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ORIGINAL ARTICLE

Identification of two distinct populations of WT1-specific cytotoxic T lymphocytes in co-vaccination of WT1 killer and helper peptides

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Abstract

Simultaneous induction of tumor antigen-specific cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (HTLs) is required for an optimal anti-tumor immune response. $WT1_{332}$, a 16-mer WT1-derived helper peptide, induce HTLs in an HLA class II-restricted manner and enhance the induction of WT1-specific CTLs in vitro. However, in vivo immune reaction to $WT1_{332}$ vaccination in tumor-bearing patients remained unclear. Here, a striking difference in WT1-specific T cell responses was shown between WT1 CTL + WT1 helper peptide and WT1 CTL peptide vaccines in patients with recurrent glioma. WT1-specific CTLs were more strongly induced in the patients who were immunized with WT1 CTL + WT1 helper peptide vaccine, compared to those who were immunized with WT1 CTL vaccine alone. Importantly, a clear correlation was demonstrated between WT1-specific CTL and $WT1_{332}$-specific HTL responses. Interestingly, two novel distinct populations of WT1-tetramer^{low} WT1-TCR^{low} CD5^{low} and WT1-tetramer^{high} WT1-TCR^{high} CD5^{high} CTLs were dominantly detected in WT1 CTL + WT1 helper peptide vaccine. Although natural WT1 peptide-reactive CTLs in the latter population were evidently less than those in the former population, the latter population showed natural WT1 peptide-specific proliferation capacity comparable to the former population, suggesting that the latter population highly expressing CD5, a marker of resistance to activation-induced cell death, should strongly expand and persist for a long time in patients. These results demonstrated the advantage of WT1 helper peptide vaccine for the enhancement of WT1-specific CTL induction by WT1 CTL peptide vaccine.

Keywords WT1 · Peptide vaccine · Helper peptide · Tetramer^{high} · CD5

Abbreviations

APC	Allophycocyanin
7-AAD	7-Amino-Actinomycin D
FITC	Fluorescein isothiocyanate
HLA	Human Leukocyte Antigen
IL	Interleukin
IFN	Interferon
PE	Phycoerythrin
TCR	T cell receptor

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TNF Tumor Necrosis Factor
TAP Transporter associated with Antigen Processing

Introduction

$CD4^+$ helper T lymphocytes (HTLs) recognize antigenic peptides presented on major histocompatibility complex (MHC) class II of antigen-presenting cells such as dendritic cells (DC). By the recognition of antigenic peptides, they activate to produce a lot of cytokines promoting cellular immunity. HTLs also activate DC and up-regulate MHC class I/II and co-stimulatory molecules such as CD80 and CD86 on the cell surface of the DC by direct interaction via CD40/40L, which is a critical step, so-called licensing, for priming/induction of cytotoxic $CD8^+$ T lymphocytes (CTLs) eliminating tumor cells [1, 2]. Besides, they also contribute to the recruitment of CTLs within a tumor and CTL effector function such as proliferation and granzyme B expression at tumor environment [3–5]. Furthermore, HTLs can enhance the generation of memory CTL and restore exhausted CTLs [6, 7]. Importantly, these benefits in CTLs by CD4 help are required for the presence of cognate-antigen-specific HTLs. Moreover, in addition to their helper functions, it is well-known that $CD4^+$ T cells can also serve as cytotoxic T cells against HLA class II-expressing tumor cells. In fact, HLA class II expression on cancer cells is associated with a good prognosis [8–10]. These findings reported previously indicate that tumor-associated antigen (TAA)-specific HTLs play a central role in anti-tumor immunity.

In cancer immunotherapy, Wilms' tumor gene 1 (WT1) is the most promising target that ranked as first among 75 TAAs [11]. Several WT1-derived HLA class I-restricted epitopes (killer peptides) that can induce CTLs capable of killing WT1-expressing tumor cells have been identified and phase I/II clinical trials using these killer peptides against hematological malignancies and solid cancers have been performing. For the simultaneous induction of WT1-specific HTLs and WT1-specific CTLs, we, in addition to the killer peptides, have identified $WT1_{332}$ (KRYFKLSHLQMHSRKH) as a WT1-derived HLA class II-restricted epitope (helper peptide) [12]. $WT1_{332}$ can bind multiple HLA class II molecules such as HLA-DRB1*01:01, DRB1*04:05, DRB1*07:01, DRB1*08:02, DRB1*08:03, DRB1*13:02, DRB1*14:03, DRB1*14:05, DRB1*15:01, DRB1*15:02, DRB3*02:02, DQB1*04:01, DPB1*05:01, and DPB1*09:01 and induce Th1-type HTLs that can promote CTL responses [12–16]. We previously demonstrated that $WT1_{332}$-specific Th1 cells and/or $WT1_{332}$ could enhance the induction of WT1-specific CTLs in vitro [12, 16]. In addition, it has been shown that $WT1_{332}$-specific Th1 responses can spontaneously be induced in cancer patients [17], suggesting that $WT1_{332}$ is an immunogenic helper peptide in human body. Thus, these results encourage us to administer WT1-derived killer peptides in combination with $WT1_{332}$ as a helper peptide to enhance WT1-specific CTL response and the resultant clinical response.

