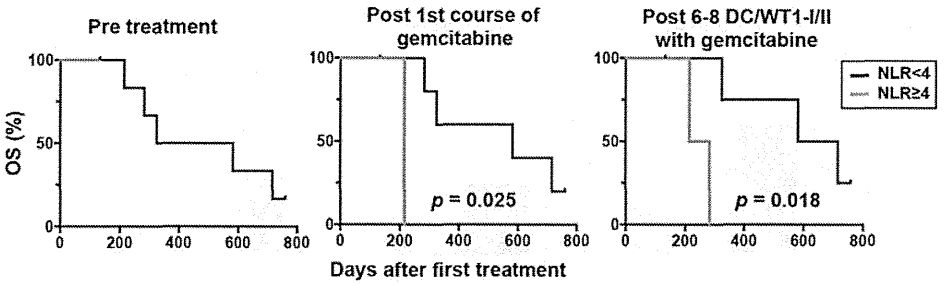

Figure 1. *Association of the neutrophil to lymphocyte ratio with overall survival. The neutrophil to lymphocyte (N/L) ratios of seven pancreatic cancer patients who received chemoimmunotherapy were analyzed prior to treatment (left panel), after the first gemcitabine course (middle panel) and after completing chemoimmunotherapy (right panel). NLR, N/L ratio.*

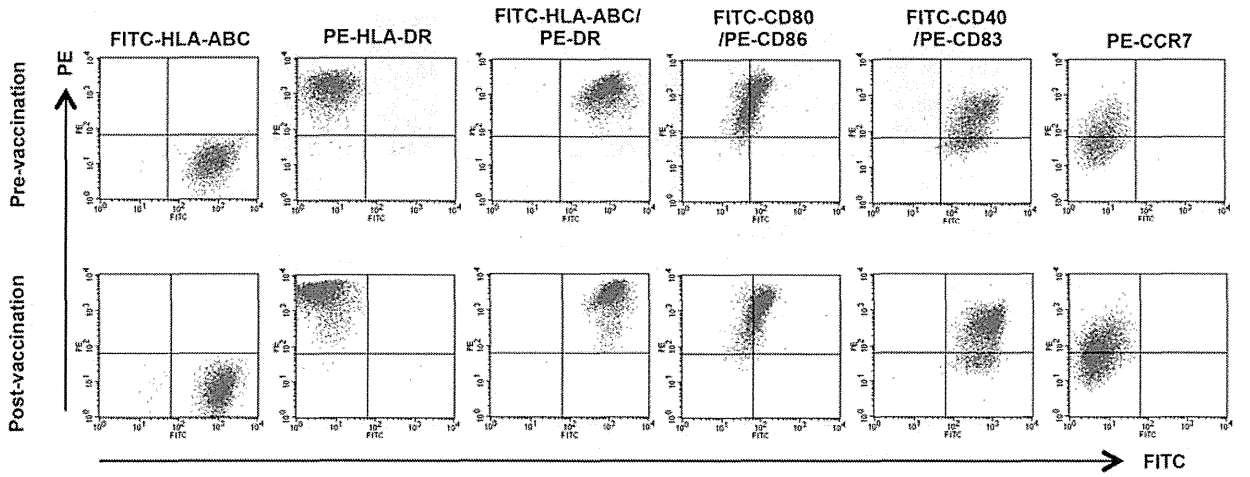

Figure 2. *Dendritic cell phenotypes. The indicated molecules expressed on the dendritic cells (DCs) from a super-responder (Patient no. 6) are shown prior to treatment and after chemoimmunotherapy.*

cultured with MHC-I- and -II-restricted WT1 peptides after which the Th1 cytokine IFN-γ and Th2 cytokine IL-10 concentrations in the supernatants were determined. In this experimental setting, no differences in the IFN-γ and IL-10 concentrations were observed between the super-responders (OS>1 year) and non-super-responders (OS≤1 year) (Figure 6). Moreover, the PBMCs produced extremely high levels of IFN-γ relative to IL-10 (Figure 6).

Discussion

The data presented herein demonstrate that a decreased N/L ratio (<4) and increased HLA-DR and CD83 MFIs may be prognostic markers of chemoimmunotherapeutic outcome.

