Impact of mature dendritic cells pulsed with a novel WT1 helper peptide on the induction of HLA-A2-restricted WT1-reactive CD8+ T cells

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Abstract. The proliferation and activation of CD4+ T helper 1 (Th1) cells and CD8+ cytotoxic T lymphocytes (CTLs) that produce interferon-γ (IFN-γ) is an essential action of effective cancer vaccines. Recently, a novel Wilms' tumor 1 (WT1) helper peptide (WT1 HP_{34-51}; amino acid sequence, WAPVLDFAPPGASAYGSL) applicable for various human leukocyte antigen (HLA) subtypes (HLA-DR, HLA-DP and HLA-DQ) was reported to increase peptide immunogenicity; however, the function of WT1 HP_{34-51} remains unclear. In the present study, mature dendritic cells (mDCs) pulsed with WT1 HP_{34-51} (mDC/WT1 HP_{34-51}) activated not only WT1-specific CD4+ T cells but also CD8+ T cells that produced IFN-γ following stimulation with immature dendritic cells (imDCs) pulsed with WT1 killer peptide (imDC/WT1 KP_{37-45}) in an HLA-A*02:01- or HLA-A*02:06-restricted manner. Furthermore, the activated WT1-reactive CD4+ Th1 cells were predominantly effector memory (EM) T cells. In 5 of 12 (41.7%) patients with cancer carrying the HLA-A*02:01 or HLA-A*02:06 allele, WT1-reactive CD8+ T cells stimulated with mDC/WT1 HP_{34-51} enhanced their levels of WT1 KP_{37-45}-specific IFN-γ production, with an increase >10%. Simultaneous activation of CD4+ and CD8+ T cells occurred more often when stimulation with mDC/WT1 HP_{34-51} was combined with imDC/WT1 KP_{37-45} restimulation. These results indicated that the novel mDC/WT1 HP_{34-51} combination induced responses by WT1-specific EM CD4+ Th1 cells and HLA-A*02:01- or HLA-A*02:06-restricted CD8+ CTLs, suggesting its potential as a WT1-targeting cancer vaccine.

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Introduction

Dendritic cells (DCs) are antigen-presenting cells that regulate the immune response via interactions between major histocompatibility complex (MHC) molecules on DCs and T-cell receptors on T cells alongside costimulatory molecules (1). Cancer vaccines using DCs loaded with antigenic peptides, tumor lysates, mRNA, DNA or whole tumor cells are an approved approach for enhancing a patient's own immune system to eradicate tumor cells (2). In clinical trials, MHC class I-restricted peptide-loaded DCs have been most commonly used to induce antigen-specific CD8+ cytotoxic T lymphocyte (CTL) responses and, in some cases, have been associated with clinical benefits; however, clinical efficacy has been limited (3,4). It has been reported that activation of CD4+ T helper type 1 (Th1) cell responses via MHC class II molecules on mature DCs (mDCs) is essential for supporting CD8+ CTL priming, memory formation, recruitment to tumor tissue and tumor cell recognition (5-7). Therefore, several long peptides targeting tumor-associated antigen (TAA)-specific CD4+ cells have been developed for the treatment of patients with cancer (5-10). Our previous study reported the clinical benefits of using MHC class I and II peptide-pulsed mDC vaccines to simultaneously induce TAA-specific CD4+ Th1 cells and CD8+ CTLs (4).

At present, numerous TAAs, such as Wilms' tumor 1 (WT1), HER-2/neu, gp100 and tyrosinase, have been isolated and incorporated into cancer vaccine preparations in the form of peptides (3-10). A promising target for cancer vaccines, WT1 was ranked as the top antigen among 75 TAAs due to its high immunogenicity (11). WT1 is highly expressed in various types of tumor and serves oncogenic roles in their formation (12-14). Additionally, WT1 has been successfully

applied to induce both $CD4^+$ Th1 cells and $CD8^+$ CTLs (15,16). WT1-specific $CD4^+$ Th1 cells contribute to the induction and maintenance of WT1-specific $CD8^+$ CTLs by establishing the required cytokine milieu via cytokine secretion, including interleukin (IL)-2 and interferon-γ (IFN-γ) (15). In our previous study, mDCs were pulsed with three types of MHC class I- or class II-restricted WT1 peptide: i) 126-134 (amino acid sequence, RMFPNAPYL) for human leukocyte antigen (HLA)-A*02:01/02:06; ii) 235-243 (amino acid sequence, CYTWNQMNL) for HLA-A*24:02; and iii) 332-347 (amino acid sequence, KRYFKLSHLQMHSRKH) for MHC class II (4). Patients with advanced pancreatic ductal adenocarcinoma (PDA) were then treated with pulsed mDCs in combination with standard chemotherapy. In the clinical trial, 4 of 7 patients with PDA exhibited strongly positive WT1-specific immunity, resulting in prolongation of overall survival. However, WT1-specific immunity was not induced in the remaining 3 patients. Therefore, a novel WT1 helper peptide with increased immunogenicity may be essential for the induction and maintenance of antitumor immunity in clinical trials to generate more successful results.

Recently, a novel WT1 helper peptide (WT1 HP_{34-51}; amino acid sequence, WAPVLDFAPPGASAYGSL) was identified (17). Of note, WT1 HP_{34-51} contains a killer WT1 peptide (WT1 KP_{37-45}; amino acid sequence, VLDFAPPGA) that has affinity for HLA-A*02:01 (18). Accordingly, the novel peptide WT1 HP_{34-51} may induce not only WT1-specific $CD4^+$ Th1 cells but also $CD8^+$ CTLs simultaneously, at least partially, in an HLA-A*02:01-restricted manner. Thus far, the function of WT1 HP_{34-51} remains unclear. In the present study, the impact of mDCs pulsed with the novel peptide WT1 HP_{34-51} (mDC/WT1 HP_{34-51}) on the induction of WT1-reactive antitumor immunity was assessed.

Materials and methods

Prediction of peptide-MHC class I binding or peptide-MHC class II molecules. WT1 peptide-MHC class I binding affinity was predicted by the NetMHC 4.0 server-prediction program (http://www.cbs.dtu.dk/services/NetMHC-4.0). Moreover, WT1 peptide-MHC class II binding affinity was predicted by the NetMHCII-2.3 server-prediction program (https://services.healthtech.dtu.dk/service.php?NetMHCII-2.3).

Patient characteristics. The present study was jointly reviewed and approved by the Ethics Committee of the Jikei Institutional Review Board, Jikei University School of Medicine and the Clinical Study Committee of Jikei University Kashiwa Hospital [approval no. 29-063 (8679)]. All patients provided written informed consent for the use of their samples in scientific research before samples were collected. To assess the stimulatory ability of mDC/WT1 HP_{34-51} for the induction of $CD8^+$ CTLs restricted by HLA-A2, 14 patients (7 male, 7 female; aged 37-88 years) with various types of cancer (cancer of the oropharynx, lungs, esophagus, stomach, breast, pancreas, ovary, gallbladder, biliary duct, prostate or uterus) expressing an HLA-A2 allele [02:01 (n=5), 02:06 (n=7) or 02:07 (n=2)] were enrolled in the Tokyo Midtown Center for Advanced Medical Science and Technology between August 2017 and April 2019 (Table I).

