Tgm immunized with HLA-DR4-binding CMV-derived peptides

Figure 3

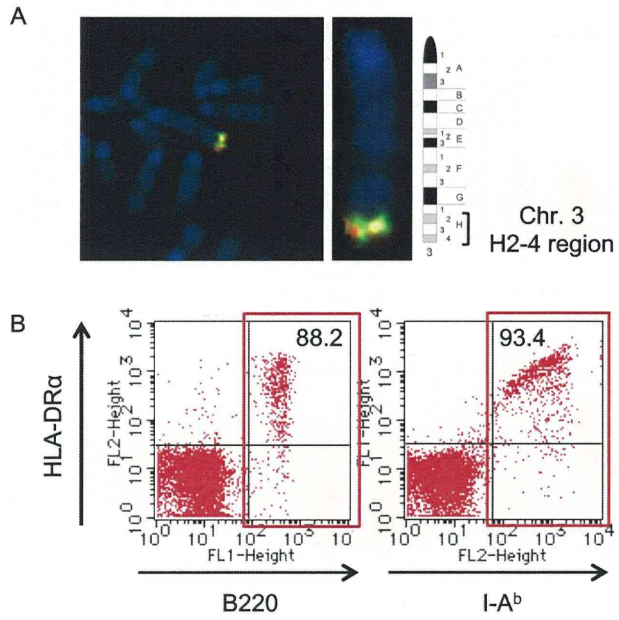

Figure 3. Chromosomal localization of transgene insertion site and cell-type specific expression of HLA-DR4/I-E^d. (A) Mapping of transgene insertion by fluorescence in situ hybridization (FISH) revealed integration in chromosome 3, H2-H4 region in line #5 Tgm. (B) PBMCs from line #5 Tgm were stained with anti-HLA-DR and anti-B220 mAbs (left) or anti-HLA-DR and anti-I-A^b mAbs (right). Numbers indicate the percentage of HLA-DR4/I-E^d-positive cells in B220-positive cells and MHC-II-positive cells indicated by the red boxes, respectively (gated on lymphocytes).

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successfully exhibited HLA-DR4-restricted and peptide-specific Th-cell responses. Thus, the Tgm were useful for screening of HLA-DR4-restricted Th-cell epitopes.

Induction of Th-cell responses specific for known HLA-DR4-binding TAA-derived peptides in Tgm

Next, we determined whether immunization of Tgm with TAA-derived peptides could induce HLA-DR4-restricted peptide-specific mouse Th-cell responses. CDCA1 (cell division cycle associated 1) is a TAA frequently overexpressed in lung cancer, head-and-neck cancer and other malignancies [16,17]. CDCA1$_{55\text{-}78}$ peptide (IVYGIRLEHFYMMPVNSEVMYPHL) is a CDCA1-derived peptide consisting of 15 amino acid residues (underlined) predicted to bind to HLA-DR4 with high affinity by a computer algorithm available at the Immune Epitope Data Base site (http://tools.immuneepitope.org/mhcii/). Tgm were immunized with CDCA1$_{55\text{-}78}$ peptide and CFA or IFA at the tail base as described in the Materials and Methods. As shown in Figure 5B, proliferation of Th cells was observed in Th cells stimulated with peptide-pulsed L-DR4 cells, but not with unpulsed L-DR4 cells or parental L-cells pulsed with or without the peptide. The data indicated that immunization of Tgm with TAA-derived peptides predicted to bind to HLA-DR4 successfully induced HLA-DR4-restricted and peptide-specific Th cells. As we previously reported [14], the CDCA1$_{55\text{-}78}$ peptide is naturally processed from CDCA1 protein by dendritic cells to induce HLA-DR4-restricted Th-cell responses in human PBMCs isolated from HLA-DR4-positive healthy donors and cancer patients. Thus, the screening of TAA-derived HLA-DR4 binding Th-cell epitopes could be performed by the combination of computer algorithm analyses and peptide immunization of Tgm.

The human CDCA1$_{55\text{-}78}$ peptide is different from the mouse ortholog peptide at 3 amino acid residues (VYGIRLEHFYMMPVNSEVMYPHL vs VYGVRLEHFYMMPMNIEVTYPHL), and therefore CDCA1$_{55\text{-}78}$ peptide is non-self and may be immunogenic in mice. However, the WT1$_{332\text{-}347}$ peptide, a known HLA-DR4 binding human Th-cell epitope derived from Wilms' Tumor 1 antigen (WT1) [13], has an identical amino acid sequence with the mouse ortholog peptide. As expected, immunization of Tgm with WT1$_{332\text{-}347}$ peptide using CFA and IFA as adjuvants failed to induce Th-cell responses (data not shown). DCs are superior antigen presenting cells and DC-based vaccination is thought to be a promising cancer immunotherapy [18-20]. Thus, to elicit stronger immune responses, we immunized Tgm with WT1$_{332\text{-}347}$ peptide-pulsed bone marrow-derived (BM)-DCs with a booster shot of WT1$_{332\text{-}347}$ peptide emulsified in CFA. In addition, to evaluate T-helper type 1 (Th1) cell-responses, which are important for the induction of potent anti-tumor immune responses [21-24], IFN-γ ELISPOT assay was performed to assess peptide-specific immune responses. As shown in Figure 5C, IFN-γ producing Th cells were increased in CD4$^+$ cells stimulated with peptide-pulsed L-DR4 cells compared with those stimulated with unpulsed L-DR4 cells. Therefore, BM-DC-based peptide immunization of Tgm in combination with a th-cell assay using IFN-γ ELISPOT is an effective way to screen TAA-derived and HLA-DR4-restricted Th-cell epitopes, especially if the peptides has low immunogenicity.

Using BM-DC-based peptide immunization, we sought to identify the Th-cell epitope in KIF20A, which is frequently overexpressed in gastric cancer [25], melanoma [26], lung cancer, pancreatic cancer [27], bladder cancer, breast cancer, and various other malignancies [28], and thus is a promising target for cancer immunotherapy [29]. The 24-mer KIF20A$_{494\text{-}517}$ peptide (TLHVAKFSAIASQLVHAPPMQLGF) consisting of overlapping 15-mer peptides with relatively high affinity binding to HLA-DR4 was predicted by a computer algorithm. In our recent study in humans, the KIF20A$_{494\text{-}517}$ peptide induced HLA-DR4-restricted Th-cell responses from PBMCs of an HLA-DR4-positive healthy donor [15].

To investigate the immunogenicity of the KIF20A$_{494\text{-}517}$ peptide in Tgm, mice were immunized with KIF20A$_{494\text{-}517}$ peptide-pulsed BM-DCs, and then with KIF20A$_{494\text{-}517}$ peptide $_{in}$ CFA emulsion. As shown in Figure 5D, Th-cells stimulated with KIF20A$_{494\text{-}517}$ peptide-$_{pulsed}$ L-DR4 cells showed a large number of IFN-γ positive spots compared with the Th-cells stimulated with unpulsed L-DR4.

Figure 4

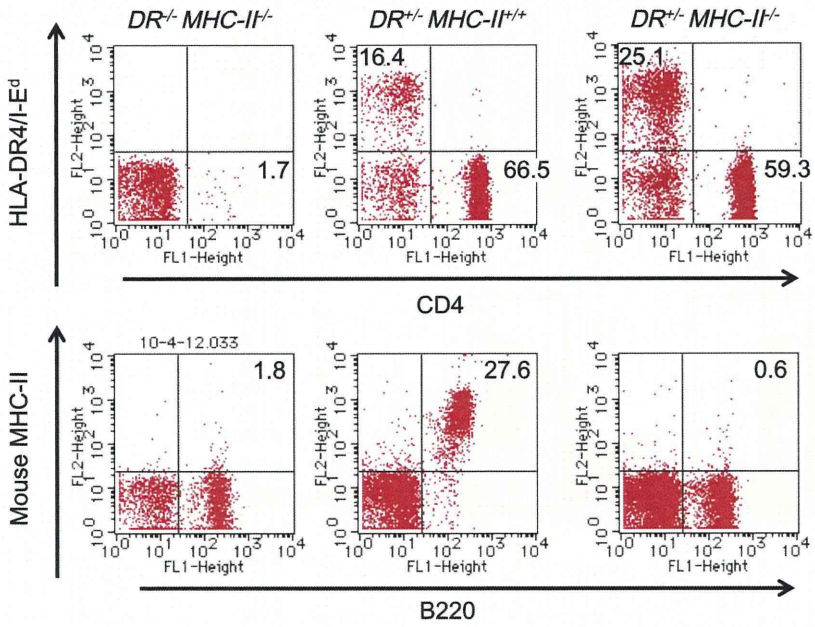

Figure 4. Chimeric HLA-DR4/I-E^d molecules induced the differentiation of mouse CD4$^+$ cells. (Upper panels) PBMCs (gated on lymphocytes) from $^{MHC-II}$ knock-out mice (DR$^{-/-}$MHC-II$^{-/-}$), Tgm (DR$^{+/-}$MHC-II$^{+/+}$) and Tgm that lack $^{MHC-II}$ (DR$^{+/-}$MHC-II$^{-/-}$) were stained with anti-HLA-DR and anti-mouse CD4 mAbs. (Lower panels) PBMC (gated on lymphocytes) from each mouse was stained with anti-B220 mAb and anti-mouse MHC-II mAb.

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Identification of a novel TAA-derived and HLA-DR4-restricted human Th-cell epitope using Tgm

DEP domain containing 1 (DEPDC1) is a novel TAA classified as cancer-testis antigen that is significantly overexpressed in a majority of bladder cancer specimens [30,31]. A clinical trial of DEPDC1-derived CTL epitope-vaccination was performed in six patients with advanced bladder cancers [32]. Four patients achieved stable disease or partial responses with the induction of CTL responses to DEPDC1$_{294-302}$ 9-mer peptide, while two cases were clinically non-responsive and negative for CTL responses. To induce more effective anti-tumor immune responses, we searched several candidate Th-cell epitope peptides in DEPDC1 using the computer algorithm and selected DEPDC1$_{191-213}$ 23-mer peptide (RYVILIYLQTILGVPSLEEVINP) consisting of overlapping 15-mer peptides with predicted high binding affinity to HLA-DR4. DEPDC1$_{60-85}$ 26-mer peptide (NSNFGPEVTRQQTIQLLRKFLKNHVI) consisting of overlapping 15-mer peptides with predicted relatively low binding affinity to HLA-DR4 was used as a control peptide. Immunization of DEPDC1$_{191-213}$ peptide emulsified in CFA and IFA in the tail base successfully induced the proliferation of CD4$^+$ cells from immunized Tgm even by *ex vivo* stimulation with the peptide, but not from WT mice (Figure 6A). Also, immunization of Tgm with control DEPDC1$_{60-85}$ peptide did not induce CD4$^+$ cell proliferation (Figure 6A), suggesting that the peptide with predicted low binding affinity to HLA-DR4 was less immunogenic than that with higher binding affinity. The responses observed in Tgm immunized with DEPDC1$_{191-213}$ peptide were blocked by the presence of anti-HLA-DR mAb (L243), while the presence of control immunoglobulin had no effect (Figure 6A). Thus, DEPDC1$_{191-213}$ peptide-specific Th-cell responses were HLA-DR4/I-E^d-restricted in Tgm.