We recently performed a phase I clinical study of a combination vaccine with WT1-derived killer and helper peptides ($WT1_{332}$) for recurrent malignant glioma such as glioblastoma multiforme and anaplastic astrocytoma [18]. In the study, we reported on the safety of the combination vaccine and observed that WT1-specific CTLs were strongly induced by the combination vaccine. Furthermore, in accordance with the strong induction of WT1-specific CTLs, cytokine production such as IFN-γ and TNF-α from $CD8^+$ T cells in response to the killer peptide was also observed. Moreover, we confirmed that $WT1_{332}$-specific cytokine production from $CD4^+$ T cells was induced after the combination vaccine. These results supported the combination vaccine to be a promising strategy for cancer immunotherapy. However, it remains unclear what the actual effect of the combination vaccine is on WT1-specific CTL responses because of the lack of comparative analysis using a control cohort who received WT1-derived killer peptide alone, and in addition, there have also been concerns that helper peptides do not always enhance CTL responses specific for co-vaccinated CTL epitope [19, 20]. In this study, we clearly demonstrate here that the combination vaccine with WT1-derived killer and $WT1_{332}$ peptides enhances WT1-specific CTL responses by inducing $WT1_{332}$-specific Th1-type HTLs. Furthermore, we identified two distinct WT1-specific CTL populations that differed in binding capacity to WT1-tetramer, CD5 and TCR expression levels, and responsiveness to WT1 peptide, indicating that these two populations play different roles in WT1-targeted immune responses in cancer patients.

Materials and methods

Patients and peripheral blood mononuclear cells (PBMCs)

In clinical studies reported previously [18, 21], peripheral blood samples were collected at pre-vaccination and post-vaccination (4–7 weeks after the first vaccination) from recurrent glioma patients who received WT1 killer peptide (modified $WT1_{235}$; CYTWNQMNL) vaccine alone or a combination vaccine with WT1 killer peptide and WT1 helper peptide ($WT1_{332}$; KRYFKLSHLQMHSRKH) vaccine. Peripheral blood mononuclear cells (PBMCs) were isolated with Lymphocyte Separation Solution (nacalai tesque, Japan) and frozen until use.

WT1 peptides

HLA-A*24:02-restricted killer peptides, natural $WT1_{235}$ (CMTWNQMNL) and modified $WT1_{235}$ (CYTWNQMNL) [22], and HLA class II-restricted helper peptide, $WT1_{332}$ (KRYFKLSHLQMHSRKH) were purchased from PEPTIDE INSTITUTE INC. (Osaka, Japan).

Antibodies

For flow cytometry analysis, the following monoclonal antibodies (mAbs) were used: anti-CD3-Pacific Blue (UCHT1), anti-CD3-V500 (SP34-2), anti-CD4-V500 (RPA-T4), anti-CD4-APC-H7 (SK3), anti-CD5-PE-Cy7 (L17F12), anti-CD8-V450 (RPA-T8), anti-CCR7-PE-Cy7 (3D12), and anti-IL-2-FITC (MQ1-17H12) purchased from BD Biosciences (San Jose, CA), anti-CD45RA-APC (MEM-56) purchased from Life Technologies (Carlsbad, CA), anti-CD8-FITC (T8) purchased from Beckman Coulter (Brea, CA), anti-αβTCR-PE (IP26), anti-TNF-α-APC (MAb11), and anti-IFN-γ-PE (4S.B3) purchased from eBioscience (San Diego, CA).

WT1-tetramer assay

Frozen PBMCs from patients were thawed and incubated for 1 h at 37 °C in X-VIVO 15 medium (Lonza, Walkersville, MD) supplemented with 10% AB serum (Gemini Bio-Products, Woodland, CA). The cells were passed through 40 µm nylon mesh to remove debris. Half of the cells were used for tetramer assay, and the other half was used for in vitro stimulation with WT1 peptides as described below.

For tetramer assay, these cells were incubated with Clear Back (MBL, Aichi, Japan) in phosphate-buffered saline containing 2% FBS and 0.02% sodium azide (FACS buffer) at room temperature for 5 min and then these cells were stained with PE-labeled HLA-A*24:02/modified $WT1_{235}$ tetramer (WT1 tetramer) (MBL, Aichi, Japan) for 1 h at 4 °C. These cells were then stained with anti-CD3, -CD8, -CD4, -CD45RA, and -CCR7 antibodies for 25 min at 4 °C in the dark, washed three times, and finally resuspended in appropriate quantities of FACS buffer and incubated with 7-AAD (eBioscience) for 5 min before analysis. The cells were analyzed with FACSAria (BD Biosciences). The data were analyzed with FlowJo software (TreeStar, San Carlos, CA). For evaluation of expansion capacity in WT1-tetramer^{high} and WT1-tetramer^{high} cells, tetramer assay was performed again after in vitro stimulation (as described below) and fold expansion was calculated based on the frequency of WT1-tetramer^{+} cells before in vitro stimulation.

In vitro stimulation with WT1 peptides

PBMCs were suspended in 2 ml of X-VIVO 15 medium supplemented with 10% AB serum, 40 IU/ml of human recombinant IL-2 (SHIONOGI & CO., LTD., Osaka, Japan), 1 µg/ml of natural $WT1_{235}$, and 20 µg/ml of $WT1_{332}$ helper peptide. The cell suspensions were plated into one well of a 24-well plate. The cells were cultured for a week and then were used for tetramer assay and intracellular cytokine staining assay.

Evaluation of WT1 peptides-specific cytokine production from T cells

Cells were incubated with or without 1 µg/ml of natural $WT1_{235}$ or 10 µg/ml of $WT1_{332}$ in the presence of 2.5 µl/ml CD28/CD49d Costimulatory Reagent (BD Biosciences) and 10 µg/ml Brefeldin A (Sigma, St Louis, USA) for 5 h. Intracellular staining for cytokines was performed using BD Cytofix/Cytoperm Buffer (BD Biosciences) according to the manufacture's procedures after surface staining of CD3, CD4 and CD8 molecules. The cells were analyzed with FACSAria. The data were analyzed with FlowJo software.