Preparation of WT1-pulsed DCs. Peripheral blood mononuclear cells (PBMCs) were collected from the 14 patients, none of whom had received cancer vaccines, via leukapheresis and cryopreserved at the Tokyo Midtown Center for Advanced Medical Science and Technology. PBMCs were isolated using a Ficoll-Plaque Premium (Cytiva) density gradient solution, cryopreserved and stored for future experiments. After cryopreserved PBMCs were thawed and washed, their viability was assessed using trypan blue solution (Sigma-Aldrich; Merck KGaA). Trypan blue staining was performed according to the manufacturer's protocols. Cell viability was assessed under a CK40-F100 light microscope (magnification, x100; Olympus Corporation). Subsequently, the cells were incubated in serum-free AIM-V medium (Gibco; Thermo Fisher Scientific, Inc.) in a 10-cm Primaria cell culture dish with surface-modified polystyrene for enhanced cell culture (Corning Inc.) under 5% CO_2 at 37˚C in a humidified incubator. After >30 min, plastic-adherent monocytes and nonadherent (NAD) cells were isolated by washing with gentle pipetting. NAD cells were cryopreserved in Bambanker solution (Nippon Genetics Co., Ltd.) and used for induction of WT1-reactive T cells. Adherent monocytes were cultured in serum-free AIM-V medium containing granulocyte macrophage colony-stimulating factor (GM-CSF; 50 ng/ml; Primmune Inc.) and IL-4 (50 ng/ml; R&D Systems, Inc.) under 5% CO_2 at 37˚C in a humidified incubator for 5 days to generate immature DCs (imDCs). Autologous imDCs were cryopreserved until they were pulsed with WT1 peptides or cocultured with NAD cells.

The viability and function of mDCs were not altered by whether fresh or cryopreserved PBMCs or imDCs were used for mDC production (19). Therefore, cryopreserved imDCs were used to prepare WT1-pulsed DCs in the present study. To generate WT1 peptide-pulsed autologous mDCs, cryopreserved imDCs ($1x10^5$) were thawed and incubated under 5% CO_2 at 37˚C in a humidified incubator for 24 h with 100 μg WT1 HP_{34-51} or WT1 KP_{37-45} (all peptides obtained from Greiner Bio-One GmbH) in the presence of GM-CSF (5 ng/ml), prostaglandin E2 (50 ng/ml; Daiichi Fine Chemical Co., Ltd.) and lyophilized preparations of a penicillin-killed, low-virulence strain of *Streptococcus pyogenes* (OK-432; 10 μg/ml; Chugai Pharmaceutical Co., Ltd.). Autologous imDCs ($1x10^4$) were also cultured with 25 μg WT1 HP_{34-51} or WT1 KP_{37-45} under 5% CO_2 at 37˚C in a humidified incubator for 24 h to generate WT1 peptide-pulsed imDCs, which were used as a second stimulator.

Phenotypes of DCs and NAD cells. To assess DC phenotypes, imDCs or mDCs ($1x10^5$/100 μl) were washed, incubated with Clear Back human Fc receptor blocking reagent (dilution 1:20; cat. no. MTG-001; MBL Life Science) for 5 min at 4˚C and then stained with the following monoclonal antibodies (mAbs) at 4˚C for 30 min: Fluorescein isothiocyanate (FITC)-conjugated anti-human HLA-ABC (dilution 1:20; cat. no. 311404; clone W6/32), anti-CD80 (dilution 1:20; cat. no. 305206; clone 2D10), anti-CD40 (dilution 1:20; cat. no. 334306; clone 5C3; all from BioLegend, Inc.); FITC-conjugated anti-CD14 (dilution 1:20; cat. no. 11-0149-42; clone 61D3; eBioscience; Thermo Fisher Scientific, Inc.); and phycoerythrin (PE)-conjugated anti-human HLA-DR (dilution 1:20; cat. no. 307606; clone L243), anti-CD11c (dilution 1:20; cat. no. 301606; clone 3.9),

Table I. Patient characteristics.

Patient	Sex	Age, years	Cancer	HLA-A
1	Male	69	Lung and esophagus	02:01/26:01
2	Female	49	Breast	02:01/11:01
3	Female	82	Stomach	02:01/24:02
4	Male	45	Pancreas	02:01/24:02
5	Male	59	Gallbladder and bile duct	02:01/24:02
6	Male	52	Oropharynx	02:06/24:02
7	Male	88	Stomach	02:06/26:03
8	Female	47	Ovary	02:06/24:02
9	Female	47	Ovary	02:06/11:01
10	Female	70	Pancreas	02:06/24:02
11	Male	65	Prostate	02:06
12	Female	60	Ovary	02:06/24:02
13	Female	37	Uterus	02:07/24:02
14	Male	46	Pancreas	02:07/24:02

HLA, human leukocyte antigen.

anti-CD83 (dilution 1:20; cat. no. 305308; clone HB15e) and anti-CD86 (dilution 1:20; cat. no. 305406; clone IT2.2; all from BioLegend, Inc.). In addition, to assess NAD phenotypes, NAD cells ($1x10^5$/100 μl) were washed, incubated with Clear Back human Fc receptor blocking reagent for 5 min at 4˚C and then stained with the following mAbs at 4˚C for 30 min: FITC-conjugated anti-human CD14 (dilution 1:20; cat. no. 11-0149-42; clone 61D3; eBioscience; Thermo Fisher Scientific, Inc.); FITC-conjugated anti-CD8 (dilution 1:60; cat. no. 301006; clone RPA-T8), PE-conjugated anti-human CD19 (dilution 1:40; cat. no. 302208; clone HIB19), allophycocyanin (APC)-conjugated anti-human CD56 (dilution 1:20; cat. no. 318310; clone HCD56) and APC/cyanine 7 (Cy7)-conjugated anti-human CD4 (dilution 1:60; cat. no. 317418; clone OKT4; all from BioLegend, Inc.). Phenotypes were analyzed using an Attune NxT flow cytometer (Thermo Fisher Scientific, Inc.) and FlowJo analysis software (version 7.6.5; Tree Star, Inc.). Prior to using the analyzer, 4 μg/ml propidium iodide (Sigma-Aldrich; Merck KGaA) was added to the samples to exclude dead cells at 4˚C for ~5 min.