Co-culture of CD4$^+$ cells from PMBCs of an HLA-DR4-positive healthy donor with DEPDC1$_{191-213}$ peptide-pulsed autologous CD14$^+$ cell-derived DCs and PBMCs induced anti-HLA-DR mAb-sensitive Th-cell responses to the peptide-pulsed L-DR4 cells but not to HLA-DR53-expressing L cells (L-DR53, Figure 6B). Thus, Tgm in combination with the computer algorithm-based analysis demonstrated its usefulness and effectiveness for the preliminary screening of HLA-DR4-restricted Th-cell epitopes.

Discussion

Many *HLA class II* Tgm have been established to identify Th-cell epitopes [33,34], as well as to analyze the pathogenesis of autoimmune diseases when susceptibility is associated with particular *HLA-II* alleles. In these Tgm, *HLA-II* alleles expressed in Tgm are those frequent in Caucasians but not in Japanese. To screen TAA-derived Th-cell epitopes for peptide-based cancer immunotherapy in as many Japanese as possible, we generated *HLA-DR4* Tgm expressing *HLA-*

Figure 5

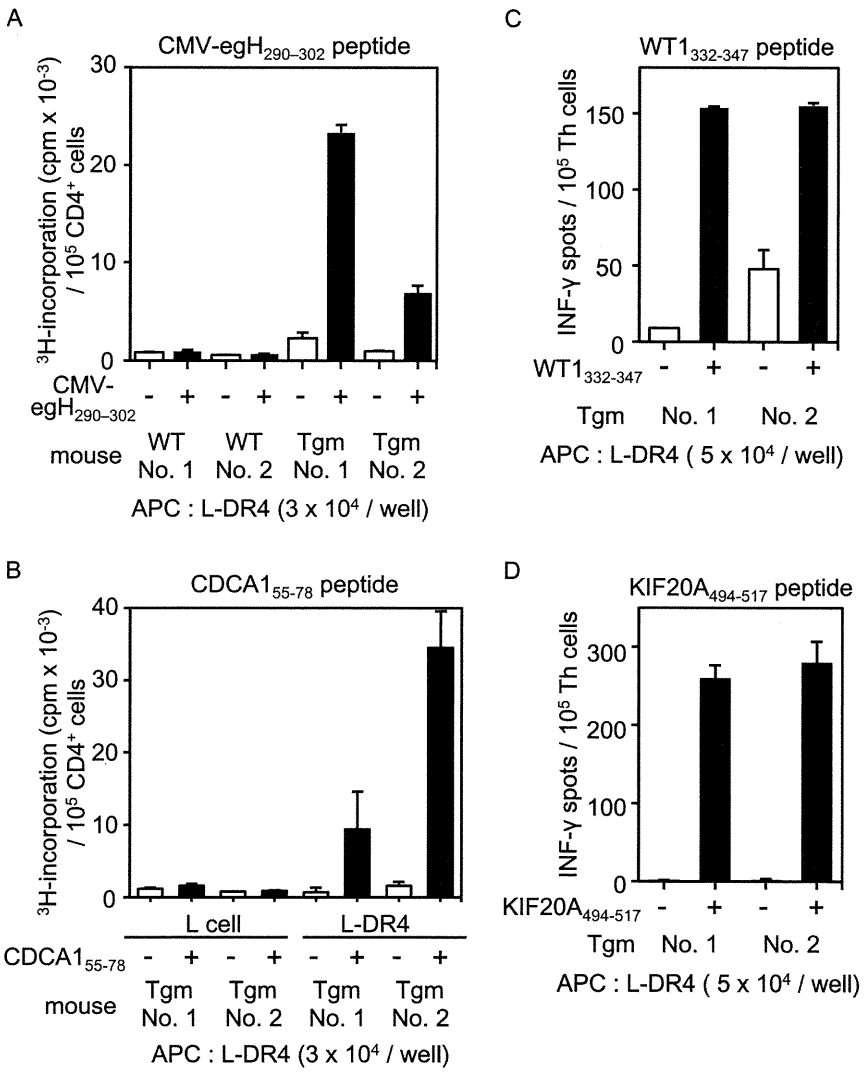

Figure 5. Immunization of TAA-derived peptides induced peptide-specific and HLA-DR4-restricted Th-cell responses in Tgm. Peptides emulsified in CFA and IFA were injected into the tail base of Tgm or C57BL/6 mice (WT) on day 0 and day 7. On day 14, the splenocytes were harvested and cultured *in vitro* for 7 days with the peptides (1 μg/ml). (A) Purified $CD4^+$ cells (1×10^5/well) were co-cultured with L-DR4 (3×10^4/well) pulsed with or without CMV-$egH_{290–302}$ peptide for 72 h and ^3H-thymidine uptake was measured. (B) Purified $CD4^+$ cells (1×10^5/well) were co-cultured with L cells (3×10^4/well) or L-DR4 (3×10^4/well) pulsed with or without $CDCA1_{55-78}$ peptide for 72 h and ^3H-thymidine uptake was measured. (C, D) Immunization of Tgm with syngeneic BM-DCs (5×10^5) pulsed with $WT1_{332-347}$ peptide (C) or $KIF20A_{494-517}$ peptide (D) followed by a booster shot of the peptide in CFA successfully induced IFN-γ production by Th cells (1×10^5/well) in response to the peptide-pulsed L-DR4 cells (5×10^4/well), but not to unpulsed L-DR4 cells.

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*DRA*01:01/HLA*DRB1*04:05* genes of which allele frequency is 13.6% in the Japanese population, that is, about one forth of Japanese have this HLA-DR molecule.

Although the overall amino acid sequence homology between human and mouse CD4 is about 80%, homology of the extracellular domain is only 55% and therefore, interspecies interactions between HLA-DR4 and mouse CD4 could be a potential problem for the induction of HLA-DR4-restricted mouse Th cells. To avoid this, Tgm were generated to express chimeric HLA-DR4/I-E^d molecules in which only the TCR-contacting and peptide-binding α1 and β1 domains were derived from HLA-DR4 but other-domains including CD4-interacting β2 domain were derived from I-E^d. The chimeric HLA-DR4/I-E^d molecules were successfully expressed on the

Figure 6

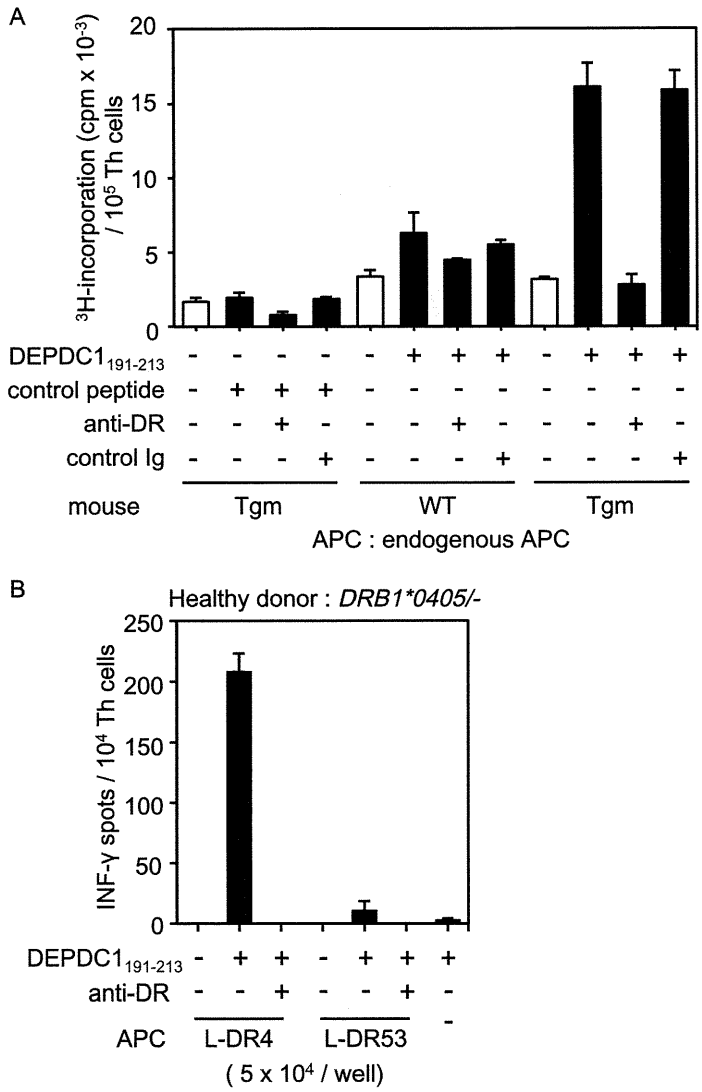

Figure 6. DEPDC1$_{191-213}$ peptide induced peptide-specific and HLA-DR4-restricted Th-cell responses in both Tgm and human PBMCs. (A) Tgm and C57BL/6 mice (WT) were immunized with DEPDC1$_{191-213}$ peptide or control peptide (DEPDC1$_{60-85}$) in CFA and IFA as described in Figure 6 legend. On day 14, inguinal lymph node cells were harvested and *ex vivo* ^{3}H-thymidine incorporation was measured. (B) Induced CD4$^+$ T cells (1 × 10^4/well) were co-cultured with peptide-pulsed L-DR4 (5 × 10^4/well) or L-DR53 cells (5 × 10^4/well) or unpulsed L-DR4 in the presence or absence of anti-HLA-DR blocking mAb L243. The CD4$^+$ T cells cultured with peptide only did not produce IFN-γ.

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surface of mouse L cells by co-transfection of the α and β transgenes as revealed by positive staining with anti-HLA-DR mAb, and L cell transfectants pulsed with WT1$_{332-347}$ peptide induced HLA-DR4-restricted IFN-γ-production and WT1$_{332-347}$ peptide-specific human Th cell clones *in vitro*. Conversely, mouse Th cells from Tgm immunized with WT1$_{332-347}$ peptide responded to HLA-DR4-expressing L-cells in a WT1$_{332-347}$ peptide-specific manner. Therefore, the chimeric HLA-DR4/I-E^d molecules were comparable with intact HLA-DR4 molecules when stimulating WT1$_{332-347}$ peptide-specific human or mouse Th-cell responses.