Results from a recent clinical trial suggest that chemotherapies, such as gemcitabine and S-1, an oral fluoropyridine, are effective chemotherapeutic agents for pancreatic cancer treatment in Japan (10). In that phase III study, the median OS was 8.8 months in the gemcitabine group, 9.7 months in the S-1 group and 10.1 months in the gemcitabine plus S-1 group. Therefore, an OS of >1 year generally indicates that the treatment was beneficial. In the present study, patients who received DC/WT1-I/II vaccinations combined with chemotherapy were classified into 2 groups: OS>1 year (super-responders) and OS≤1 year (non-super-responders). We first analyzed the pre-treatment laboratory data, including the albumin levels (data not shown), CRP levels, neutrophil numbers and lymphocyte numbers. There were no differences between the super-responders and non-super-responders in terms of these factors in our study. Our results support those from a recent report that indicated that the albumin level, CRP level, neutrophil number and lymphocyte number were not prognostic factors for the outcomes in 255 patients who had received standard

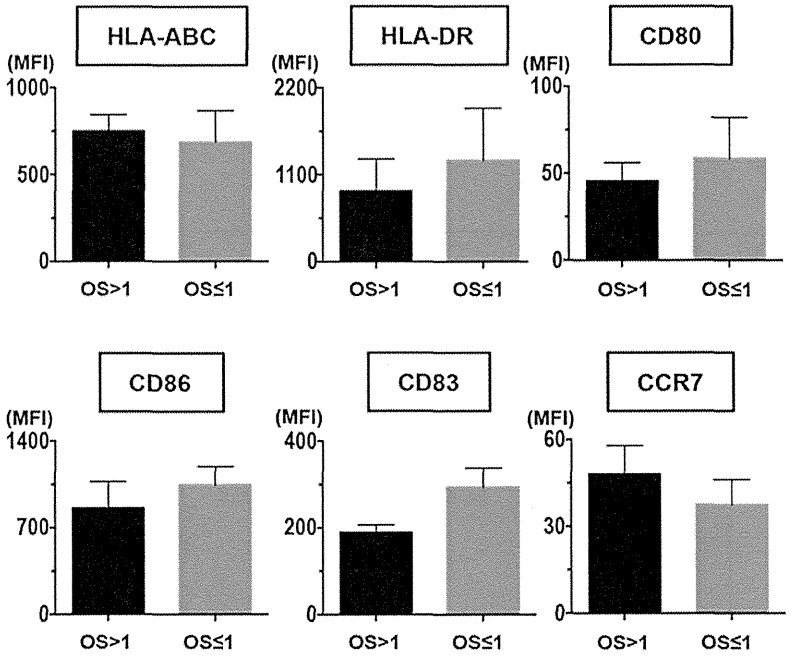

Figure 3. *The mean fluorescent intensities of surface molecules on dendritic cells. The pretreatment mean fluorescent intensities (MFIs) of HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 on dendritic cells (DCs) were compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means±SD.*

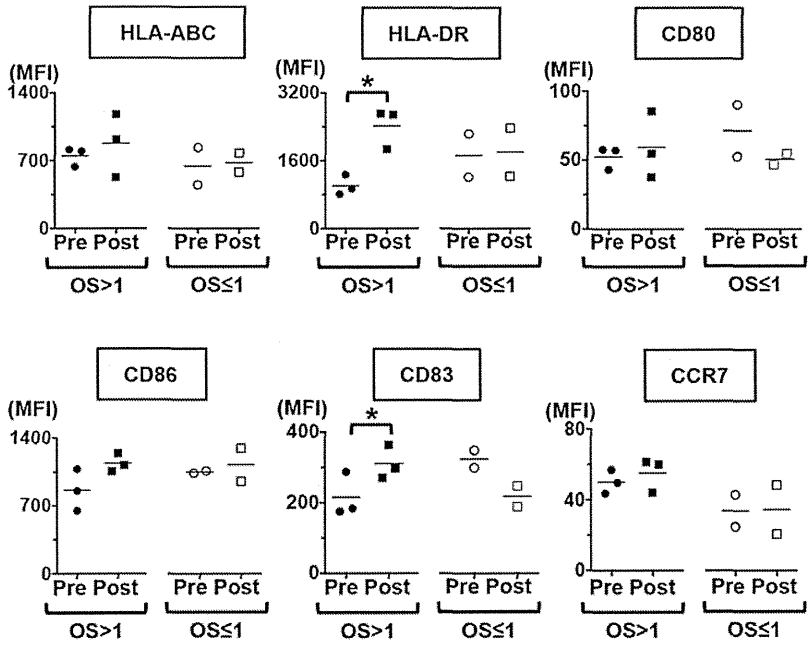

Figure 4. *The pre- and post-chemoimmunotherapy mean fluorescent intensities of surface molecules on dendritic cells. The pre- and post-chemoimmunotherapy mean fluorescent intensities (MFIs) of the indicated molecules on the surfaces of dendritic cells (DCs) were compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means. *p<0.05.*