Induction of WT1-reactive CD4⁺ and CD8⁺ T cells. Cryopreserved NAD cells were thawed, washed and incubated under 5% CO_2 at 37˚C in a humidified incubator for 24 h in 24-well plates with RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 1% minimum essential medium nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol (all from Gibco; Thermo Fisher Scientific, Inc.) and 10% heat-deactivated fetal calf serum (Cytiva) in the presence of IL-2 (20 U/ml; Shionogi & Co., Ltd.) and IL-7 (20 ng/ml; PeproTech, Inc.). The next day (day 1), WT1 $HP_{34\text{-}51}$-pulsed mDCs (mDC/WT1 $HP_{34\text{-}51}$; $1x10^5$) or WT1 $KP_{37\text{-}45}$-pulsed mDCs (mDC/WT1 $KP_{37\text{-}45}$; $1x10^5$) were cocultured with NAD cells ($1x10^6$) in RPMI-1640 medium containing 1% minimum essential medium nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 10% heat-deactivated fetal calf serum in the presence of low doses of IL-2 (10 U/ml) and IL-7 (10 ng/ml) under 5% CO_2 at 37˚C in a humidified incubator for 7 days. At day 8, the stimulated cells ($1x10^6$) were washed and cocultured again with freshly prepared mDC/WT1 $HP_{34\text{-}51}$ ($1x10^5$) or mDC/WT1 $KP_{37\text{-}45}$ ($1x10^5$) in the fresh culture medium containing IL-2 (10 U/ml) and IL-7 (10 ng/ml) for 7 days. To generate WT1-reactive T cells effectively, the stimulation was repeated in the same manner for a total of 4 times (day 1, 8, 15 and 22). In addition, as a control (no stimulation group), NAD cells ($1x10^6$) were maintained with IL-2 (10 U/ml) and IL-7 (10 ng/ml) in 7-day intervals for a total of 4 times under 5% CO_2 at 37˚C in a humidified incubator.

Microscopic analysis of cell clusters. On day 4 of the final 7-day simulation interval (day 26), cell cluster formation was examined under a EVOS™ XL core imaging system (magnification, x4; cat. no. AMEX1000; Thermo Fisher Scientific, Inc.).

Detection of CD4⁺ or CD8⁺ T cells producing IFN-γ in response to WT1-$KP_{37\text{-}45}$. To assess the impact of mDC/WT1 $HP_{34\text{-}51}$ on the activation of WT1-specific T cells, WT1-reactive T cells ($1x10^5$) induced by 4 total stimulations with mDC/WT1 $HP_{34\text{-}51}$ or mDC/WT1 $KP_{37\text{-}45}$ were restimulated once with WT1 $HP_{34\text{-}51}$-pulsed imDCs (imDC/WT1 $HP_{34\text{-}51}$), WT1 $KP_{37\text{-}45}$-pulsed imDCs (imDC/WT1 $KP_{37\text{-}45}$) or imDCs ($1x10^4$) alone in 96-well U-bottomed plates (Corning Inc.) under 5% CO_2 at 37˚C in a humidified incubator for 6 h using BD GolgiStop (BD Biosciences). After stimulation, the cells were washed, incubated with Clear Back human Fc receptor blocking reagent for 5 min at 4˚C and then stained with a PE/Cy5-conjugated anti-human CD8 mAb (dilution 1:20; cat. no. 15-0088-71; clone RPA-T8; eBioscience; Thermo Fisher Scientific, Inc.) and an APC/Cy7-conjugated anti-human CD4 mAb (dilution 1:60; cat. no. 317418; clone OKT4; BioLegend, Inc.). Thereafter, they

were washed, fixed at 4˚C for 20 min and then permeabilized using a BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit with BD GolgiStop (BD Biosciences). The cells were then incubated with an APC-conjugated anti-human IFN-γ mAb (dilution 1:25; cat. no. 506510; clone B27; BioLegend, Inc.). Lymphocytes were gated on an forward scatter-side scatter (FSC-SSC) dot plot, and $CD4^+$ or $CD8^+$ T cells were regated on a CD4/IFN-γ or CD8/IFN-γ dot plot, respectively. The percentages of $CD4^+$IFN-γ^+ cells and $CD8^+$IFN-γ^+ cells among the $CD4^+$ or $CD8^+$ T cell population, respectively, were analyzed using the same equipment and software as were used to analyze DC/NAD phenotypes.

WT1-specific memory cells in $CD4^+$ or $CD8^+$ T cell populations induced by mDC/WT1 HP_{34-51}. To assess the memory cell subsets of activated WT1-reactive $CD4^+$ or $CD8^+$ T cells, NAD cells were first stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} for 4 times, followed by restimulation with imDC/WT1 KP_{37-45} at a ratio of 10:1 under 5% CO_2 at 37˚C in a humidified incubator. NAD cells without any stimulation were maintained and used as a control. After stimulation, the cells were washed, incubated with Clear Back human Fc receptor blocking reagent as previously described and then stained with an FITC-conjugated anti-human exon 4 splice variant of the tyrosine phosphatase (CD45RA) mAb (dilution 1:40; cat. no. 304106; clone HI100; BioLegend, Inc.), a PE-conjugated anti-human chemokine receptor type 7 (CCR7) mAb (dilution 1:10; cat. no. FAB-197P; clone 150503; R&D Systems, Inc.), or a PE/Cy5-conjugated anti-human CD8 mAb and an APC/Cy7-conjugated anti-human CD4 mAb at 4˚C for 30 min. Lymphocytes were gated on an FSC-SSC dot plot, $CD4^+$ or $CD8^+$ T cells were regated, and CD45RA and CCR7 were then analyzed using the same equipment and software as were used to analyze DC/NAD phenotypes. Activated $CD4^+$ or $CD8^+$ T cells can be classified into 4 groups based on CD45RA and CCR7: $CD45RA^+CCR7^+$ naïve, $CD45RA^-CCR7^+$ central memory (CM), $CD45RA^-CCR7^-$ effector memory (EM) and $CD45RA^+CCR7^-$ terminally differentiated effector memory (EMRA) cells.

Statistical analysis. Data are presented as the mean ± SD. Comparisons of the difference in the percentage of cell surface markers between imDCs and mDCs were performed with a paired t-test or a Wilcoxon signed-rank test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using Microsoft Office Excel 2011 (Microsoft Corporation) with the add-in software Statcel 3 (OMS Publishing Inc.).

Results

Binding of WT1 peptides to MHC class I or MHC class II molecules. According to the NetMHC 4.0 server-prediction program, WT1 KP_{37-45} within WT1 HP_{34-51} exhibited high affinity for HLA-A*02:01 and A*02:07. Moreover, according to the NetMHCII-2.3 server-prediction program, WT1 HP_{34-51} and WT1 KP_{37-45} exhibited high affinity for MHC class II (data not shown).

Characterization of imDCs, mDCs or NAD cells. Frozen and stored PBMCs were used, and the yield of mDCs at the end of the production process was 2.47±1.86% (n=3). As not enough cells were available for patients 4 and 9, the immunophenotype results of the imDCs and mDCs from the 12 cases are presented in Tables SI and SII. Both imDCs and mDCs generated from 12 of the analyzed cancer patients displayed a characteristic phenotype comprising MHC class I (HLA-ABC), MHC class II (HLA-DR), CD14, CD11c, CD80, CD86, CD40 and CD83 expression (Fig. S1, Tables SI and SII). In addition, the percentages of mDCs expressing CD80, CD86, CD40 and CD83 were significantly increased compared with imDCs ($P<0.001$, $P=0.002$, $P=0.021$ and $P<0.001$, respectively), indicating that mDCs were activated by exposure of imDCs to OK-432 (Tables SI and SII). In contrast, the NAD cell composition (n=5) included lymphocytes ($CD8^+$ cells, 27.14±8.44%; $CD4^+$ cells, 54.49±10.38%; $CD19^+$ cells, 0.78±0.42%; $CD56^+$ cells, 9.80±3.86%) and monocytes ($CD14^+$ cells, 2.24±1.20%; data not shown).