Although mice lacking endogenous I-A^b expression had very few, if any, CD4$^+$ T cells in the periphery, HLA-DR4/I-E^d expression without endogenous I-A^b expression restored a fraction of CD4$^+$ T cells in mice, comparable to mice expressing both endogenous I-A^b and HLA-DR4/I-E^d molecules. This indicated that chimeric HLA-DR4/I-E^d molecules were functionally equivalent to I-A^b molecules as they could

positively select $CD4^+$ thymocytes. Since the transgenes contained endogenous I-$E^d\alpha$ and I-$E^d\beta$ promoter regions spanning 3.2 kb and 5.2 kb of 5'-untranslated regions, respectively, the expression of HLA-DR4/I-E^d was expected to be cell type- and organ-specific similar to the endogenous I-A^b. Of total PBMCs from Tgm, more than 80% of B220-positive cells and approximately 90% of endogenous I-A^b-positive cells were positive for HLA-DR4/I-E^d, confirming the correct cell type-specific expression of the transgenes.

For FISH analyses, fluorescence-labeled α and β transgenes were used as probes to detect the positions of inserted transgenes. Both transgenes were co-localized in the telomeric H2-H4 region of chromosome 3 in line #5 Tgm or in the B region of the Y chromosome in line #7 Tgm with higher fluorescence intensity compared to the faint fluorescence detected on chromosome 17 where the mouse MHC region exists. This indicated that many transgenes were tandemly inserted in both Tgm lines. Nonetheless, the levels and cell-type specificity of expression of HLA-DR4/I-E^d and endogenous I-A^b were comparable between these two Tgm lines by the flow-cytometric analyses.

CMV-$egH_{290–302}$ peptide is a cytomegalovirus envelope glycoprotein H (egH)-derived peptide reported to bind to HLA-DR4 and induced CMV-$egH_{290-302}$ peptide-specific Th-cell responses[12]. Thus, CMV-$egH_{290–302}$ peptide is a natural HLA-DR4 binding Th-cell epitope that could be used as a positive control to induce specific and HLA-DR4-restricted Th-cell responses in Tgm. As expected, immunization of CMV-$egH_{290–302}$ peptide emulsified with CFA and IFA induced HLA-DR4-restricted and peptide-specific Th-cell responses. Again, mouse Th cells restricted by HLA-DR4/I-E^d molecules responded to CMV-$egH_{290–302}$ peptide presented by intact HLA-DR4, indicating that HLA-DR4/I-E^d and HLA-DR4 were interchangeable and that Tgm could be used for screening and identification of HLA-DR4-restricted Th-cell epitope peptides.

Since the $CDCA1_{55-78}$ peptide was predicted to be a strong HLA-DR4 binder but is a non-self peptide in mice, it was a good immunogenic peptide to induce mouse Th-cell responses in Tgm. A similar protocol to that used for CMV-$egH_{290–302}$ peptide immunization induced $CDCA1_{55-78}$ peptide-specific and HLA-DR4-restricted Th-cell responses. However, the $WT1_{332-347}$ peptide has an identical amino acid sequence to the mouse ortholog peptide, and immunization with CFA and IFA adjuvants was not successful, while immunization using peptide-pulsed DC was effective for the induction of peptide-specific and HLA-DR4-restricted Th-cell responses. Therefore, to screen the computer algorithm-predicted peptides that were expected to be less immunogenic for mice, the utilization of DCs prepared from syngeneic BM-DCs could be an alternative protocol for immunization. The $KIF20A_{494-517}$ peptide includes a 15-mer amino acid residue predicted to bind to HLA-DR4 with low affinity, and there are differences in three amino acid residues between the human peptide and mouse ortholog. However, the predicted core binding amino acid sequences to HLA-DR4 are similar between humans and mice (AKFSAIASQ vs AKFSALASQ), in which the only different amino acid residue, P6, is not in contact with the TCR but MHC-II. Thus, the $KIF20A_{494-517}$ peptide could be less immunogenic in mice to induce apparent Th-cell responses presented by HLA-DR4/I-E^d. However, immunization of $KIF20A_{494-517}$ $peptide_{-pulsed}$ BM-DCs followed by a booster shot using the same peptide in CFA emulsion successfully induced peptide-specific and HLA-DR4-restricted Th-cell responses. Therefore, the screening efficiency could be improved if peptide-pulsed DCs were used for immunization.

The $DEPDC1_{191-213}$ peptide has a different amino acid sequence to the mouse ortholog peptide (RYVILIYLQTILGVPSLEEVINP vs RYVIMIYLQTILSLPSIEELLNP), thus immunization using CFA and IFA as adjuvants was successful. DEPDC1 is over-expressed in various cancers, especially in bladder cancer and a clinical trial using DEPDC1-derived short CTL epitope peptide-vaccination was performed for 6 patients with advanced bladder cancer [30-32], although the clinical response was marginal. In a mouse model, co-vaccination of CTL- and Th-epitope peptides from HER-2 increased the numbers of specific CTL and Th cells and decreased the number of regulatory T cells compared with CTL-epitope vaccination alone [35]. In other clinical trials, Slingluff and colleagues recently reported that combination of CTL epitope- and Th cell epitope-vaccination in melanoma patients induced clinical benefit compared with CTL epitope-vaccination alone [36], and Woods and Cebon argued that tumor-specific T-cell help was associated with improved survival in melanoma [37]. Thus, co-vaccination of the DEPDC1 CTL peptide and Th epitope identified by using Tgm may induce additional or synergistic immune responses and clinical effects for bladder cancer patients.

In conclusion, the HLA-DR4 Tgm established in this study are useful for preliminary screening of HLA-DR4-restricted Th-cell epitope peptides derived from TAAs among candidate peptides predicted to bind to HLA-DR4 by the computer algorithm. If the Th cells from Tgm immunized with TAA peptides were reactive to syngenic BM-DCs incorporated whole TAA protein, the screened peptides could be proven to be naturally processed and such experimental system could be a powerful tool for the identification of Th-cell epitope peptides that could be applicable for vaccines for cancer immunotherapy.

Supporting Information

Figure S1. Chromosomal localization of transgene insertion site and Cell-type specific expression of HLA-DR4/I-E^d (line #7 Tgm). (A) Mapping of transgene insertion by FISH revealed integration in chromosome Y, B-region in line #7 Tgm. (B) PBMCs from line #7 Tgm were stained with anti-HLA-DR and anti-B220 mAbs (left) or anti-HLA-DR and anti-I-A^b mAbs (right). Numbers indicate the percentage of HLA-DR4/I-E^d-positive cells in B220-positive cells and MHC-II-positive cells indicated by the red boxes, respectively (gated on lymphocytes).
(TIF)

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Author Contributions

Conceived and designed the experiments: JY AI YN. Performed the experiments: JY AI KH YM NT FF. Analyzed the data: JY AI HT S. Senju YN. Contributed reagents/materials/analysis tools: YT AY MH MAS IS S. Sogo HS ME. Wrote the manuscript: JY AI YN.

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Identification of CDCA1-derived long peptides bearing both CD4$^+$ and CD8$^+$ T-cell epitopes: CDCA1-specific CD4$^+$ T-cell immunity in cancer patients

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We recently identified a novel cancer-testis antigen, cell division cycle associated 1 (CDCA1) using genome-wide cDNA microarray analysis, and CDCA1-derived cytotoxic T lymphocyte (CTL)-epitopes. In this study, we attempted to identify CDCA1-derived long peptides (LPs) that induce both CD4$^+$ helper T (Th) cells and CTLs. We combined information from a recently developed computer algorithm predicting HLA class II-binding peptides with CDCA1-derived CTL-epitope sequences presented by HLA-A2 (*A*02:01*) or HLA-A24 (*A*24:02*) to select candidate CDCA1-LPs encompassing both Th cell epitopes and CTL-epitopes. We studied the immunogenicity of CDCA1-LPs and the cross-priming potential of LPs bearing CTL-epitopes in both human *in vitro* and HLA-class I transgenic mice *in vivo*. Then we analyzed the Th cell response to CDCA1 in head-and-neck cancer (HNC) patients before and after vaccination with a CDCA1-derived CTL-epitope peptide using IFN-γ enzyme-linked immunospot assays. We identified two CDCA1-LPs, CDCA1$_{39-64}$-LP and CDCA1$_{55-78}$-LP, which encompass naturally processed epitopes recognized by Th cells and CTLs. CDCA1-specific CTLs were induced through cross-presentation of CDCA1-LPs *in vitro* and *in vivo*. In addition, CDCA1-specific Th cells enhanced induction of CDCA1-specific CTLs. Furthermore, significant frequencies of CDCA1-specific Th cell responses were detected after short-term *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with CDCA1-LPs in HNC patients (CDCA1$_{39-64}$-LP, 74%; CDCA1$_{55-78}$-LP, 68%), but not in healthy donors. These are the first results demonstrating the presence of CDCA1-specific Th cell responses in HNC patients and underline the possible utility of CDCA1-LPs for propagation of both CDCA1-specific Th cells and CTLs.

Tumor Immunology

We recently used genome-wide cDNA microarray analysis to identify a novel cancer testis antigen, cell division cycle associated 1 (CDCA1), which is frequently overexpressed in lung cancer, head-and-neck cancer (HNC), and various other malignancies.[1,2] We also identified highly immunogenic CDCA1-derived short peptides (SPs) that can induce HLA-A2 (*A*02:01*)-restricted CTLs from peripheral blood mononuclear cells (PBMCs) of lung cancer patients.[1] It is known

Key words: helper T-cell epitope, CDCA1, cancer testis antigen, cross-priming, head and neck cancer
Abbreviations: APCs: antigen-presenting cells; CDCA1: cell division cycle associated 1; CTL: cytotoxic T lymphocyte; DC: dendritic cell; ELISPOT: enzyme-linked immunospot; HD: healthy donor; HLA: human leukocyte antigen; HNC: head and neck cancer; IL: interleukin; LP: long peptide; mAb: monoclonal antibody; PBMCs: peripheral blood mononuclear cells; SP: short peptide; Tgm: transgenic mice; Th: CD4+ helper T; Th1: T-helper type 1
Additional Supporting Information may be found in the online version of this article.
*Y.T. and A.Y. contributed equally to this work.
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What's new?