For the assessment of cytokine production from WT1-specific CTL clones, TAP-deficient and HLA-A*24:02-positive cell line, T2-2402 (kindly provided by Kiyotaka Kuzushima, Aichi Cancer Center Research, Aichi, Japan) was used as a stimulator. T cells were stimulated for 4 h with or without WT1 peptides in the presence of T2-2402 (1×10^5 cells) and Brefeldin A. Then, intracellular cytokine staining was performed as described above after surface staining of CD8 molecule.

Establishment of WT1-specific CTL clones from WT1-tetramer^{high} and WT1-tetramer^{low} cells

Frozen PBMCs from Patient 007 at post-vaccination were thawed and stained with WT1-tetramer as described above. Patient 007 showed stable disease and a long overall survival (> 320.1 weeks) in our previous clinical study [18]. WT1-tetramer^{high} and WT1-tetramer^{low} cells were single-cell sorted and expand at 96-well U-bottomed plates in the presence of 1×10^5 irradiated allogeneic PBMCs, 100 IU/ml IL-2, and 2 µg/ml HA16 phytohaemagglutinin (PHA, Remel Inc., Lenexa, KS).

Statistics

Data were analyzed using Prism 6 or 8. Normally and non-normally distributed data were analyzed by parametric (unpaired *t* test) and nonparametric (Mann–Whitney test and Wilcoxon test) tests, respectively. Wilcoxon test and Fisher's exact test were used for comparison between paired samples

(i.e.; pre and post sample) and evaluation of difference in response rates, respectively.

Results

$WT1_{332}$-specific $CD4^+$ T cell responses in patients immunized with WT1 killer and WT1 helper peptide combination vaccine

$WT1_{332}$-specific $CD4^+$ T cell responses were evaluated in patients immunized with WT1 killer and WT1 helper peptide combination (K + H) vaccine or WT1 killer peptide (K) vaccine alone. Th1-type cytokine (IFN-γ, TNF-α, and IL-2) production from the $WT1_{332}$-specific $CD4^+$ T cells by the stimulation with $WT1_{332}$ peptide was measured. As expected, Th1-type $WT1_{332}$-specific cytokine-producing $CD4^+$ T cells were strongly induced in K + H vaccine, whereas they were not induced in K vaccine alone (Fig. 1 and Table 1).

WT1-specific CTL responses at higher frequencies and rates in K + H vaccine compared to K vaccine alone

WT1-specific CTL responses were examined. Frequencies of WT1-tetramer$^+$ $CD8^+$ T cells in $CD8^+$ T cells significantly increased in both K and K + H vaccines at post-WT1 vaccination (Fig. 2a). It was likely that K + H vaccine could induce WT1-specific CTLs at higher frequencies than K vaccine alone although it was not statistically significant ($p = 0.066$). In fact, the induction of WT1-tetramer$^+$ $CD8^+$ T cells at frequencies of ≥ 0.33% (mean + SD) was observed in 7 out of 11 patients (induction rates: 63.6%) immunized with K + H vaccine whereas it was observed in only one out of 11 patients (induction rates: 9.0%) immunized with K vaccine alone (Table 1). Next, cytokine production from $CD8^+$ T cells in response to natural $WT1_{235}$ peptide, which was endogenously expressed in WT1-expressing tumors and could be recognized by WT1-specific CTLs, was examined. Frequencies of natural $WT1_{235}$ peptide-specific IFN-γ-producing $CD8^+$

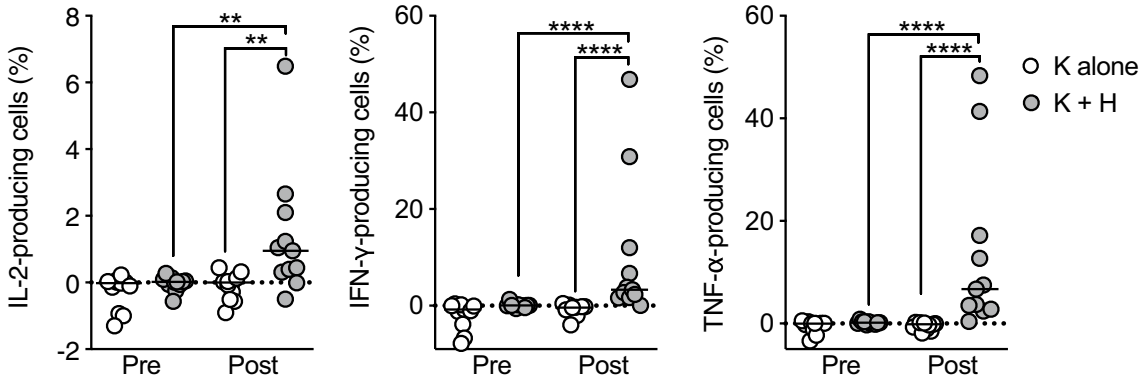

Fig. 1 Induction of $WT1_{332}$-specific Th1-type $CD4^+$ T cells. PBMCs were cultured in the presence of WT1 peptides as described in "Materials and methods" section. One week later, the cells were re-stimulated with/without $WT1_{332}$ for 5 h and evaluated for cytokine (IL-2, IFN-γ, and TNF-α) production from $CD4^+$ T cells by intracellular cytokine staining. The frequencies (%) subtracted the frequencies of cytokine-producing cells in non-stimulated samples from those in $WT1_{332}$-stimulated samples are shown. $^{**}p < 0.01$; $^{***}p < 0.001$