WT1-reactive T cells generated by mDC/WT1 HP_{34-51} stimulation. To assess the stimulatory capacity of mDC/WT1 HP_{34-51} for induction of HLA-A*02:01-restricted $CD8^+$ T cells, NAD cells from patient 4 (HLA-A*02:01/24:02; Table I) were first stimulated 4 times in 7-day intervals with mDC/WT1 HP_{34-51}. As a control, NAD cells were also first cultured with mDC/WT1 KP_{37-45}, or not stimulated but maintained with low doses of IL-2 and IL-7 4 times in 7-day intervals. The cells were then restimulated with imDC/WT1 HP_{34-51}, imDC/WT1 KP_{37-45} or imDCs alone (Fig. 1A). At 4 days after the final stimulation with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} (Fig. 1A), cell clusters had formed (Fig. 1B). In contrast, there was no cluster formation by cells in the no stimulation group (Fig. 1B). These results indicated that both mDC/WT1 HP_{34-51} and mDC/WT1 KP_{37-45} exhibited the ability to stimulate these cells.

Then, it was evaluated as to whether mDC/WT1 HP_{34-51} induced WT1-reactive T cells with the functional capacity to produce IFN-γ by monitoring the activity of $CD4^+$ and $CD8^+$ T cells in response to imDC/WT1 HP_{34-51} (Fig. 2A). The percentages of $CD4^+$IFN-γ^+ T cells among total $CD4^+$ T cells were 0.58% [0.29/(0.29+49.4)] and 0.58% [0.28/(0.28+48.4)] after restimulation with imDC/WT1 HP_{34-51} or imDCs alone, respectively (Fig. 2B). In contrast to $CD4^+$ T cells, the percentages of $CD8^+$IFN-γ^+ T cells among total $CD8^+$ T cells after restimulation with imDC/WT1 HP_{34-51} or imDCs alone were 7.26% [3.10/(3.10+39.6)] and 0.05% [0.02/(0.02+43.9)], respectively (Fig. 2B). These results suggested that mDC/WT1 HP_{34-51} possessed the ability to stimulate $CD8^+$ T cells that can produce IFN-γ.

Whether mDC/WT1 HP_{34-51} had the capacity to induce IFN-γ-producing $CD8^+$ T cells similar to that of mDC/WT1 KP_{37-45} was also evaluated (Fig. 3A). The percentages of $CD4^+$IFN-γ^+ T cells among total $CD4^+$ T cells stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} or left unstimulated, all of which were subsequently restimulated once with imDC/WT1 KP_{37-45}, were 0.68% [0.34/(0.34+49.3)], 1.00% [0.51/(0.51+50.3)], and 0.19% [0.08/(0.08+41.9)], respectively (Fig. 3B). Moreover, imDCs were used as a control for the second stimulator, and the percentages of $CD4^+$IFN-γ^+ T cells among total $CD4^+$ T cells stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} following restimulation with imDCs alone were 0.58% [0.28/(0.28+48.4); Fig. 2B] and

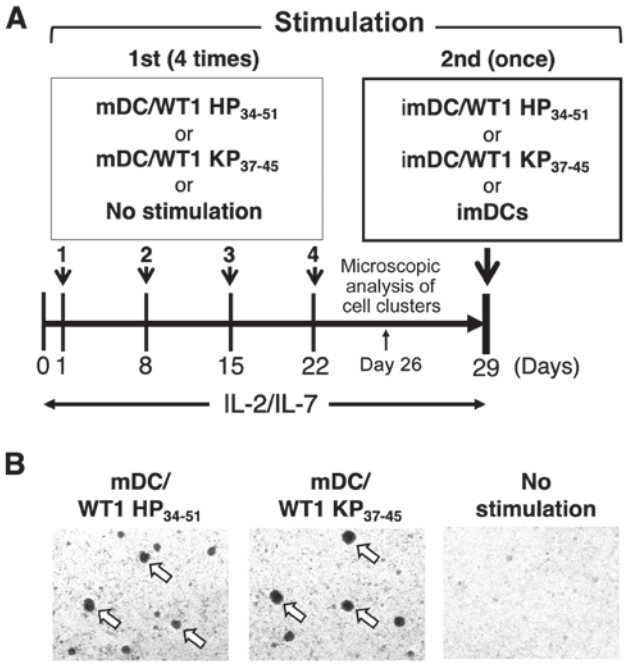

Figure 1. Stimulation of T cells with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45}. (A) Schematic representation of methods to induce WT1-reactive $CD4^+$ and $CD8^+$ T cells. (B) At 4 days after 4 times stimulation with mDC/WT1 HP_{34-51} (left) or mDC/WT1 KP_{37-45} (middle) in 7-day intervals (day 26), but not incubation without stimulation (right), the formation of cell clusters (white arrows) was observed (magnification, x4). Representative images of two independent experiments are presented. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; KP_{37-45}, killer peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; mDC/WT1 KP_{37-45}, mDCs pulsed with WT1 KP_{37-45}; IL, interleukin.

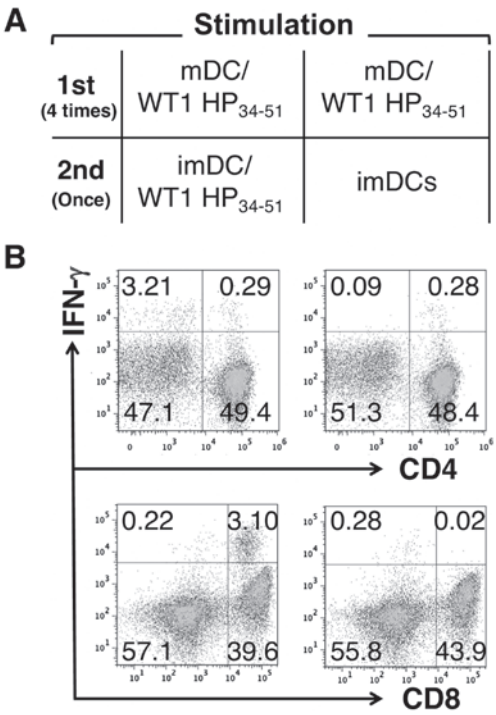

Figure 2. Characterization of T cells stimulated with mDC/WT1 HP_{34-51}. (A) Nonadherent cells were first stimulated with mDC/WT1 HP_{34-51}. The activated T cells were restimulated with imDC/WT1 HP_{34-51} or imDCs alone. (B) Dot plots of IFN-γ-producing $CD4^+$ (top) and $CD8^+$ T cells (bottom) derived from a patient with pancreatic ductal adenocarcinoma after stimulation with mDC/WT1 HP_{34-51} and restimulation with imDC/WT1 HP_{34-51} (left) or imDCs alone (right). Representative plots of two independent experiments are presented. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; imDC/WT1 HP_{34-51}, imDCs pulsed with WT1 HP_{34-51}; IFN-γ, interferon-γ.