Tumor immunotherapy often focuses on the induction of cancer-specific cytotoxic T cells (CTLs). However, T helper cells (Th1) play a critical role in the efficient and long-lasting induction of functional antitumor CTLs. Here, the authors describe two new peptides derived from the cell division cycle associated 1 (CDCA1) tumor antigen that are efficient in stimulating both a Th1 and a CTL response through cross-presentation. The two peptides, $CDCA1_{39-64}$-LP and $CDCA1_{55-78}$-LP are longer than conventionally used vaccination peptides and elicited efficient T cell responses after in vitro stimulation of peripheral blood mononuclear cells isolated from head and neck cancer patients but not from healthy individuals. These findings will help refine clinical trials currently ongoing with CDCA1 peptide-based immunotherapy for lung, breast, prostate and head and neck cancer patients.

that CDCA1 has a specific function at the kinetochores to stabilize microtubule attachment in cancer cells and CDCA1 is involved in the process of carcinogenesis. The patients with lung cancer strongly expressing CDCA1 showed poorer prognosis in comparison to patients with low CDCA1 expression.[2] Therefore, CDCA1 is an attractive target molecule for cancer immunotherapy. Phase I/II clinical trials of cancer immunotherapy for lung cancer, HNC, breast cancer and prostate cancer using CDCA1-derived CTL-epitopes are underway.

Tumor-specific $CD4^+$ helper T (Th) cells, particularly T-helper type 1 (Th1) cells, play a critical role in efficient induction of CTL-mediated antitumor immunity. IFN-γ produced by Th1 cells is critical for induction and maintenance of long-lived CTL responses through multiple interactions.[3–5] IFN-γ secreted by Th1 cells also mediates direct antitumor or anti-angiogenic effects.[6] Furthermore, Th cells pave the way for entry of CTLs at the tumor site.[7] Therefore, identification of Th-cell epitopes that can activate tumor-specific Th1 cells is important for induction of effective tumor immunity in tumor-bearing hosts.

Melief *et al.* recently reported a long peptide (LP) naturally including CTL-epitope as an attractive vaccine compound. Following injection of LP, a patient's dendritic cells (DC) take up the LP, process it and present all possible CTL-epitopes and Th-cell epitopes in the context of various HLA class I and class II molecules.[8] In addition, recent clinical studies using a promiscuous telomerase-derived helper-epitope vaccine called GV1001 bearing CTL-epitopes, increased survival of cancer patients when combined with radiotherapy and chemotherapy.[9,10] Thus, we proposed that an ideal peptide vaccine for cancer immunotherapy may be a single polypeptide containing epitopes for both Th1 cells and CTLs, which are naturally proximal to each other and can be induced simultaneously.[11]

In this study, a computer algorithm predicting HLA class II-binding peptides and known CTL-epitope sequences recognized by HLA-A24 or -A2-restricted CTLs were used to select candidate CDCA1-LPs encompassing both Th cell epitopes and the CTL-epitopes. Our results show that the predicted LPs triggered Th1 responses in individuals expressing several common *HLA-DR*, *-DP*, or *-DQ* alleles and that the efficient cross-presentation of the CDCA1-LPs induced CDCA1-specific CTLs.

Material and Methods

Patients

Blood samples were collected from 19 HNC patients enrolled in two peptide vaccine trials, and the immune responses of Th cells reactive to CDCA1-LPs were investigated. These phase I/II clinical trials of cancer immunotherapy using three HLA-A24-binding SPs, (clinical-grade 9–10 amino acids peptides) derived from CDCA1 (CDCA1-$A24_{56-64}$, reported in this study, Supporting Information Fig. S1), IMP-3 (IMP-3-$A24_{508-516}$) and LY6K (LY6K-$A24_{177-186}$)[12] were reviewed and approved by the Institutional Review Board of Kumamoto University, Kumamoto, Japan. Peptides (1 mg each antigen) are emulsified in 500 μL Montanide ISA51 and injected subcutaneously (s.c.) on days 0, 7, 14, 28, 42, 56, 63 and 70, then monthly until tumor progression or toxicity are observed. All HNC patients were selected based on HLA-A24 possession after providing written informed consent. The patients suffered from inoperable advanced HNC with recurrent or metastatic tumors and were resistant to standard therapy; they were enrolled in the trial under University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) number 000008379 (CTR-8379). HNC patients with radical resection were enrolled in the trial under UMIN-CTR number 000008380 (CTR-8380). In the latter trial, HNC patients were treated with postoperative peptide vaccine immunotherapy combined with S-1, ifosfamide, or doxorubicin. These clinical trials and analyses are ongoing.

Cell lines

Mouse fibroblast cell lines (L-cells), genetically engineered to express DR4 (*DRB1*04:05*), L-DR4; DR8 (*DRB1*08:03*), L-DR8; DR15 (*DRB1*15:02*), L-DR15; or DR53 (*DRB4*01:03*), L-DR53 were used to assess HLA-class II molecules involved in antigen presentation. The TAP-deficient and $HLA\text{-}A2^+$ T2 cells were purchased from Riken Cell Bank. The C1R-A2402 cells, an HLA-A24 transfectant of human B lymphoblastoid cell line expressing a trace amount of intrinsic HLA class I molecule were a gift from Dr. Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan). T2 cells and C1R-A2402 cells were used as target cells. These cells were maintained *in vitro* in DMEM (L-cells) or RPMI 1640 (T2 and C1R-A24 cells) supplemented with 10% FCS in a 5% CO_2 at 37°C.

Prediction of HLA class II-binding peptides

To predict possible HLA-class II binding human CDCA1-derived LPs, the amino acid sequence of the human CDCA1 protein was analyzed by a recently developed computer algorithm (IEDB analysis resource, consensus method, http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html).[13,14] The program analyzed 15 amino-acid-long sequences offset to encompass the entire protein. The 24 and 26 amino-acid-long peptides, $CDCA1_{39-64}$-LP (NPKPEVLHMIYMRALQIVYGIRLEHF) and $CDCA1_{55-78}$-LP (IVYGIRLEHFYMMPVNSEVMYPHL), with overlapping high consensus percentile ranks for multiple HLA-class II molecules encoded by *DRB1*04:05*, *DRB1*15:02*, or *DPB1*02:01* alleles and that encompass CDCA1-derived 9-mer CTL epitopes ($CDCA1\text{-}A24_{56-64}$ and $CDCA1\text{-}A2_{65-73}$) were selected (Supporting Information Fig. S2 and Supporting Information Table S1). Because $CDCA1_{55-78}$-LP encompasses the sequences of both $CDCA1\text{-}A2_{65-73}$ SP and $CDCA1\text{-}A24_{56-64}$ SP, we tested only $CDCA1_{55-78}$-LP in some experiments.

Synthetic peptides and recombinant proteins

Three human CDCA1-derived SPs presented by HLA-A2 ($CDCA1\text{-}A2_{65-73}$ and $CDCA1\text{-}A2_{351-359}$)[1] or HLA-A24 ($CDCA1\text{-}A24_{56-64}$) and two overlapping LPs ($CDCA1_{55-78}$-LP and $CDCA1_{39-64}$-LP) were synthesized (Biomatik, Canada; purity >90%). Two human immunodeficiency virus (HIV)-SPs that bind to HLA-A24 (HIV-A24) and HLA-A2 (HIV-A2) were used as negative control SPs.[15,16] A WT1-derived LP presented by HLA-DR4 (WT1-peptide) and a promiscuous HIV-derived LP were used as negative control LPs.[17,18] Peptides were dissolved in dimethylsulfoxide at 10 μg/μL, and stored at −80°C. The recombinant whole CDCA1 protein and truncated CDCA1 protein lacking both CDCA1-derived Th epitopes recognized by $CDCA1_{55-78}$-LP- and $CDCA1_{39-64}$-LP-specific Th cells were expressed by *Escherichia coli* BL21 with a pET28a vector (Novagen). The truncated CDCA1 protein was used as a control. Each recombinant protein was purified and assessed by SDS-PAGE.

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

The research protocol for collecting and using PBMCs from healthy donors was approved by the Institutional Review Board of Kumamoto University. We obtained PBMCs from 12 healthy donors with written informed consent. We isolated the PBMCs from the heparinized blood of Japanese healthy donors by means of Ficoll-Conray density gradient centrifugation. Genotyping of *HLA-A*, *DRB1* and *DPB1* was performed at the HLA Laboratory (Kyoto, Japan; Supporting Information Table S2). With some modifications, induction of antigen-specific $CD4^+$ T-cells was performed as described previously.[19] $CD4^+$ T-cells were purified from PBMCs by positive selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA). Monocyte-derived dendritic cells (DCs) were generated from $CD14^+$ cells by *in vitro* culture, as described previously,[1] and used as antigen-presenting cells (APCs) to induce antigen-specific $CD4^+$ T-cells. DCs (1×10^4/well) were pulsed with 10 μg/mL LP for 3 hr and irradiated (45 Gy), and subsequently mixed with $CD4^+$ T-cells (3×10^4/well) in 200 μL AIM-V supplemented with 5% human decomplemented plasma in each well of a 96-well, flat-bottomed culture plate. After 7 days, half of the medium was removed from each culture, and fresh medium (100 μL/well) containing irradiated (50 Gy) autologous PBMCs (1×10^5) pulsed with peptide (10 μg/mL) and 5 ng/mL recombinant human interleukin 7 (rhIL-7) was added. Two days after the second stimulation with peptide, rhIL-2 was added to each well (10 IU/mL). A week later, the stimulated $CD4^+$ T-cells in each well were analyzed for specificity in IFN-γ ELISPOT assays. The T-cells showing a specific response to the cognate peptide were transferred to 24-well plates and restimulated at weekly intervals with irradiated autologous PBMCs (1×10^6/well) pulsed with the peptide in medium supplemented with rhIL-2 (20 IU/mL) and rhIL-7 (5 ng/mL). In some instances, T-cells were cloned by limiting dilution for further studies as described previously.[20]

Assessment of T-cell responses to peptides and proteins

The immune responses of T-cells to PBMC, L-cells and murine BM-DC pulsed with peptides (10 μg/mL) or CDCA1 proteins-loaded human DCs (50 μg/mL) were assessed by IFN-γ enzyme-linked immunospot (ELISPOT) assays (BD Biosciences, San Jose, CA) according to manufacturer's instructions and described previously.[19] In briefly, peptide-pulsed PBMCs (3×10^4/well), L-cells (5×10^4/well), T2 cells (2×10^4/well), C1R-A24 cells (2×10^4/well), bone marrow-derived DCs (BM-DCs, 2×10^4/well), or protein-loaded DCs (5×10^3/well) were seeded in triplicate or duplicate in the ELISPOT plates as APCs or target cells. To determine the HLA molecules involved in antigen presentation, antigen-induced IFN-γ production was blocked by adding anti-HLA-DR monoclonal antibody (mAb) (L243, BioLegend), anti-HLA-DP mAb, (B7/21, Abcam), anti-human HLA-DQ mAb (SPV-L3, Abcam), or anti-HLA class I mAb, (W6/32, Abcam) after seeding APCs or target cells. All mAbs were used at a final concentration of 5 μg/mL. The APCs or target cells were incubated with mAbs for 1 hr at room temperature. Then, the responder T-cells were harvested, washed and transferred to the ELISPOT plate in the indicated number of the figures. After incubation for 18 hr, spot numbers were counted. HIV-A2, HIV-A24, or WT1-derived LP were used as negative control peptides. In some experiments, unpulsed PBMCs or L-cells were used as negative controls. Cells cultured with PMA (100 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Sigma-Aldrich) were used as positive controls in all assessments of IFN-γ ELISPOT assays. Results are presented as means ± SD.