Table 1 Differences between K + H and K alone group in WT1-specific T cell responses

Responses	Response rate		p value[f]
	K + H group	K alone group	
$WT1_{332}$-specific CD4 response[a]	10/11 (90.9%)	0/11 (0%)	*< 0.0001*
Increase in WT1-tetramer + cells[b]	7/11 (63.6%)	1/11 (9.0%)	*0.0237*
Natural $WT1_{235}$-specific TNF-α response[c]	8/11 (72.7%)	5/11 (45.4%)	0.3807
Natural $WT1_{235}$-specific IFN-γ response[d]	9/11 (81.8%)	9/11 (81.8%)	1.0000
Increase in WT1-tetramerhigh cells[e]	8/11 (72.7%)	2/11 (18.1%)	*0.0300*

Positive responses were defined by the following criteria: [a] The percentage is ≥ 1% and increases ≥ 1% from pre-vaccination in Fig. 1, [b] The percentage is ≥ 0.33% (mean + SD of all pre-samples) and increases at post-vaccination in Fig. 2a, [c] The percentage is ≥ 0.76% (mean + SD of all pre-samples) and increases at post-vaccination in Fig. 2b, [d] The percentage is ≥ 0.50% (mean + SD of all pre-samples) and increases at post-vaccination in Fig. 2b, [e] The percentage increases ≥ 0.1% at post-vaccination in Fig. 4b, [f] Fisher's exact test

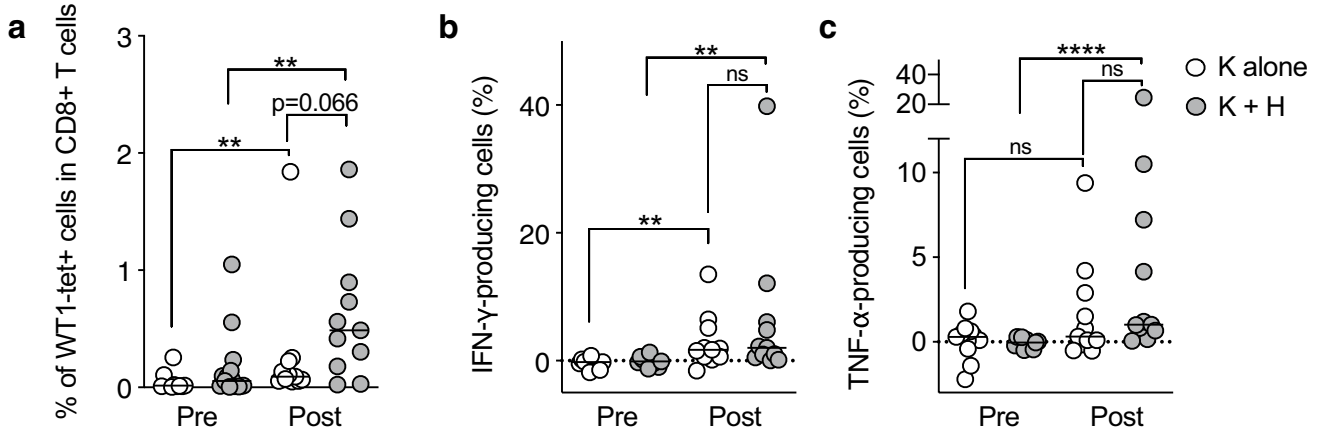

Fig. 2 Induction of WT1-specific CTLs. **a** Frozen PBMC samples were thawed and measured for frequencies of WT1-tetramer$^+$ cells by flow cytometer. Frequencies (%) of WT1-tetramer$^+$ cells in CD8$^+$ T cells are shown. **b**, **c** The thawed PBMCs were cultured in the presence of WT1 peptides as described in "Materials and methods" section. One week later, the cells were re-stimulated with/without natural $WT1_{235}$ for 5 h and cytokine (IFN-γ and TNF-α)-producing CD8$^+$ T cells were evaluated by intracellular cytokine staining. The frequencies (%) subtracted the frequencies of cytokine-producing cells in non-stimulated samples from those in natural $WT1_{235}$-stimulated samples are shown. *$p<0.05$; **$p<0.01$; ***$p<0.001$. *ns* not significant

T cells significantly increased in accordance with the increase in WT1-tetramer$^+$ CD8$^+$ T cells in both K + H and K vaccines although there was no significant difference in both the frequencies and response rates between K + H and K vaccines (Fig. 2b, Table 1). Interestingly, the frequency of WT1-specific TNF-α-producing CD8$^+$ T cells significantly increased in only K + H vaccine (Fig. 2c), and the response rates were higher in K + H vaccine (72.7%) than K vaccine alone (54.4%), although it was not statistically significant (Table 1). Thus, these results indicated that K + H vaccine could more strongly induce WT1-specific CTL responses at both their frequencies and rates, compared to K vaccine alone.