0.68% [0.34/(0.34+49.6); Fig. S2], respectively. These results suggested that mDC/WT1 HP_{34-51} and mDC/WT1 KP_{37-45} exhibited a capacity to induce IFN-γ-producing $CD4^+$ T cells after restimulation with imDC/WT1 KP_{37-45} when compared with no stimulation. In contrast to $CD4^+$ T cells, the percentages of $CD8^+IFN\text{-}\gamma^+$ T cells among total $CD8^+$ T cells stimulated 4 times with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} and restimulated once with imDC/WT1 KP_{37-45} were 7.96% [3.39/(3.39+39.2)] and 5.13% [2.64/(2.64+48.8)], respectively (Fig. 3B). Furthermore, the percentages of $CD8^+IFN\text{-}\gamma^+$ T cells among total $CD8^+$ T cells stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} following restimulation with imDCs alone were 0.05% [0.02/(0.02+43.9); Fig. 2B] and 0.10% [0.05/(0.05+52.2); Fig. S2], respectively. In addition, 0.60% [0.30/(0.30+50.0)] of total $CD8^+$ T cells were $CD8^+IFN\text{-}\gamma^+$ following no stimulation during 29 days of culture and then one stimulation with imDC/WT1 KP_{37-45} (Fig. 3B). Of note, $CD8^+$ T cells stimulated 4 times with mDC/WT1 HP_{34-51} produced IFN-γ at higher levels than those stimulated 4 times with mDC/WT1 KP_{37-45} after one restimulation with imDC/WT1 KP_{37-45} (Fig. 3B). These results suggested that the combination treatment of mDC/WT1 HP_{34-51} and imDC/WT1 KP_{37-45} exhibited the capacity to induce IFN-γ-producing $CD8^+$ T cells more frequently than that of mDC/WT1 KP_{37-45} and imDC/WT1 KP_{37-45}.

WT1-specific memory cells in activated $CD4^+$ or $CD8^+$ T cells stimulated with mDC/WT1 HP_{34-51}. As mDC/WT1 HP_{34-51} and mDC/WT1 KP_{37-45} stimulated WT1 KP_{37-45}-reactive $CD4^+$ T cells, patient 4 was selected for an analysis of the memory cell subsets of $CD4^+$ T cells (Fig. 4A). The percentages of EM cells among total $CD4^+$ T cells stimulated with mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} or left unstimulated were 78.3, 73.7 and 30.8%, respectively (Fig. 4B and C). The percentages of EMRA cells among total $CD4^+$ T cells stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} were 8.29 and 6.67%, respectively (Fig. 4B and C). These percentages were higher than those in the no stimulation group (3.54%). The results suggested that mDC/WT1 HP_{34-51} and mDC/WT1 KP_{37-45} induced EM and EMRA CD4+ T cells. Additionally, the percentage of naïve T cells among total $CD4^+$ T cells was decreased after stimulation with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} (Fig. 4B and C).

As mDC/WT1 HP_{34-51} and mDC/WT1 KP_{37-45} also stimulated WT1 KP_{37-45}-reactive $CD8^+$ T cells, patient 4 was also selected for an analysis of the memory cell subsets of $CD8^+$ T cells (Fig. 5A). In contrast to $CD4^+$ T cells, the percentages of EM cells among total $CD8^+$ T cells stimulated with mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} or left unstimulated after one restimulation with imDC/WT1 KP_{37-45} were 51.3, 53.2 and 56.4%, respectively (Fig. 5B and C). The percentages of EMRA cells among total $CD8^+$ T cells stimulated with mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} or left unstimulated following one restimulation with imDC/WT1 KP_{37-45} were

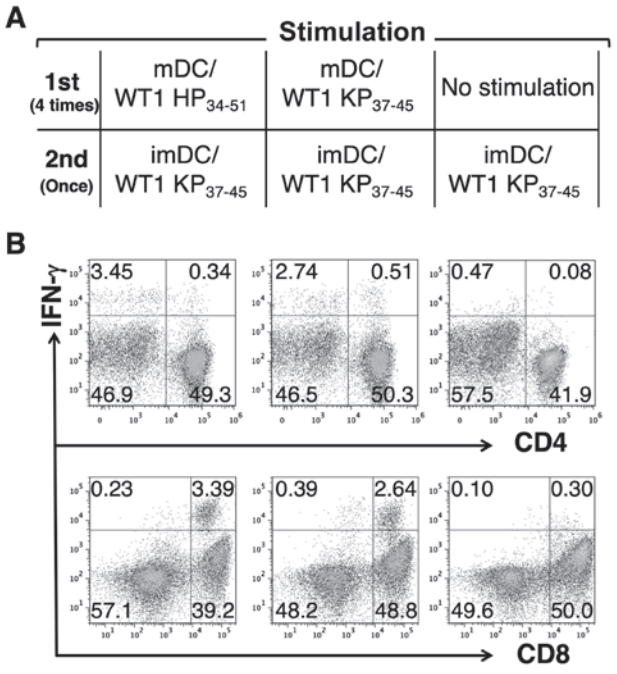

Figure 3. Comparison of the efficiencies of mDC/WT1 HP_{34-51} and mDC/WT1 KP_{37-45} in activating WT1-specific $CD4^+$ or $CD8^+$ T cells. (A) Nonadherent cells were first stimulated with mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} or IL-2/IL-7 alone. The activated T cells were restimulated with imDC/WT1 KP_{37-45}. (B) Dot plots of IFN-γ-producing $CD4^+$ (top) and $CD8^+$ T cells (bottom) derived from a patient with pancreatic ductal adenocarcinoma after stimulation with mDC/WT1 HP_{34-51} (left), mDC/WT1 KP_{37-45} (middle) or no stimulation (right) and restimulation with imDC/WT1 KP_{37-45}. Representative plots of two independent experiments are presented. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; KP_{37-45}, killer peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; mDC/WT1 KP_{37-45}, mDCs pulsed with WT1 KP_{37-45}; imDC/WT1 KP_{37-45}, imDCs pulsed with WT1 KP_{37-45}; IL, interleukin; IFN-γ, interferon-γ.