In the ELISPOT assays for HNC patients, after 1 week of cell culture with CDCA1-LPs, the cells were collected, washed

and cultured in ELISPOT plates (1×10^5/well) with $CDCA1_{55-78}$-LP, $CDCA1_{39-64}$-LP, or control LP (HIV-LP) for 18 hr. The number of CDCA1-LP-specific Th cells expressed as spot-forming cells/10^5 cells was calculated after subtracting control values (background). Responses were scored as positive when the mean number of IFN-γ spots numbered more than 15 and more than twofold over background. The ELISPOT assays on HNC patients' cells were conducted in single, duplicate, or triplicate wells because of the limited number of available cells.

Propagation of CDCA1-$A24_{56-64}$ SP-specific CTLs by stimulation with $CDCA1_{55-78}$-LP in healthy donor

Induction of CDCA1-$A24_{56-64}$ SP-reactive CTLs by stimulation of purified $CD8^+$ T-cells with CDCA1-$A24_{56-64}$ SP was performed as described previously.[16,21] To assess the expansion capacity of CDCA1-$A24_{56-64}$ SP-specific CTLs by stimulation with $CDCA1_{55-78}$-LP-pulsed DCs, the CDCA1-$A24_{56-64}$ SP-specific bulk CTLs obtained from an HLA-$A24^+$ donor (HD2; 2×10^6/well, 24-well plates) were stimulated with 16 μM $CDCA1_{55-78}$-LP or control-LP-pulsed autologous DCs (2×10^5/well). The LP-pulsed mature DCs (3 hr) were irradiated and washed, then used as antigen-presenting cells (APCs). On days 1 and 7, rh IL-2 (20 IU/mL) and rhIL-7 (5 ng/mL) were added. Before LP stimulation (day 0) and on days 5, 7, 8 and 10 after stimulation, an aliquot of cultured cells (1×10^5 cells) was stained with a PE-labeled tetramer of the HLA-A24(*A*24:02*)/CDCA1-$A24_{56-64}$-complex (MBL, Nagoya, Japan) with an FITC-labeled anti-human CD8 mAb (clone T8, Beckman Coulter, Brea, CA). A PE-labeled tetramer of the HLA-A24 (*A*24:02*)/HIV-A24 (RYLRDQQLL) complex was used as negative control. In this experiment, we tested only $CDCA1_{55-78}$-LP.

Expansion of CDCA1-$A24_{56-64}$ SP-specific CTLs by stimulation with CDCA1-LPs in HNC patients vaccinated with CDCA1-$A24_{56-64}$ SP

PBMCs from 5 HNC patients (HNC26, 29, 31, 39, 109) vaccinated with CDCA1-$A24_{56-64}$ SP were cultured with a mixture of $CDCA1_{55-78}$-LP and $CDCA1_{39-64}$-LP (10 μg/mL each) in a 24-well plate (2×10^6/well); rhIL-2 and rhIL-7 were added on day 0 and day 2. On day 0 (*ex vivo*) and day 7, the PBMCs were stained with HLA-A24/$CDCA1_{56-64}$ tetramer.

In vitro cross-presentation assay

An HLA-$A24^+$ donor (HD2)-derived DCs were kept alive or fixed for 3 min in 0.1% glutaraldehyde (Sigma-Aldrich), pulsed with peptides (16 μM) for 3 hr, and washed three times. OK432 (0.1 Klinische Einheit (KE)/mL, Chugai Pharmaceutical Co, Tokyo, Japan) was added to induce the maturation of DCs during and after the peptide pulse. CDCA1-$A24_{56-64}$-reactive bulk CTLs were added at a 2:1 ratio for 6 hr in medium containing 10 μg/mL brefeldin A (Sigma-Aldrich). The brefeldin A was added to inhibit protein secretion during the stimulation. IFN-γ production by the CDCA1-$A24_{56-64}$-specific CTLs was measured by intracellular labeling. The cells were stained with a FITC-labeled anti-human IFN-γ mAb (BioLegend) in combination with a PerCP-labeled anti-human CD8 mAb (BioLegend) and a PE-labeled HLA-A24/$CDCA1_{56-64}$ tetramer. Data acquisition was performed on a FACSCalibur (BD Biosciences) and data files were analyzed with FlowJo software (Tree Star, Ashland, OR).

Induction of human CDCA1-specific CTLs by stimulation with $CDCA1_{55-78}$-LP

To assess the induction of CDCA1-$A2_{65-73}$ SP or CDCA1-$A24_{56-64}$ SP-reactive CTLs by cross-presentation of $CDCA1_{55-78}$-LP by DCs, the DCs generated from HLA-A2 or A24-positive donors (HD1 and HD5) were used as APCs. Stimulation of purified human $CD8^+$ T-cells with LP-pulsed DCs (20 μg/mL) was performed as described previously.[21,22] After three rounds of stimulation of $CD8^+$ T-cells with $CDCA1_{55-78}$-LP-pulsed DCs, the number of IFN-γ producing $CD8^+$ T-cells in response to stimulation with the CDCA1-$A2_{65-73}$ SP-pulsed T2 cells or CDCA1-$A24_{56-64}$ SP-pulsed C1R-A2402 cells was counted by an IFN-γ ELISPOT assay. In this experiment, we tested only $CDCA1_{55-78}$-LP.

In vivo cross-priming assay

HLA-A2 (HHD) and HLA-A24 (HHH) transgenic mice (Tgm) were kindly provided by Dr. F.A. Lemonnier.[23,24] Mice were intradermally injected at the base of the tail with CDCA1-LP (HLA-A2 Tgm, 50 μg/mouse; HLA-A24 Tgm 100 μg/mouse) emulsified in incomplete Freund's adjuvant (IFA) at 7-day intervals. Seven days after the second or third vaccination with CDCA1-derived LPs, $CD8^+$ T-cells were isolated from inguinal lymph nodes by positive selection with magnetic microbeads (Miltenyi Biotec, Auburn, CA). The number of IFN-γ producing $CD8^+$ T-cells in response to stimulation with SP-pulsed BM-DCs or C1R-A2402 cells was counted by *ex vivo* ELISPOT assay.

The synergistic effect of $CDCA1_{55-78}$-LP on induction of CDCA1-specific CTLs

PBMCs obtained from HLA-$A2^+$/$DR4^+$ HD1, from whom the $CDCA1_{55-78}$-LP-specific Th cell clones (Th-clone) were generated, were plated in 24-well plates (3×10^6/well), followed by addition of SP alone (CDCA1-$A2_{351-359}$, 20 μg/mL), SP + LP ($CDCA1_{55-78}$-LP, 20 μg/mL), SP + Th-clone (5×10^5/well), or SP + LP + Th-clone in a final volume of 2 mL. After culture for 7 days, these peptides and IL-2 (20 U/mL) were added, then IL-15 (5 ng/mL) was added on day 9. On day 11, cells were stained with a PE-labeled tetramer of the HLA-A2(*A*02:01*)/CDCA1-$A2_{351-359}$-complex with an FITC-labeled anti-human CD8 mAb. Lytic activity was tested in standard chromium release assays.[25,26] In this experiment, we tested only $CDCA1_{55-78}$-LP.

Assessment of CDCA1-LP specific CD4$^+$ T-cell responses in HNC patients immunized with CDCA1-A24$_{56-64}$ SP

We isolated the PBMCs from the heparinized blood of HNC patients by means of Ficoll-Conray density gradient centrifugation. Fresh PBMCs from HNC patients or healthy donors were cultured with a mixture of CDCA1$_{39-64}$-LP and CDCA1$_{55-78}$-LP (10 μg/mL each) in a final volume of 2 ml AIM-V supplemented with 5% human decomplemented plasma at 37°C (2 × 10^6/well, 24-well plates); IL-2 and IL-7 were added on day 0 and day 2. After 1 week of cell culture, the number of antigen-specific IFN-γ producing T-cells was counted by ELISPOT assay. This study was conducted in a laboratory that operates under exploratory research principles, and was performed using investigative protocols. We acknowledge the recommendations of the Minimal Information About T-cell Assay (MIATA) reporting framework for human T-cell assays.[27]

Statistical analysis

Data were compared by the two-tailed Student's *t*-test (bar graphs), Fisher's exact test, or nonparametric Mann–Whitney *U* test (scatter-dot graph). Differences with a p value < 0.05 were considered statistically significant for all tests.

Results

CDCA1-A24$_{56-64}$ SP induce HLA-A24-restricted CTLs

We recently identified two highly immunogenic CDCA1-derived HLA-A2-restricted CTL-epitopes. These peptides-specific CTLs generated from lung cancer patients effectively killed the cancer cells naturally expressing both CDCA1 and HLA-A2.[1] Next, we identified a new HLA-A24-restricted CTL-epitope, CDCA1-A24$_{56-64}$ SP (Supporting Information Fig. S1). Detailed methods are provided in Supporting Information and Methods. Purified HLA-A24-positive CD8$^+$ T-cells were stimulated with autologous DCs pulsed with CDCA1-A24$_{56-64}$ SP. After three rounds of stimulation with CDCA1-A24$_{56-64}$ SP, peptide-specific IFN-γ production was analyzed by IFN- γ enzyme-linked immunosorbent assay as described previously.[28] We established CDCA1-A24$_{56-64}$ SP-specific bulk CTL lines and CTL clones from three healthy donors. The CTL clones specifically responded to COS7 cells genetically engineered to express HLA-A24 and CDCA1, but did not respond to COS7 cells expressing either HLA-A24 or CDCA1, suggesting CDCA1-A24$_{56-64}$ SP is naturally processed and presented by HLA-A24.

These results suggest CDCA1-derived CTL-epitopes would be applicable for peptide-based cancer immunotherapy, and we have started phase I/II clinical trials of peptide vaccine for HNC, lung cancer, breast cancer and prostate cancer using CDCA1-derived HLA-A2 and HLA-A24-restriced CTL-epitopes in Europe and Japan respectively.