A clear correlation between WT1-specific CTL and $WT1_{332}$-specific CD4$^+$ T cell responses

Whether or not there was a correlation between WT1-specific CTL and $WT1_{332}$-specific CD4$^+$ T cell responses was investigated in K + H vaccine. As shown in Fig. 3a, there was a clear and significant correlation between frequencies of WT1-tetramer$^+$ CD8$^+$ T cells and those of $WT1_{332}$-specific

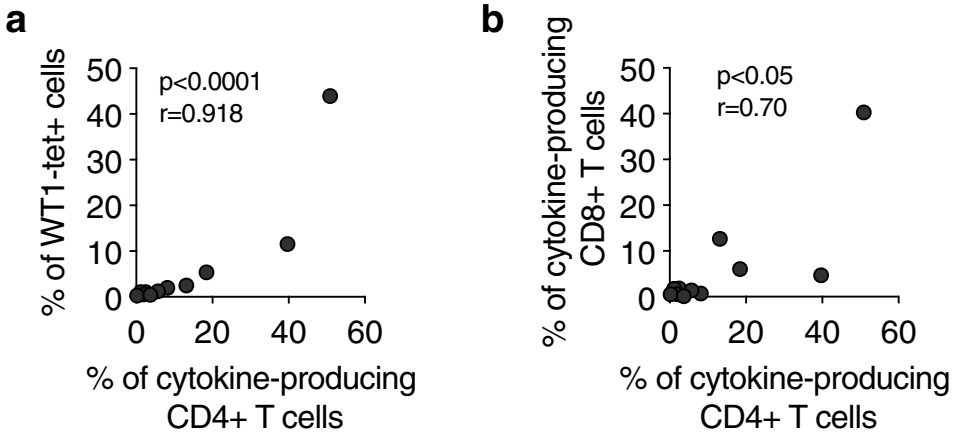

Fig. 3 A clear correlation between $WT1_{332}$-specific HTL and WT1-specific CTL responses. PBMC samples at post-vaccination were cultured with natural $WT1_{235}$ and $WT1_{332}$ peptides for 1 week and examined for the frequencies of WT1-tetramer$^+$ CD8$^+$ T cells, natural $WT1_{235}$-specific cytokine-producing CD8$^+$ T cells, and $WT1_{332}$-specific cytokine-producing CD4$^+$ T cells. Correlation between the frequencies of WT1-tetramer$^+$ CD8$^+$ T cells and $WT1_{332}$-specific cytokine-producing CD4$^+$ T cells (**a**) and between the frequencies of natural $WT1_{235}$-specific cytokine-producing CD8$^+$ T cells and $WT1_{332}$-specific cytokine-producing CD4$^+$ T cells (**b**) was examined. Spearman correlation was used for statistical analysis

IFN-γ and/or TNF-α-producing $CD4^+$ T cells. Furthermore, we also observed a correlation between frequencies of natural $WT1_{235}$-specific IFN-γ and/or TNF-α-producing $CD8^+$ T cells and those of $WT1_{332}$-specific IFN-γ and/or TNF-α-producing $CD4^+$ T cells (Fig. 3b). Thus, these results indicated that $WT1_{332}$-specific $CD4^+$ T cells induced by $WT1_{332}$ helper peptide vaccine strongly promoted the induction and cytokine response of WT1-specific CTLs.

Efficient induction of WT1-tetramer^{high} $CD8^+$ T cells by K + H vaccine

Interestingly, WT1-tetramer^{high} $CD8^+$ T cells were induced at higher frequencies in K + H vaccine than K vaccine alone although it was not statistically significant (Fig. 4a, b and Table 1). Our previous clinical trials showed that there was a correlation between the frequencies of WT1-tetramer^{high} $CD8^+$ T cells and clinical response [23, 24]. However, it remained unclear what roles the WT1-tetramer^{high} $CD8^+$ T cells played in WT1-specific immune responses because they were normally too low in PBMCs to investigate their function. Thus, the enhanced induction of WT1-tetramer^{high} $CD8^+$ T cells by the K + H vaccine allowed us to investigate their properties.

To characterize the WT1-tetramer^{low} and WT1-tetramer^{high} (hereafter, described as WT1-tet^{high} and WT1-tet^{low}, respectively) cells in detail, a number of clones were established by single-cell sorting from WT1-tet^{high} and WT1-tet^{low} $CD8^+$ T cells of PBMCs of Patient 007 and then evaluated for a binding capacity to WT1-tetramer. As shown in Fig. 4c, there was a clear and significant difference in the binding capacity between WT1-tet^{high} $CD8^+$ T cell- and WT1-tet^{low} $CD8^+$ T cell-derived clones. Since the expression levels of WT1-specific TCRs are one of causes to determine WT1-tet^{high} or WT1-tet^{low}, they were examined on WT1-tet^{high} and WT1-tet^{low} clones. As shown in Fig. 4d, the expression levels of WT1-specific TCRs were significantly higher in WT1-tet^{high} clones than WT1-tet^{low} clones. However, there was no correlation ($r = -0.19$) or only weak correlation ($r = 0.54$) between the binding capacity to WT1-tetramer and TCR expression level in WT1-tet^{high} or WT1-tet^{low} clones, respectively (data not shown), indicating that the binding capacity to WT1-tetramer can not be explained only by the expression level of TCR. Next, since the target antigen of WT1-specific $CD8^+$ T cells was natural $WT1_{235}$ peptide that expressed on the patients' tumor cells in a complex with HLA-A*24:02 molecules, the responsiveness to the natural $WT1_{235}$ peptide was examined for WT1-tet^{high} and WT1-tet^{low} clones at low (10 ng/ml) and high (1000 ng/ml) concentrations of the $WT1_{235}$ peptide. Surprisingly, there was a remarkable difference in the responsiveness between the two clones. In WT1-tet^{low} clones, the positive response, which was defined as cytokine production in $\geq 10\%$ $CD8^+$ T cells with stimulation of natural $WT1_{235}$ peptide, was observed in 28 (87.5%) out of the 32 clones at both low and high concentrations (Fig. 4e, upper). On the other hand, in a striking contrast, only 12 (21.0%) and 15 (26.3%) out of the 57 clones showed a positive response at the low and high concentrations, respectively, in WT1-tet^{high} clones (Fig. 4e, lower). These results showed that the cytokine-productive responsiveness to natural $WT1_{235}$ peptide was stronger in WT1-tet^{low} clones than WT1-tet^{high} clones. Importantly, many of WT1-tet^{high} clones that could not produce cytokines in response to natural $WT1_{235}$ peptide showed modified $WT1_{235}$ peptide-specific responses in which frequencies of cytokine-producing $CD8^+$ T cells at the low concentration were almost same with those at the high concentration (data not shown). This finding suggests that they were too high-avidity to modified $WT1_{235}$ peptide to cross-react to natural $WT1_{235}$ peptide.