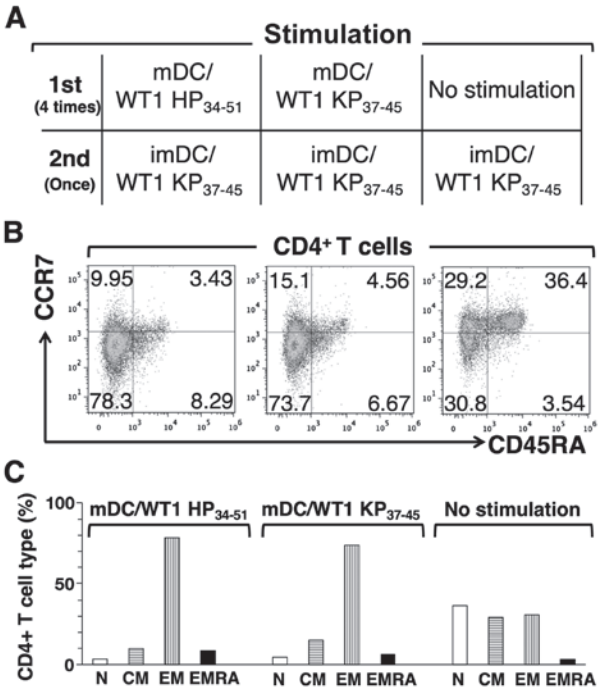

Figure 4. Memory cell subsets of $CD4^+$ T cells stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45}. (A) Nonadherent cells were first stimulated 4 times with mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} or IL-2/IL-7 alone. The activated T cells were restimulated with imDC/WT1 KP_{37-45}. (B) Dot plots of the memory cell subsets in $CD4^+$ T cells derived from a patient with pancreatic ductal adenocarcinoma activated by mDC/WT1 HP_{34-51} (left), mDC/WT1 KP_{37-45} (middle) or no stimulation (right), followed by restimulation with imDC/WT1 KP_{37-45}. (C) Percentage of four subgroups (N, CM, EM or EMRA) of memory $CD4^+$ T cells activated by mDC/WT1 HP_{34-51} (left), mDC/WT1 KP_{37-45} (middle) or no stimulation (right), and restimulation with imDC/WT1 KP_{37-45}. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; KP_{37-45}, killer peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; mDC/WT1 KP_{37-45}, mDCs pulsed with WT1 KP_{37-45}; imDC/WT1 KP_{37-45}, imDCs pulsed with WT1 KP_{37-45}; IL, interleukin; N, naïve; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory; CCR7, chemokine receptor 7; CD45RA, human exon 4 splice variant of the tyrosine phosphatase.

45.0, 42.4 and 37.6%, respectively (Fig. 5B and C). These results suggested that the frequencies of $CD8^+$ memory T cell subsets from a patient with PDA showed no notable differences between the mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} and no stimulation groups.

Induction of WT1-reactive $CD4^+$ or $CD8^+$ T cells derived from patients with different types of cancer by mDC/WT1 HP_{34-51}. The ability of mDC/WT1 HP_{34-51} to induce WT1-reactive $CD4^+$ or $CD8^+$ T cells obtained from patients with different types of cancer and HLA-A2 alleles [02:01 (n=5), 02:06 (n=7), 02:07 (n=2)] was then subsequently assessed. For 10 (patients 1, 3, 5, 6, 7, 10, 11, 12, 13 and 14) of the 14 patients with cancer (71.4%), >1% of the total $CD4^+$ T cell population expressed IFN-γ after four rounds of stimulation with mDC/WT1 HP_{34-51} followed by one restimulation with imDCs (Table II). Furthermore, for 10 (patients 1, 2, 4, 6, 7, 8, 9, 10, 13 and 14) of the 14 patients examined (71.4%), >10% of the total $CD4^+$ T cell population expressed IFN-γ after four rounds of stimulation with mDC/WT1 HP_{34-51} and restimulation once with imDC/WT1 KP_{37-45}, which was higher compared with the percentage after restimulation once with imDCs alone (Table II). In contrast to the results for WT1-reactive $CD4^+$ T cells, >1% of the total $CD8^+$ T cell population from 10 (patients 1, 3, 5, 6, 7, 8, 10, 11, 12 and 13) of the 14 patients (71.4%) examined produced IFN-γ after four rounds of stimulation with mDC/WT1 HP_{34-51} followed by one stimulation with imDCs (Table III). For 6 (patients 2, 3, 4, 7, 9 and 14) of the 14 patients with cancer (42.9%), a >10% increase in the IFN-γ-producing $CD8^+$ T cell population among total $CD8^+$ T cells was detected after restimulation once with imDC/WT1 KP_{37-45} compared with restimulation once with imDCs alone (Table III). WT1-reactive $CD8^+$ T cells from patient 14 with the HLA-A*02:07 allele exhibited greater IFN-γ production than the cells from the other patient with the HLA-A*02:07 allele. In summary, for 5 of the 12 patients with cancer (41.7%) with the HLA-A*02:01 or HLA-A*02:06 allele, WT1-reactive $CD8^+$ T cells stimulated with mDC/WT1 HP_{34-51} exhibited enhanced levels of WT1 KP_{37-45}-induced IFN-γ production, with a >10% increase following one restimulation with imDC/WT1 KP_{37-45} in this experimental setting. These results suggested that mDC/WT1 HP_{34-51} generated from patients with different types of cancer may induce WT1-specific $CD8^+$ T cells at least in part in an HLA-A*02:01-, HLA-A*02:06- or HLA-A*02:07-restricted manner.

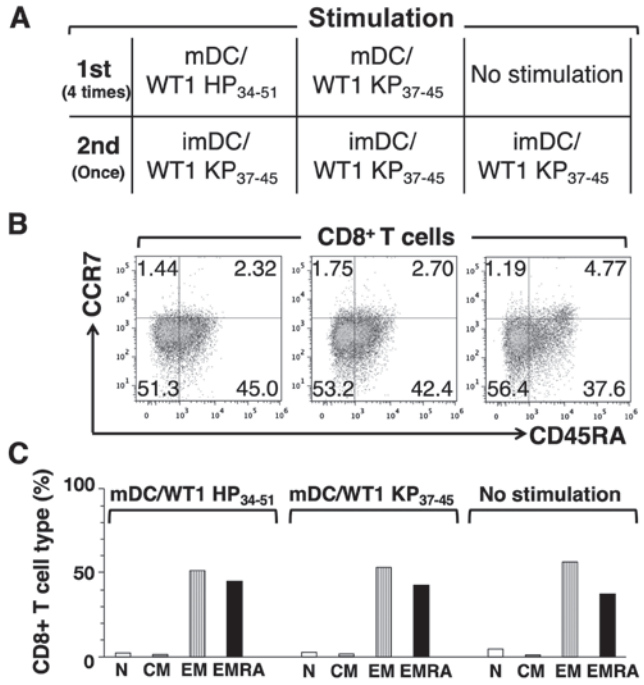

Figure 5. Memory cell subsets of CD8+ T cells stimulated with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45}. (A) Nonadherent cells were first stimulated 4 times with mDC/WT1 HP_{34-51}, mDC/WT1 KP_{37-45} or IL-2/IL-7 alone. The activated T cells were restimulated with imDC/WT1 KP_{37-45}. (B) Dot plots of the memory cell subsets in CD8+ T cells derived from a patient with pancreatic ductal adenocarcinoma activated by mDC/WT1 HP_{34-51} (left), mDC/WT1 KP_{37-45} (middle) or no stimulation (right), followed by restimulation with imDC/WT1 KP_{37-45}. (C) Percentage of four subgroups (N, CM, EM or EMRA) of memory CD8+ T cells activated by mDC/WT1 HP_{34-51} (left), mDC/WT1 KP_{37-45} (middle) or no stimulation (right), and restimulation with imDC/WT1 KP_{37-45}. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; KP_{37-45}, killer peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; mDC/WT1 KP_{37-45}, mDCs pulsed with WT1 KP_{37-45}; imDC/WT1 KP_{37-45}, imDCs pulsed with WT1 KP_{37-45}; IL, interleukin; N, naïve; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory; CCR7, chemokine receptor 7; CD45RA, human exon 4 splice variant of the tyrosine phosphatase.