Prediction and selection of possible HLA class II-binding CDCA1-LPs encompassing both Th cell epitopes and CTL-epitopes

To identify possible immunogenic CDCA1-LPs that can induce Th cells, we first examined the amino acid sequence of CDCA1 using a recently developed computer algorithm (Supporting Information Fig. S2*a* and Supporting Information Table S1).[13,14] One region, CDCA1$_{39-78}$-peptide, predicted by the computer algorithm to be a potent HLA class II-binding peptide, was identified proximal to the 9-mer HLA-A2 or HLA-A24-restricted CTL-epitopes (Supporting Information Fig. S2*b*). Therefore, 2 candidate overlapping LPs, CDCA1$_{39-64}$-LP and CDCA1$_{55-78}$-LP, predicted to have strong binding affinity to HLA-class II molecules HLA-DR4, HLA-DR15 and HLA-DP2 (*DPB1*02:01*), were synthesized for subsequent analyses.

Identification of immunogenic CDCA1-derived LPs encompassing Th-cell epitopes

CD4$^+$ T-cells isolated from PBMCs of healthy donors were stimulated at weekly intervals with autologous DCs and PBMCs pulsed with CDCA1$_{55-78}$-LP. After at least three rounds of stimulation, CDCA1$_{55-78}$-LP-specific responses of cultured CD4$^+$ T-cells were examined by IFN-γ ELISPOT assays. In an HLA-DR4-positive healthy donor (HD1), the generated Th cells produced a significant amount of IFN-γ in response to CDCA1$_{55-78}$-LP-pulsed PBMCs in an HLA-DR-dependent manner. The bulk Th cells specifically recognized L-DR4 cells pulsed with CDCA1$_{55-78}$-LP in an HLA-DR-dependent manner, but not irrelevant peptide-pulsed L-DR4 cells or CDCA1$_{55-78}$-LP-pulsed L-DR53 cells (Fig. 1*a*). The similar results were obtained from other two DR4$^+$ donors (Supporting Information Table S2; HD4 and HD5). These results suggest that CDCA1$_{55-78}$-LP encompasses an HLA-DR4-restricted Th cell epitope.

To investigate whether CDCA1$_{55-78}$-LP induces responses in Th cells restricted by other HLA class II molecules, CD4$^+$ T-cells from HLA-DR4-negative healthy donors were tested. We confirmed that CDCA1$_{55-78}$-LP generates HLA-DR15-restricted Th cells (Fig. 1*b*). CDCA1$_{55-78}$-LP also generates HLA-DP2-restricted Th cells (Supporting Information Fig. S3*a*). L-cells transduced with *HLA-DP2* were unavailable; therefore, we established a CDCA1$_{55-78}$-LP-reactive Th cell clone (Th-clone) and used allogeneic PBMCs from five different donors as APCs to determine restriction by shared HLA-DP molecules. Thus, CDCA1$_{55-78}$-LP binds to HLA-DR4, HLA-DR15 and HLA-DP2, which suggest that CDCA1$_{55-78}$-LP encompasses Th cell epitopes presented by frequent HLA class II molecules in the Japanese/Pacific-Asian populations.[29,30]

Next, we assessed and confirmed that CDCA1$_{39-64}$-LP can generate HLA-DR9 and HLA-DR15-restricted Th cells using the method described above (Supporting Information Fig. S3*b* and Fig. 1*c*). Taken together, these results clearly demonstrate these overlapping-LPs can stimulate HLA-DR4, -DR9, -DR15 and -DP2-restricted Th cells. In this study, CDCA1-LP-specific Th cells generated from healthy donors did not respond to the CDCA1-A24$_{56-64}$ SP embedded in CDCA1-LPs (Fig. 1*a*–1*c*, left panels).

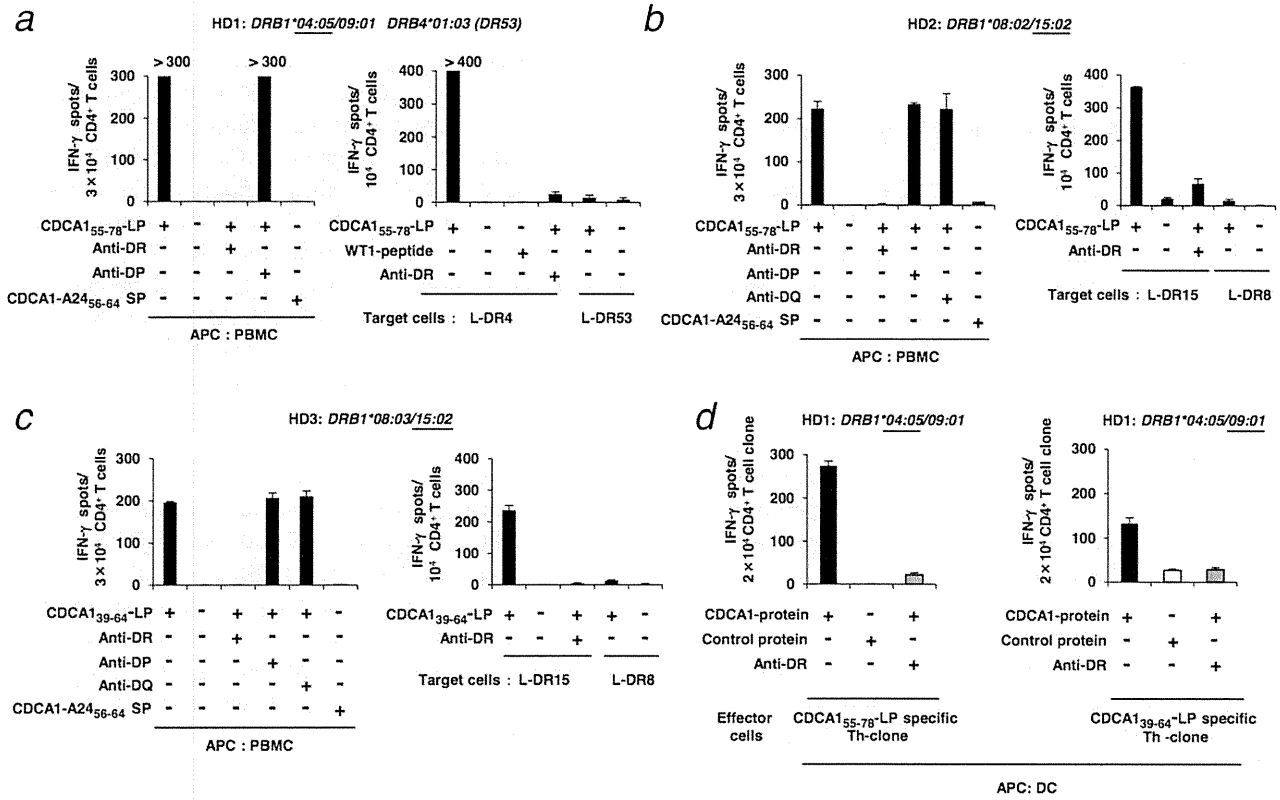

Figure 1. Induction of CDCA1-specific Th cells from healthy donors. *a*: CDCA1-specific Th cells were generated from a DR4$^+$ healthy donor (HD1) by stimulation with CDCA1$_{55-78}$-LP. The generated Th cells were re-stimulated with autologous PBMCs or L-cells pulsed with CDCA1$_{55-78}$-LP. A WT1-peptide was used as a control peptide. The number of IFN-γ-producing Th cells was analyzed by ELISPOT assay. Representative data from at least three independent experiments with similar results obtained from HD1 are shown. The similar results were obtained from other two DR4$^+$ donors (Supporting Information Table S2; HD4 and HD5). The HLA class-II genotype of donor HD1 is indicated above the panels. The underlined HLA-class II alleles encode HLA-class II-molecule presenting the peptides to Th cells. Blocking effect by HLA-DQ mAb was not tested in HD1 (*a*, left panel). *b*: CDCA1-specific Th cells were generated from a DR4-negative, DR15-positive healthy donor (HD2) by stimulation with CDCA1$_{55-78}$-LP. Representative data from at least five independent experiments with similar results are shown. *c*: CDCA1$_{39-64}$-LP-specific Th cells were generated from a DR15$^+$ healthy donor (HD3) by stimulation with CDCA1$_{39-64}$-LP. *d*: CDCA1-LPs encompass naturally processed Th cell epitopes. CDCA1$_{55-78}$-LP-specific HLA-DR4-restricted Th-clone (left panel), and CDCA1$_{39-64}$-LP-specific HLA-DR9-restricted Th clone (right panel) established from HD1 recognized autologous DCs loaded with recombinant CDCA1 protein. Representative data from at least three independent experiments with similar results are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CDCA1$_{55-78}$-LP and CDCA1$_{39-64}$-LP encompass naturally processed Th cell epitopes

We proceeded to assess whether DCs take up and process the CDCA1 protein to stimulate CDCA1-LP-specific Th cells. DCs loaded with recombinant CDCA1 protein were prepared and used as APCs in IFN-γ ELISPOT assays.[1,16] An HLA-DR4-restricted CDCA1$_{55-78}$-LP-reactive Th-clone efficiently recognized DC loaded with CDCA1 protein in an HLA-DR-dependent manner, but did not recognize control protein-loaded DC, indicating CDCA1$_{55-78}$-LP encompasses naturally processed HLA-DR4-rstricted Th cell epitope (Fig. 1*d*, left panel). Similar analyses were performed using CDCA1$_{55-78}$-LP-specific HLA-DR15-restricted bulk Th cells, CDCA1$_{55-78}$-LP-specific HLA-DP2-restricted Th-clone (Supporting Information Fig. S4), and CDCA1$_{39-64}$-LP-reactive HLA-DR9-restricted Th-clone (Fig. 1*d*, right panel). These CDCA1-LP-specific Th cells recognized CDCA1 protein-loaded DCs and produced IFN-γ, confirming CDCA1-LPs encompass several naturally processed Th cell epitopes.

CDCA1$_{55-78}$-LP stimulates Th1-type CD4$^+$ T-cells

To further characterize CDCA1-LP-reactive Th cells, we used the Bio-Plex system to measure the levels of several cytokines released in response to stimulation by the cognate peptide. Detailed method is provided in Supporting Information Materials and Methods. CDCA1$_{55-78}$-LP-specific bulk Th cells from healthy donors (HD1, HD2, HD3 and HD4) produced a large amount of IFN-γ, TNF-α, GM-CSF and MIP-1β, but less IL-4 and IL-17 after restimulation with cognate peptide-pulsed L-DR4 or autologous PBMCs, indicating Th1 polarized Th cell characteristics (Supporting Information Fig. S5*a*–S5*d*). Interestingly, the CD107a as a marker for degranulation was detected on the CDCA1-LP-specific bulk Th cells stimulated with cognate peptide (Supporting Information Fig.

S5*e*–S5*h*; HD1, HD2 and HD3), as was previously demonstrated for antiviral $CD4^+$ effectors and tumor-infiltrating lymphocytes.[31–34]

CDCA1-LPs induce efficient expansion of CDCA1-$A24_{56-64}$ SP-specific CTLs.