Next, expansion capacities in response to natural $WT1_{235}$ peptide were compared between WT1-tet^{low} and WT1-tet^{high} cells. There was no significant difference between them (Fig. 4f). Interestingly, CD5 expression was higher in WT1-tet^{high} clones and unsorted WT1-tet^{high} cells than WT1-tet^{low} clones and unsorted WT1-tet^{low} cells, respectively (Fig. 4g, h). Since it had been reported that $CD5^{high}$ $CD8^+$ T cells were protected from apoptosis and that they clonally expanded more efficiently than $CD5^{low}$ $CD8^+$ T cells [25–27], WT1-tet^{high} $CD8^+$ T cells may have higher expansion capacities than WT1-tet^{low} $CD8^+$ T cells in response to natural $WT1_{235}$ peptide (see "Discussion" section about these issues).

Discussion

Although we previously reported the in vitro high immunogenicity of $WT1_{332}$ helper peptide, the immunogenicity in the clinical settings remained unknown until this study. Here, we reported for the first time the high induction rates of $WT1_{332}$ helper peptide-specific $CD4^+$ T cells in the patients who were vaccinated with $WT1_{332}$ helper peptide. We previously reported that $WT1_{332}$ helper peptide-specific $CD4^+$ T cells were spontaneously induced in some cancer patients prior to WT1 vaccination, and the spontaneous induction, or presence of the $CD4^+$ T cells before WT1 vaccination correlated with good clinical responses to K vaccine. These results indicated the high immunogenicity of the $WT1_{332}$ peptide antigen. Therefore, the present results will be compatible with the above findings.

Slingluff et al. reported that response rates to the helper peptides in the patients who were vaccinated with 12 MHC class I-restricted melanoma peptides and a mixture of six melanoma helper peptides (6MHP) or with 6MHP alone were 40% and 41%, respectively [20, 28]. HTL response