Table II. Percentage of IFN-γ-producing CD4+ T cells among total CD4+ T cells by stimulation with mDC/WT1 HP_{34-51}.

		Restimulation	
Case	HLA-A	imDC/WT1 KP_{37-45}	imDCs
1	02:01	4.97[a]	3.78
2	02:01	1.01[a]	0.64
3	02:01	6.93	6.99
4	02:01	0.68[a]	0.58
5	02:01	1.03	1.11
6	02:06	3.69[a]	2.34
7	02:06	10.26[a]	7.96
8	02:06	0.69[a]	0.58
9	02:06	0.85[a]	0.23
10	02:06	3.31[a]	2.81
11	02:06	2.5	2.43
12	02:06	3.17	3.72
13	02:07	1.79[a]	1.46
14	02:07	3.23[a]	1.48

[a]Indicates a >10% increase in the IFN-γ-producing CD4+ T cell frequency among total CD4+ T cells induced by four rounds of stimulation with mDC/WT1 HP_{34-51} followed by one stimulation with imDC/WT1 KP_{37-45}, compared with imDCs alone. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; KP_{37-45}, killer peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; imDC/WT1 KP_{37-45}, imDCs pulsed with WT1 KP_{37-45}; IFN-γ, interferon-γ; HLA, human leukocyte antigen.

Discussion

The present study indicated that mDC/WT1 HP_{34-51} exhibited the ability to induce not only WT1-reactive EM CD4+ Th1 cells but also CD8+ CTLs producing IFN-γ in an HLA-A*02:01- or HLA-A*02:06-restricted manner in an *in vitro* human model. The combination of mDC/WT1 HP_{34-51} and imDC/WT1 KP_{37-45} induced higher levels of IFN-γ-producing CD8+ T cells than stimulation with mDC/WT1 KP_{37-45} and imDC/WT1 KP_{37-45}.

The impact of mDC/WT1 HP_{34-51} on the activation of WT1-specific T cells was assessed by monitoring IFN-γ production following restimulation with imDC/WT1 HP_{34-51} or imDC/WT1 KP_{37-45}. As restimulation with mDCs alone induced a degree of nonspecific IFN-γ production in the present study, WT1-loaded or unloaded imDCs were used as restimulators. CD4+ and CD8+ T cells were stimulated with mDC/WT1 HP_{34-51} together at the same time, resulting in IFN-γ production after restimulation with imDC/WT1 HP_{34-51} or imDC/WT1 KP_{37-45}, but not imDCs alone. Our previous phase I study with mDCs pulsed with a mixture of three WT1 peptides, including WT1 $HP_{332-347}$ and WT1 KP, observed that simultaneous induction of WT1-specific CD4+ and CD8+ T cells was associated with improved long-term survival (4). The novel peptide WT1 HP_{34-51} was subsequently developed to enhance WT1 peptide immunogenicity, and early clinical trials of a cocktail WT1 peptide vaccine containing the novel peptide WT1 HP_{34-51} and WT1 KP have been conducted in patients with pediatric brain cancer or myelodysplastic syndromes (20,21). The results demonstrated WT1-specific CTL induction in clinical responders, suggesting that WT1 HP_{34-51} has effective activity. Moreover, vaccination of a mouse model with both WT1 HP_{34-51} and WT1 KP also induced strong infiltration of WT1-specific CD4+ Th1 cells and CD8+ CTLs into tumor microenvironments (17).

In general, simultaneous activation of WT1-specific CD4+ Th1 cells and CD8+ CTLs may be essential when treating patients with advanced-stage cancer. Notably, the novel WT1 HP_{34-51} amino acid sequence includes $WT1_{37-45}$, which has binding affinity for HLA-A*02:01 molecules (18). Therefore, the novel peptide WT1 HP_{34-51} may stimulate not only WT1-specific CD4+ T cells but also CD8+ T cells in a manner, at least in part, restricted by HLA-A*02:01 molecules. To evaluate this hypothesis, a patient with PDA possessing HLA-A*02:01/24:02 alleles was initially selected for further analysis. Notably, mDC/WT1 HP_{34-51} activated not only

Table III. Percentage of IFN-γ-producing $CD8^+$ T cells among total $CD8^+$ T cells by stimulation with mDC/WT1 HP_{34-51}.

		Restimulation	
Case	HLA-A	imDC/WT1 KP_{37-45}	imDCs
1	02:01	3.11	3.87
2	02:01	1.84[a]	0.7
3	02:01	32.96[a]	11.07
4	02:01	7.96[a]	0.05
5	02:01	0.92	1.11
6	02:06	10.18	12.39
7	02:06	18.16[a]	12.61
8	02:06	0.72	1.22
9	02:06	0.62[a]	0.46
10	02:06	4.39	4.15
11	02:06	2.31	2.78
12	02:06	5.86	7.8
13	02:07	3.53	3.57
14	02:07	1.0[a]	0.8

[a]Indicates a >10% increase in the IFN-γ-producing $CD8^+$ T cell frequency among total $CD8^+$ T cells induced by four rounds of stimulation with mDC/WT1 HP_{34-51} followed by one stimulation with imDC/WT1 KP_{37-45}, compared with imDCs alone. mDC, mature dendritic cell; imDC, immature dendritic cell; WT1, Wilms' tumor 1; HP_{34-51}, helper peptide; KP_{37-45}, killer peptide; mDC/WT1 HP_{34-51}, mDCs pulsed with WT1 HP_{34-51}; imDC/WT1 KP_{37-45}, imDCs pulsed with WT1 KP_{37-45}; IFN-γ, interferon-γ; HLA, human leukocyte antigen.

$CD4^+$ T cells but also $CD8^+$ T cells to produce IFN-γ after restimulation with imDC/WT1 KP_{37-45}, which according to the NetMHC 4.0 server-prediction program has a high affinity for HLA-A*02:01 but not for HLA-A*24:02. Thus, WT1-specific $CD8^+$ T cells from patient 4 induced by mDC/WT1 HP_{34-51} may be HLA-A*02:01-restricted.

Stimulation of T cells by 4 incubations with mDC/WT1 HP_{34-51}, followed by one restimulation with imDC/WT1 HP_{34-51} or imDC/WT1 KP_{37-45} resulted in IFN-γ production by $CD8^+$ T cells. When imDCs are pulsed with WT1 HP_{34-51}, WT1 HP_{34-51} may be taken up by imDCs, which can be activated by OK-432. Both imDCs and mDCs may be able to process WT1 HP_{34-51} and load WT1 KP_{37-45} onto HLA-A*02:01 molecules in the endoplasmic reticulum, resulting in their surface expression of WT1 KP_{37-45}/HLA-A*02:01 complexes for presentation to $CD8^+$ T cells. Endogenously processed WT1 in DCs has relatively good access to MHC class I and II molecules, resulting in the efficient induction of antitumor immunity (22). Thus, mDC/WT1 HP_{34-51}, imDC/WT1 HP_{34-51} or imDC/WT1 KP_{37-45} may also stimulate $CD8^+$ T cells through WT1 KP_{37-45}/HLA-A*02:01 complexes, resulting in the production of IFN-γ.