Next, we assessed whether the CDCA1-LPs can induce expansion of CDCA1-specific bulk CTLs. CDCA1-$A24_{56-64}$ SP-specific bulk CTLs generated from purified $CD8^+$ T-cells of HD2 (HLA-$A24^+$/$DR15^+$) were cultured for 1 week with $CDCA1_{55-78}$-LP-pulsed autologous DCs. As shown in Figure 2*a*, the population of HLA-A24/$CDCA1_{56-64}$-$tetramer^+$ $CD8^+$ T-cells was expanded by stimulation with $CDCA1_{55-78}$-LP-pulsed DCs, but decreased when bulk CTLs were stimulated with control-LP-pulsed DCs. A similar result was obtained from HLA-$A24^+$/$DR4^+$ HD5 in IFN-γ ELSPOT assay (Supporting Information Fig. S6). Detailed method of this experiment is provided in Supporting Information Materials and Methods.

We also tested whether CDCA1-LPs could induce *in vitro* expansion of CDCA1-$A24_{56-64}$ SP-specific CTLs in the PBMCs of HNC patients vaccinated with CDCA1-$A24_{56-64}$ SP. PBMCs from vaccinated HNC patients were cultured with a mixture of $CDCA1_{55-78}$-LP and $CDCA1_{39-64}$-LP. When fresh PBMCs isolated from HNC29 were stained with a HLA-A24/$CDCA1_{56-64}$ tetramer before *in vitro* culture (*ex vivo*), the frequency of $tetramer^+$ cells was only 0.09% of $CD8^+$ T-cells. Interestingly, $tetramer^+$ $CD8^+$ T-cell in PBMCs from HNC29 were significantly expanded by 1-week *in vitro* stimulation of PBMCs with a mixture of $CDCA1_{55-78}$-LP and $CDCA1_{39-64}$-LP. The frequency of CDCA1-$A24_{56-64}$ SP-specific CTLs increased to 3.07% of $CD8^+$ T-cells (Fig. 2*b*; Day7). CDCA1-$A24_{56-64}$ SP-specific IFN-γ production was also detected when the cultured cells were stimulated with CDCA1-$A24_{56-64}$ SP (Fig. 2*b*; bar graph, Day7). The similar results were also obtained in HNC26, 31, 39 and 109 (Supporting Information Fig. S7). These results suggest expansion of CDCA1-$A24_{56-64}$ SP-specific CTLs may be induced by cross-presentation of CDCA1-LPs by DCs.

Cross-presentation of CDCA1-LPs efficiently primes CDCA1-specific $CD8^+$ T-cells *in vitro* and *in vivo*

The cross-presentation of CDCA1-LPs by DCs was evaluated in the context of the IFN-γ response of HLA-A24/$CDCA1_{56-64}$ $tetramer^+$ $CD8^+$ T-cells. Fixed DCs, unable to cross present but able to present CDCA1-$A24_{56-64}$ SP as efficiently as live DCs (Fig. 2*c*, Fixed DC + SP), were used to exclude or evaluate the contribution of exogenous presentation of LP degradation products in the T-cell response. $CDCA1_{55-78}$-LP (Fig. 2*c*) and $CDCA1_{39-64}$-LP (Supporting Information Fig. S8) induced a significant proportion of IFN-γ secreting $tetramer^+$ $CD8^+$ T-cells only when they were cross presented by unfixed DCs (DC + LP). CDCA1-LP-pulsed fixed DCs could not stimulate CDCA1-$A24_{56-64}$-specific CTLs, similar to irrelevant LP-pulsed unfixed DCs (Fixed DC + LP and DC + control LP).

To investigate the cross-priming of CDCA1-specific CTLs, we examined whether $CDCA1_{55-78}$-LP-loaded DCs could induce CDCA1-$A2_{65-73}$ SP-specific CTLs by cross-presentation of the $CDCA1_{55-78}$-LP. Purified human $CD8^+$ T-cells obtained from HLA-$A2^+$ donor (HD2) were stimulated with $CDCA1_{55-78}$-LP-pulsed DCs. After three rounds of stimulation, the $CD8^+$ T-cells specifically produced IFN-γ in response to restimulation with T2-cells pulsed with the CDCA1-$A2_{65-73}$ SP, thus indicating that $CDCA1_{55-78}$-LP-loaded DCs primed CDCA1-$A2_{65-73}$ SP-specific CTLs through cross-presentation of $CDCA1_{55-78}$-LP by DCs *in vitro* (Fig. 3*a*). $CDCA1_{55-78}$-LP-loaded DCs also could prime CDCA1-$A24_{56-64}$ SP-specific CTLs and CDCA1-$A2_{65-73}$ SP-specific CTLs in an HLA-$A24^+$/$A2^+$/$DR4^+$ donor (HD5; Supporting Information Fig. S9*a*).

Next, the capacity of CDCA1-LPs to prime CDCA1-$A2_{65-73}$ or CDCA1-$A24_{56-64}$-specific CTLs was examined by an *ex vivo* IFN-γ ELISPOT assay. HLA-A2 and -A24 Tgm were immunized twice or three times with $CDCA1_{55-78}$-LP or $CDCA1_{39-64}$-LP. The $CD8^+$ T-cells of HLA-A2 Tgm vaccinated with $CDCA1_{55-78}$-LP produced IFN-γ specifically in response to stimulation with BM-DCs pulsed with the CDCA1-$A2_{65-73}$ SP, and the number of specific spots was increased by repeated vaccinations (Fig. 3*b*). The $CD8^+$ T-cells of HLA-A24 Tgm vaccinated with $CDCA1_{55-78}$-LP also produced IFN-γ specifically in response to stimulation with BM-DCs and C1R-A2402 cells pulsed with the CDCA1-$A24_{56-64}$ SP (Fig. 3*c*, left panel). A similar result was obtained when HLA-A24 Tgm were vaccinated with $CDCA1_{39-64}$-LP (Fig. 3*c*, right panel). HLA-A24 Tgm were immunized with twice the amount of CDCA1-LPs, because the number of CDCA1-$A24_{56-64}$ SP-specific spots of HLA-A24 Tgm was lower than that of HLA-A2 Tgm. Furthermore, vaccination with CDCA1-LPs was superior to CDCA1-$A24_{56-64}$ SP in induction of SP-specific CTLs (Fig. 3*d*). These results demonstrate that after uptake of CDCA1-LPs, APCs can cross-prime CDCA1-specific CTLs *in vitro* and *in vivo*.

Enhanced induction of CDCA1-specific CTLs by $CDCA1_{55-78}$-LP and Th-clone

Next, we tested whether $CDCA1_{55-78}$-LP could enhance induction of CDCA1-specific CTL (Fig. 4*a*). When PBMCs from an HLA-$A2^+$/$DR4^+$ donor were stimulated with CDCA1-$A2_{351-359}$-SP alone (SP), the frequency of HLA-A2/$CDCA1_{351-359}$ $tetramer^+$ cells was 0.05% of $CD8^+$ T-cells (Fig. 4*b*). Addition of $CDCA1_{55-78}$-LP or Th-clone into the SP culture induced a slight increase in the frequency of $tetramer^+$ cells. In contrast, when the PBMCs were co-stimulated with SP, $CDCA1_{55-78}$-LP and $CDCA1_{55-78}$-LP-specific Th-clone (SP + LP + Th-clone), the frequency of CDCA1-$A2_{351-359}$-specific CTLs increased to 0.6% (12-fold increase). We also observed $CDCA1_{55-78}$-LP and Th-clone enhanced induction of HLA-A2/$CDCA1_{351-359}$ $tetramer^+$ T-cells by adding CDCA1-$A2_{65-73}$ SP (data not shown). These results indicate that the activated Th cells enhanced induction of CDCA1-specific CTLs.