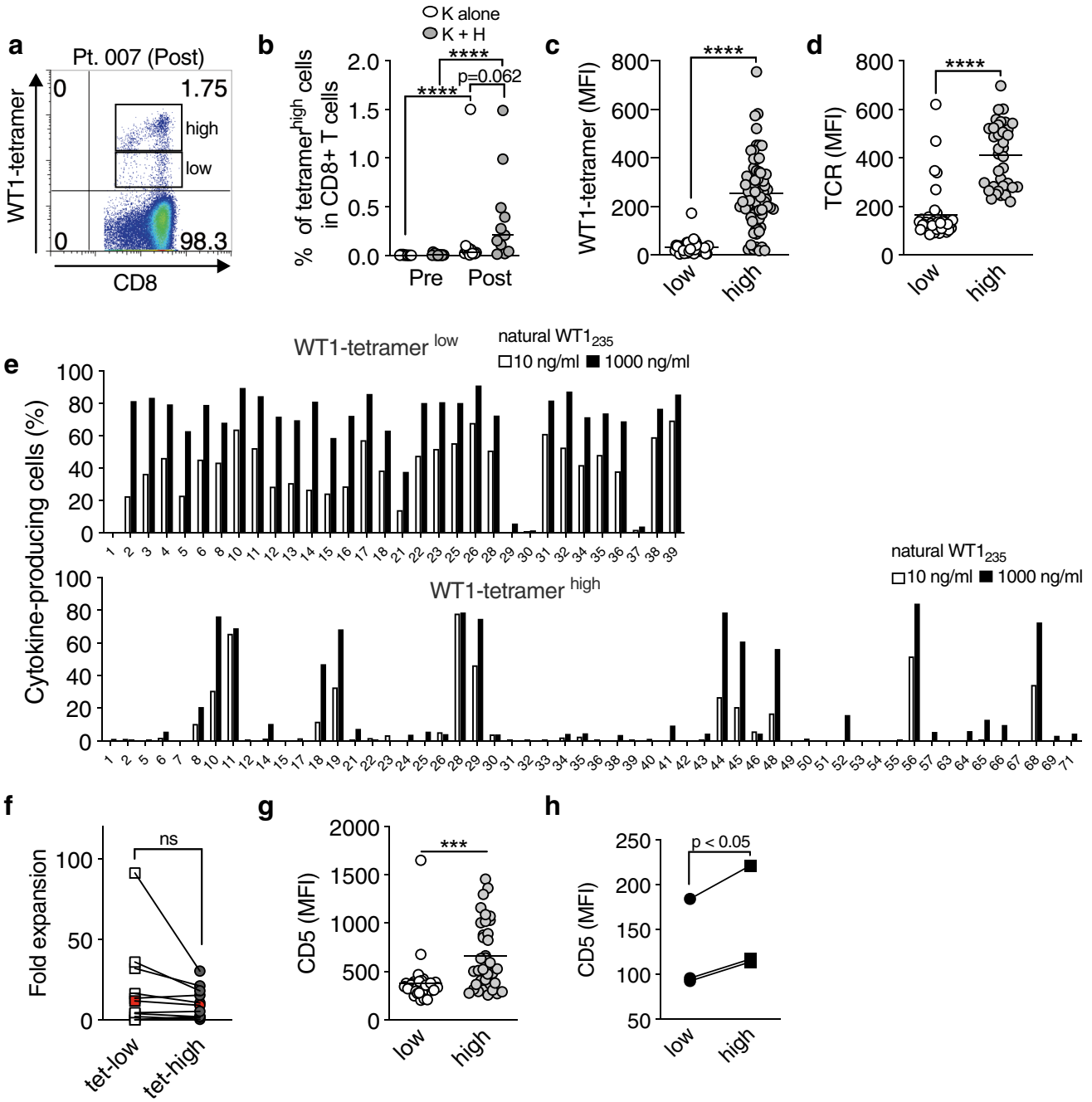

Fig. 4 Difference in function and phenotypes between WT1-tetramer-^{high} and WT1-tetramer^{low} CD8$^+$ T cells. **a** A representative dot plots showing WT1-tetramer^{high} and WT1-tetramer^{low} CD8$^+$ T cells. **b** Frequencies (%) of WT1-tetramer^{high} cells in CD8$^+$ T cells are shown. The binding intensity of WT1-tetramer (**c**) and the expression level of TCR (**d**) in WT1-tetramer^{low} and WT1-tetramer^{high} clones. **e** Cytokine (IFN-γ and/or TNF-α) production in WT1-tetamer^{low} (*upper*) and WT1-tetamer^{high} (*lower*) clones after the stimulation with 10 or 1000 ng/ml of natural $WT1_{235}$ peptide. **f** Fold expansion of WT1-tetramer^{high} and WT1-tetramer^{low} CD8$^+$ T cells after in vitro stimulation with natural $WT1_{235}$ peptide in each patient. The fold expansion was calculated by dividing frequencies of WT1-tetramer-^{high} and WT1-tetramer^{low} CD8$^+$ T cells after in vitro stimulation by those before in vitro stimulation. Red shows Pt. 007. **g** Expression levels of CD5 in WT1-tetramer^{low} and WT1-tetramer^{high} CD8$^+$ T cell clones. **h** CD5 expression levels in WT-tetramer^{low} and -tetramer^{high} CD8$^+$ T cells of fresh PBMCs from three patients (K + H group). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$

rate to a tetanus toxoid-derived helper peptide was only 59% although it was generally a foreign antigen with strong immunogenicity. Thus, it is likely that $WT1_{332}$ helper peptide is higher immunogenic than foreign antigen such as a tetanus toxoid. This finding raises the question of why $WT1_{332}$ helper peptide can be so high immunogenic.

Kobayashi et al. had demonstrated that $WT1_{332}$ helper peptide could bind to several HLA class II molecules such as HLA-DRB1*01:01, 04:05, and 09:01 more stably than the class II invariant chain-associated peptide (CLIP) [15], which occupied the peptide-binding groove of HLA class II molecules and prevented the binding of endogenous self-peptides to the molecules. This strong binding property to HLA class II molecules may determine high immunogenicity of $WT1_{332}$. In fact, it has been shown that the stability of a peptide/MHC class II complex is the principal determinant of the peptide immunogenicity in vivo [29]. Accordingly, high immunogenicity of $WT1_{332}$ helper peptide should be in part ascribed to the stronger binding capability of the peptide to HLA class II molecules.

Preclinical studies using mouse models have demonstrated that CD4 help confers various benefits (for example, resistance to apoptosis, tolerance induction, and exhaustion and enhancement of effector/memory development and migratory potential to inflammation sites) to CTLs [5], supporting the concept that simultaneous induction of both TAAs-specific CTLs and HTLs should dramatically improve anti-tumor immune responses [30, 31]. Consequently, many efforts have been spent to identify human TAA-derived helper peptides capable of binding multiple HLA class II molecules and then inducing HTLs [12, 32, 33]. However, the enhancement of CTL responses by the addition of helper peptide was not induced in human clinical studies [19, 20, 28]. On the other hand, DC vaccines succeeded in enhancing anti-tumor CTL responses by the combination with helper peptides [34–36]. The reason is as follows: It is generally thought that CD4 help plays critical roles in the enhancement of CTL responses, especially at a priming phase, at which HTLs can condition DCs to express CD70 via CD40-CD40L interaction through the recognition of peptide/HLA class II complex by HTLs [37] and that subsequently, the conditioned DCs induce fully functional and exhaustion-resistant $CD8^+$ T cells via CD70-CD27 and peptide/HLA class I complex. Accordingly, the indirect three-cell-interaction via DCs that presented simultaneously both CTL and helper epitopes on their HLA class I and class II molecules, respectively, is essential for optimal induction of CTLs. This recommends us to vaccinate simultaneously with both WT1 CTL and helper peptides. In this study, the enhancement of CTL responses by helper peptide was for the first time demonstrated in a peptide vaccine setting.