Indeed, a >10% increase in IFN-γ-producing cells among total $CD8^+$ T cells was detected in 3 of 5 patients with the HLA-A*02:01 allele and 2 of 7 patients with the HLA-A*02:06 allele when their NAD cells were stimulated 4 times with mDC/WT1 HP_{34-51} followed by restimulation with imDC/WT1 KP_{37-45} compared with restimulation with imDCs alone. Therefore, WT1 HP_{34-51} may have the ability to induce WT1-specific $CD8^+$ CTLs in patients with cancer carrying the HLA-A*02:01 or HLA-A*02:06 allele. Nonetheless, 1 of 2 cancer patients with the HLA-A*02:07 allele exhibited IFN-γ production by $CD8^+$ T cells. According to the NetMHC 4.0 server-prediction program, WT1 KP_{37-45} within WT1 HP_{34-51} has a relatively high affinity for HLA-A*02:07. However, as HLA-A*02:07^+ patients are not a major population according to the national marrow donor program (23), only two patients with HLA-A*02:07 were enrolled in the present study. Accordingly, further studies are required to assess whether mDC/WT1 HP_{34-51} consistently induces HLA-A*02:07-restricted WT1-specific $CD8^+$ T cells in patients with cancer.

Among the total $CD8^+$ T cell populations from patients with cancer, WT1-specific IFN-γ-producing $CD8^+$ T cells were more frequently induced by four rounds of stimulation with mDC/WT1 HP_{34-51} followed by one stimulation with imDC/WT1 KP_{37-45} compared with four rounds of stimulation with mDC/WT1 KP_{37-45} followed by one stimulation with imDC/WT1 KP_{37-45}. Previous reports indicate that $CD4^+$ and $CD8^+$ T cell stimulation occurs when peptide vaccination with combined WT1 HP and WT1 KP is performed; however, vaccination with WT1 HP alone does not strongly activate T cells (4,17). It has also been reported that activation of $CD4^+$ T cells is essential for $CD8^+$ CTL induction, potentiating $CD8^+$ CTL proliferation and maintaining effector functions in the tumor microenvironment (5,24). Therefore, effective cancer vaccines must include a mechanism to activate $CD4^+$ T cells. Simultaneous activation of $CD4^+$ and $CD8^+$ T cells by DCs pulsed with both WT1 HP_{34-51} and WT1 KP_{37-45} may improve the efficient induction of WT1-specific $CD8^+$ T cells that produce IFN-γ. In fact, for 10 of the 14 patients in the present study, a >10% increase in the IFN-γ-producing $CD4^+$ T cell population among total $CD4^+$ T cells was induced by four rounds of stimulation with mDC/WT1 HP_{34-51} followed by one stimulation with imDC/WT1 KP_{37-45}. According to the NetMHCII-2.3 server-prediction program, WT1 KP_{37-45}, which is a part of WT1 HP_{34-51}, has a relatively high affinity for MHC class II. Thus, WT1 KP_{37-45} may also be present in MHC class II with imDC/WT1 KP_{37-45}, resulting in stimulation of $CD4^+$ T cells producing IFN-γ. As one of the major subtypes of HLA-A molecule is HLA-A*24:02 (23), mDCs may be pulsed with a cocktail of WT1 HP_{34-51} and WT1 $KP_{235-243}$ for HLA-A*24:02 (4) to maximize the induction and maintenance of $CD4^+$ Th1 cells and $CD8^+$ CTLs, at least in part, in an HLA-A*02:01-, HLA-A*02:06- or HLA-A*24:02-restricted manner for application in a wider cohort of patients with cancer.

WT1-reactive $CD4^+$ T cells from patient 4 induced by mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45} stimulation were almost all EM T cells, whereas those maintained with low doses of IL-2 and IL-7 were not. In contrast, cancer patient $CD4^+$ T cells in the no stimulation group were almost all naïve. Naïve T cells differentiated into EM T cells via the effector T cell route (25) by undergoing several rounds of stimulation with mDC/WT1 HP_{34-51} or mDC/WT1 KP_{37-45}. It has been reported that peptide-specific memory T cell

frequencies are increased in vaccine responders with a positive delayed-type hypersensitivity (DTH) test (4,26-28). Our previous study also demonstrated that in super-responders with WT1-specific DTH induced by vaccination with mDCs pulsed with WT1 HP and WT1 KP, continuous elicitation of WT1-specific long-lived EM and CM T cells occurred and could be boosted; these long-lived WT1-specific EM T cells patrol and recognize tumor cells and are therefore associated with a clinical benefit (4). Although EM $CD4^+$ T cells were induced by mDC/WT1 HP_{34-51} stimulation, compared with EM CD4+ T cells, the EM $CD8^+$ T cell frequency was not strongly increased. As some WT1 overexpression occurs in various types of solid tumor, WT1-specific $CD8^+$ T cells are spontaneously induced (11-13), suggesting that some memory $CD8^+$ T cells may be detected without any stimulation *in vitro*. As memory $CD4^+$ T cells particularly support the priming and maintaining of antigen-specific $CD8^+$ T cells (29,30), the increase in memory $CD4^+$ T cell frequency induced by mDC/WT1 HP_{34-51} stimulation may support an increase in WT1-reactive $CD8^+$ T cell frequency. In summary, T cells stimulated with mDC/WT1 HP_{34-51} may have the capacity to elicit WT1-specific EM $CD4^+$ T cells and $CD8^+$ CTLs, which was at least in part restricted by HLA-A*02:01 or HLA-A*02:06, both of which produce IFN-γ. However, the memory cell subsets were only analyzed in a single case, a patient with PDA, as a sufficient number of cells was not available. The induction and maintenance of the durable memory type of $CD4^+$ Th1 cells by mDC/WT1 HP_{34-51} may produce promising clinical activity to prolong overall survival in advanced-stage cancer patients.

As a sufficient number of PBMCs and autologous tumor cells were not available, the killing activity of WT1-ractive T cells induced by mDC/WT1 HP_{34-51} could not be assessed in this preclinical study. A phase I clinical trial involving patients with advanced pancreatic cancer who express HLA-A*02:01, HLA-A*02:06 or HLA-A*24:02 is being performed using mDCs pulsed with a novel peptide cocktail containing WT1 HP_{34-51} and HLA-A*24:02-restricted WT1 $KP_{235-243}$ combined with standard chemotherapy; the results of this clinical trial will be available soon. Additionally, future studies should optimize cancer vaccines incorporating mDCs loaded with an immunogenic novel WT1 peptide cocktail in combination with an immune checkpoint-blocking antibody.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SKa and TB performed the experiments. SKa, TB and SKo analyzed the data. MS and JT collected patient samples, and analyzed and interpreted data. SKa, SS, TO, HS and SKo conceived and designed the experimental study. SKa and SKo wrote the manuscript. All authors discussed, read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Ethics Committee of the Jikei Institutional Review Board, Jikei University School of Medicine and the Clinical Study Committee of Jikei University Kashiwa Hospital [approval no. 29-093 (8679)].

Patient consent for publication

Not applicable.

Competing interests

HS is an inventor on the patents for the WT1 peptides used in the present study (PCT/JP02/02794 and PCT/2010/057149), which are held by the International Institute of Cancer Immunotherapy.

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