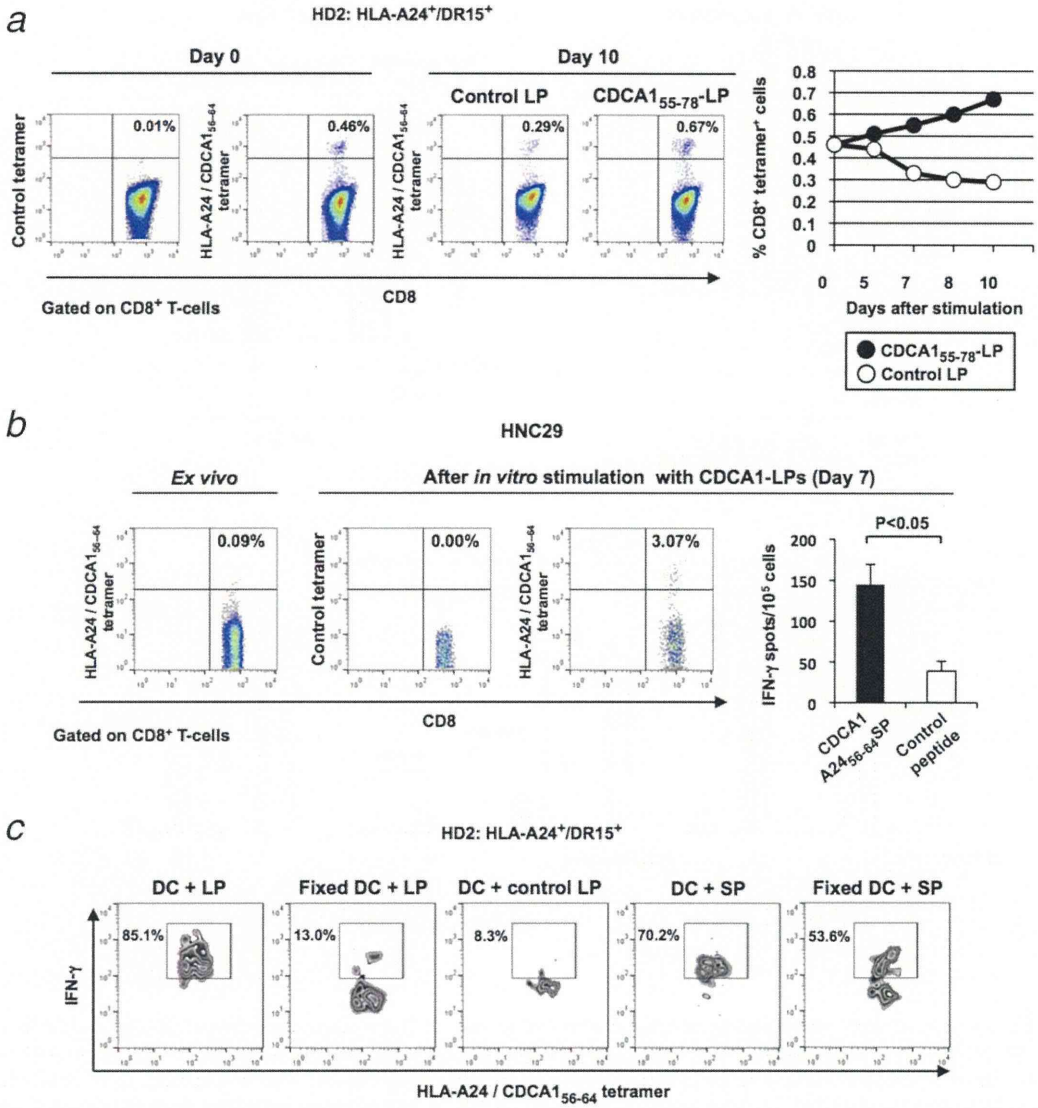

Figure 2. CDCA1-LPs induce efficient expansion of CDCA1-A24$_{56-64}$ SP-specific CD8$^+$ T-cells *in vitro*. *a*: CDCA1-A24$_{56-64}$-specific bulk CTLs established from HD2 (HLA-A24$^+$ and DR15$^+$) were stimulated with CDCA1$_{55-78}$-LP (closed circle) or irrelevant LP (open circle)-pulsed autologous DCs *in vitro*. Before LP-stimulation (day 0) and on days 5, 7, 8 and 10 after stimulation, an aliquot of cultured cells (1×10^5 cells) CD8$^+$ T-cells was stained with a HLA-A24/CDCA1$_{56-64}$ tetramer in combination with an anti-human CD8 mAb. Representative data on day 0 and day 10 from three independent experiments are shown (left panels). Events are gated for CD8$^+$ T-cells. The percentage of tetramer$^+$ cells in CD8$^+$ T-cells is depicted with lines (right panel). *b*: PBMCs from the HNC patient (HNC29) vaccinated with CDCA1-A24$_{56-64}$ SP were cultured with a mixture of CDCA1$_{55-78}$-LP and CDCA1$_{39-64}$-LP. On day 0 (*ex vivo*) and day 7 (after *in vitro* stimulation with CDCA1-LPs), the PBMCs were stained with a HLA-A24/CDCA1$_{56-64}$ tetramer or control tetramer (gated on CD8$^+$ T-cells). On day 7, the frequency of CDCA1-A24$_{56-64}$-SP-specific CTLs was also detected by IFN-γ ELISPOT assay (right panel, bar graph). Data are presented as the mean ± SD of triplicate assays. *c*: Uptake and cross-presentation of CDCA1$_{55-78}$-LP by DCs. Unfixed or fixed DCs were pulsed for 3 hr with CDCA1$_{55-78}$-LP or CDCA1-A24$_{56-64}$ SP. The bulk CDCA1-A24$_{56-64}$-specific CTLs were co-cultured for 6 hr and responses were measured by IFN-γ labeling. Events were gated for CD8$^+$ tetramer$^+$ T-cells and the numbers inside the plots indicate the percentage of IFN-γ$^+$ T-cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The CDCA1-specific CTL line induced by stimulation with SP, CDCA1$_{55-78}$-LP and Th-clone (Fig. 4*b*, SP + LP + Th-clone) was re-stimulated with SP and CDCA1$_{55-78}$-LP on day 14 (third stimulation) and IL-2 was added to expand CDCA1-specific CTLs. The frequency of HLA-A2/CDCA1$_{351-359}$ tetramer$^+$ cells increased (5.4%) on day 18 (Fig. 4*c*, left). Antigen-specific IFN-γ production (Fig. 4*c*, middle) and the cytotoxicity marker CD107a exposed on the cell surface (data not shown) were detected when these cells were re-stimulated with CDCA1-A2$_{351-359}$ SP. These cells lysed specifically CDCA1-A2$_{351-359}$ SP-pulsed T2-cells (Fig. 4*c*, right), indicating the antigen-specific bulk CTLs induced in the presence of Th clone and CDCA1-LP were functional.

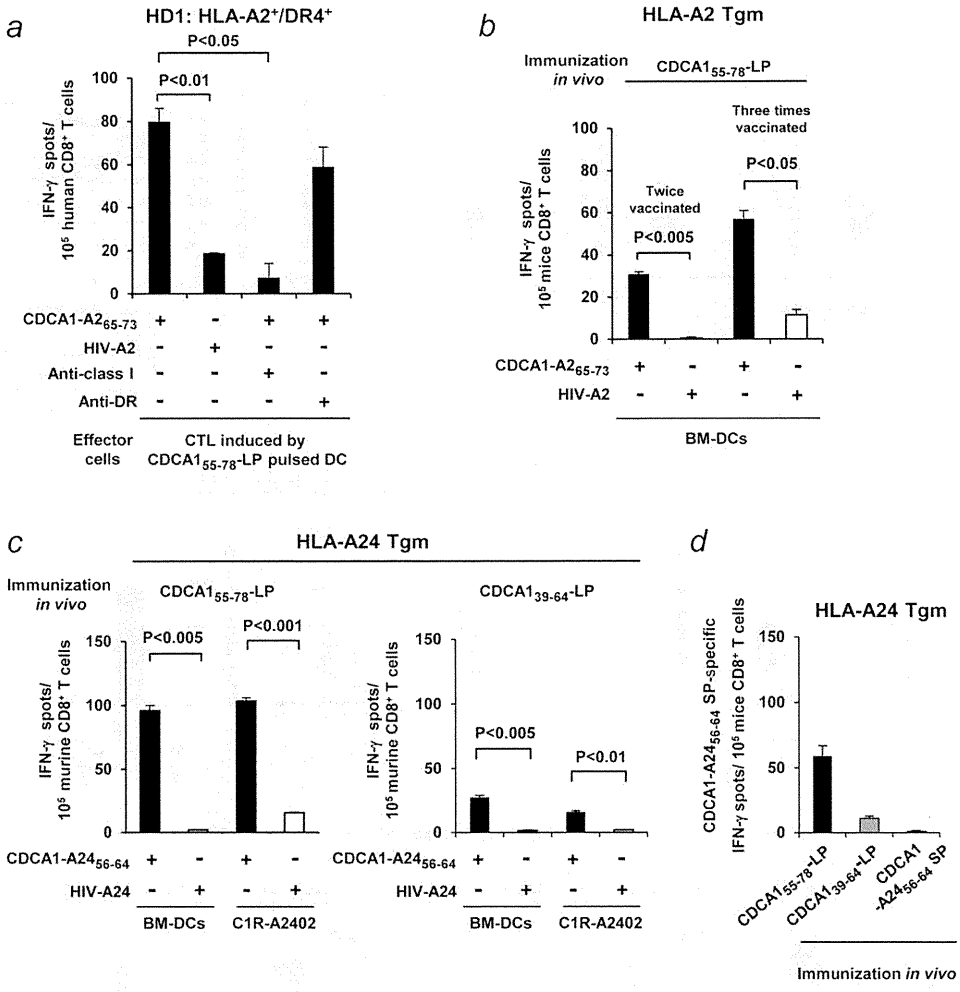

Figure 3. CDCA1-LPs induce efficient cross-priming of CTLs *in vitro* and *in vivo*. *a*: The cross-presentation of $CDCA1_{55-78}$-LP by DC primes CDCA1-$A2_{65-73}$ SP-specific CTL *in vitro*. Purified $CD8^+$ T-cells isolated from HLA-$A2^+$ HD1 were stimulated with DCs loaded with $CDCA1_{55-78}$-LP. After three rounds of stimulation, the generated CTLs were restimulated with T2-cells pulsed with CDCA1-$A2_{65-73}$ SP or HIV-A2-pulsed T2-cells, and the numbers of IFN-γ-producing CTL were analyzed by ELISPOT assay. A representative data from three independent experiments with similar results is shown. *b*: Induction of CDCA1-$A2_{65-73}$ SP-specific CTLs in mice immunized with $CDCA1_{55-78}$-LP. HLA-A2 Tgm were immunized with $CDCA1_{55-78}$-LP. After the second or third vaccination with $CDCA1_{55-78}$-LP, murine $CD8^+$ T-cells in the inguinal lymph nodes were stimulated with BM-DCs pulsed with CDCA1-$A2_{65-73}$ SP or HIV-A2 SP. The number of IFN-γ-producing murine $CD8^+$ T-cells was analyzed by *ex vivo* ELISPOT. Representative data from at least five independent experiments with similar results are shown. *c*: HLA-A24 Tgm were immunized with $CDCA1_{55-78}$-LP (left panel) or $CDCA1_{39-64}$-LP (right panel). After the second vaccination with CDCA1-LPs, murine $CD8^+$ T-cells in the inguinal lymph nodes were stimulated with BM-DC or C1R-A2402 pulsed with CDCA1-$A24_{56-64}$ SP or HIV-A24 SP. *d*: Superior induction of CDCA1-specific CTLs by CDCA1-LPs vaccines. HLA-A24 Tgm were immunized with $CDCA1_{55-78}$-LP, $CDCA1_{39-64}$-LP, or CDCA1-$A24_{56-64}$ (300 nmol/mouse). After the second vaccination with CDCA1-derived peptides, murine $CD8^+$ T-cells in inguinal lymph nodes were stimulated with BM-DCs pulsed with CDCA1-$A24_{56-64}$ SP or HIV-A24 SP (background). The results represent specific IFN-γ spots after background subtraction (right panel). Data are presented as the mean ± SD of triplicate assays. A representative of three independent experiments with similar results is shown.

The synergistic effect on induction of CDCA1-specific CTLs were also tested in HLA-$A24^+$/$DR15^+$ HD2 using HLA-A24/$CDCA1_{56-64}$ tetramer. $CDCA1_{55-78}$-LP-specific bulk $CD4^+$ T-cells and CDCA1-$A24_{56-64}$ SP-specific bulk $CD8^+$ T-cells were cultured with autologous DCs in the presence of CDCA1-$A24_{56-64}$ SP alone (SP), CDCA1-$A24_{56-64}$ SP + Control LP (Control LP + SP), or CDCA1-$A24_{56-64}$ SP + $CDCA1_{55-78}$-LP ($CDCA1_{55-78}$-LP + SP) without any cytokine. After 1-week *in vitro* culture with peptides, the cultured cells were stained with HLA-A24/$CDCA1_{56-64}$ tetramer and anti-human CD8 mAb as described in the Supporting Information Materials and Methods section. As shown Supporting Information Figure S9*b*, the addition of CDCA1-$A24_{56-64}$ SP + $CDCA1_{55-78}$-LP ($CDCA1_{55-78}$-LP + SP) significantly increased the absolute number of CDCA1-$A24_{56-64}$ SP-specific $CD8^+$ T-cells compared with the addition of SP