In this study, we identified two different populations, WT1-tet^{low} and -tet^{high} $CD8^+$ T cells. Interestingly, WT1-tet^{high} $CD8^+$ T cells expressed CD5 molecule higher than WT1-tet^{low} $CD8^+$ T cells. CD5 expression is known to indicate the potential of clonal expansion and the resistance to activation-induced cell death (AICD) [25–27]. Accordingly, WT1-tet^{high} $CD8^+$ T cells were expected to expand greater than WT1-tet^{low} $CD8^+$ T cells. In fact, although WT1-tet^{high} $CD8^+$ T cells contained natural $WT1_{235}$ peptide-unresponsive cells at higher frequency compared to WT1-tet^{low} $CD8^+$ T cells, there was no significant difference in expansion capacity in response to natural $WT1_{235}$ peptide between the two populations. Importantly, Tabbekh et al. have demonstrated that CD5-deficient tumor antigen-specific $CD8^+$ T cells elicited stronger effector function, such as the production of cytokine and cytotoxicity, than CD5-positive ones, but were more sensitive to AICD than CD5-positive ones, indicating that CD5 protected $CD8^+$ T cells from AICD by attenuating their excess activation [26]. These findings including ours suggested that WT1-tet^{low} $CD8^+$ T cells contributed to induce strong but transient anti-tumor immune responses, while WT1-tet^{high} $CD8^+$ T cells played an important role in moderate but persistent anti-tumor immune responses. Since persistence of tumor antigen-specific $CD8^+$ T cells was crucial for tumor eradication both in mouse and human [38, 39], WT1-tet^{high} $CD8^+$ T cells might be strongly involved in the induction of clinical response to WT1 peptide vaccine. These results should be compatible with the clinical findings that the frequencies of WT1-tet^{high} $CD8^+$ T cells positively correlated with the clinical effect of WT1 peptide vaccine.

Importantly, we observed a clear correlation ($r = 0.872$, $p < 0.001$) between frequencies of WT1-tet^{high} $CD8^+$ T cells and those of WT1-tet^{low} $CD8^+$ T cells (data not shown). In addition, although WT1-tet^{high} $CD8^+$ T cells were thought to be predominantly modified $WT1_{235}$ peptide-specific $CD8^+$ T cells, the frequencies of WT1-tet^{high} $CD8^+$ T cells also correlated with frequencies of natural $WT1_{235}$ peptide-specific cytokine-producing $CD8^+$ T cells ($r = 0.836$, $p < 0.01$, data not shown). These findings demonstrated that the induction of natural $WT1_{235}$ peptide-specific $CD8^+$ T cells deeply links to the induction of modified $WT1_{235}$ peptide-specific $CD8^+$ T cells, which is consistent with the concept that a modified (analog) peptide can used as a vaccine to induce a natural (tumor-expressing) peptide-specific CTLs. In the present study, $WT1_{332}$ helper peptide enhanced the induction of CTLs specific for not only modified $WT1_{235}$ peptide but also natural $WT1_{235}$ peptide and the resultant immune responses revealed two different populations, WT1-tet^{low} and -tet^{high} $CD8^+$ T cells.

The relationship among the binding capacity of TCRs to peptide/MHC multimers, and TCR and CD5 expression levels have been investigated, notably, in murine T cells, but the results were different between experimental models [27, 40]. Mandl et al. showed that the binding capacity of TCRs to peptide/MHC multimers correlated with CD5 expression levels, but did not correlate with TCR expression levels [40]. On the other hand, Fulton et al. had reported that there was no relationship between CD5 expression levels and binding capacity of TCRs to peptide/MHC multimers [27]. To our knowledge, there was no study to investigate

the relationship between CD5 expression levels and binding capacity of TCRs to peptide/MHC multimers in human T cells. Our results showed that WT1-tet^{high} T cells were also TCR^{high} and CD5^{high}, demonstrating clearly a positive correlation among the binding capacity of TCRs to peptide/MHC multimers, and TCR and CD5 expression levels. This inconsistency with previous studies might come from the difference in species (human or mouse) and the specificity of T cells (self-antigen or foreign-antigen). It should be noted that these WT1-tet^{high} CD5^{high} T cells were directly isolated from PBMCs of cancer patients without in vitro culture, demonstrating that this was not an artifact produced by in vitro culture. Thus, a novel population of WT1-tet^{high} CD5^{high} (and TCR^{high}) T cells was described here for the first time.

In this study, we demonstrated for the first time that $WT1_{332}$ was highly immunogenic and enhances the induction of WT1-specific CTLs, promising improvement of clinical outcomes in cancer patients. It should be noted that $WT1_{332}$ could induce not only HTLs but also CD4$^+$ cytotoxic T cells [15, 16, 41]. Accordingly, $WT1_{332}$ could serve as both an enhancer and a direct inducer of anti-tumor immune responses, especially, in patients with cancer expressing both HLA class I and II. A randomized clinical study is needed to clarify the accurate effects of $WT1_{332}$ on clinical responses in cancer patients and should be performed in the near future.

Precis WT1 killer + helper peptide vaccine-induced greater WT1-specific CTL response than WT1 killer peptide vaccine in recurrent glioma patients, thereby revealing the existence of two distinct populations, WT1-tetramer^{high} TCR^{high} CD5^{high} and WT1-tetramer^{low} TCR^{low} CD5^{low} T-cells.

Author contributions FF and HS organized this study and prepared this manuscript. AK and NH recruited the patients, carried out the clinical investigations and collected patients' clinical data. FF, SM, and MI performed the experiments. HN, JN, SN, KH, NH, YoO, and YuO contributed to the acquisition of the patient samples and conception of the study. SS supported this study and gave useful suggestions.

Compliance with ethical standards

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Conflict of interest Shinji Sogo is an employee of Otsuka Pharmaceutical Co., Ltd.

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