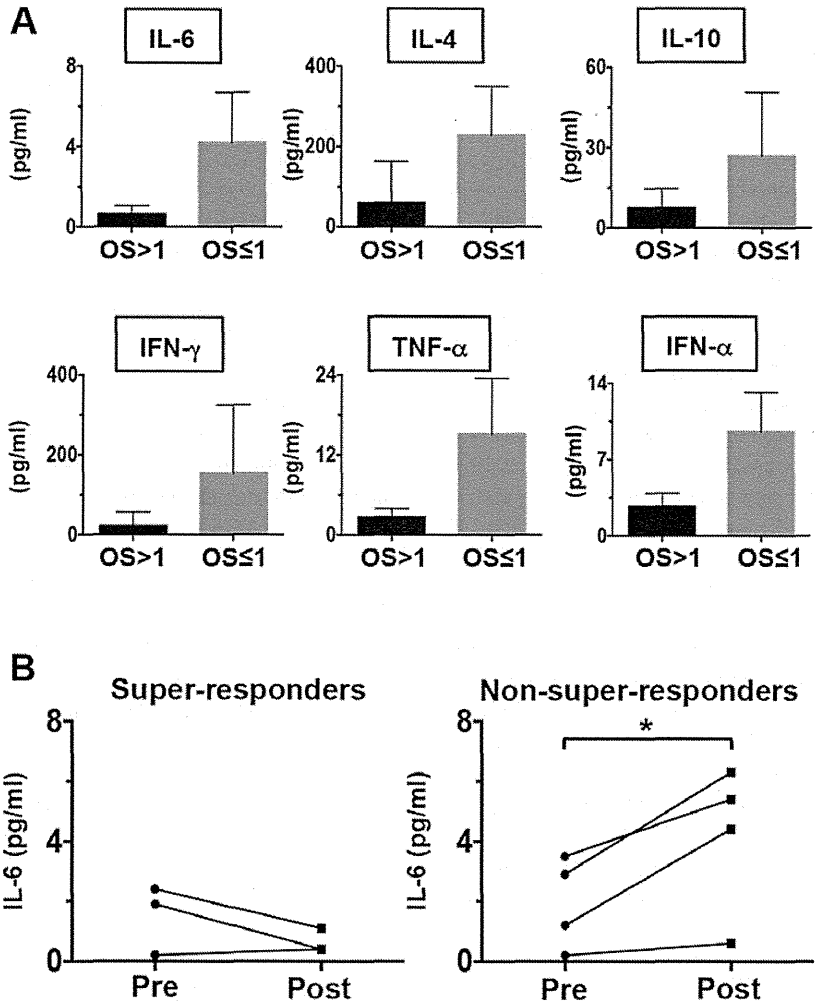

Figure 5. *Plasma cytokine profiles. A. The levels of cytokines (IL-6, IL-4, IL-10, IFN-γ, TNF-α and IFN-α) shown in plasma samples from patients who received 6-8 vaccinations and chemotherapy are compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year). B. IL-6 levels in plasma samples (prior to treatment and after 6-8 vaccinations and chemotherapy) are compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means±SD. *p<0.05.*

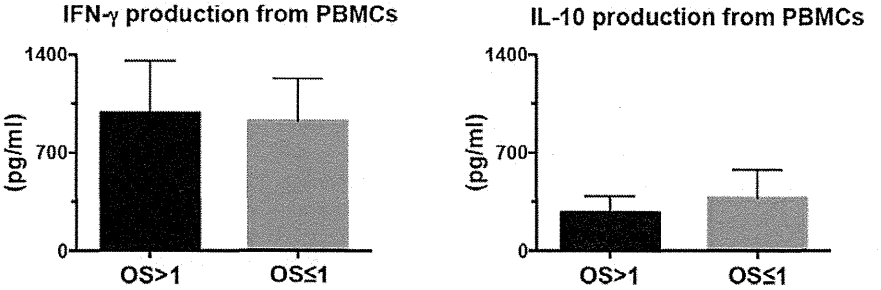

Figure 6. *IFN-γ and IL-10 production by peripheral blood mononuclear cells. IFN-γ and IL-10 production by peripheral blood mononuclear cells after 6 vaccinations is compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year).*

chemotherapy combined with MHC-I-restricted peptide-pulsed DCs (11). Interestingly, a low post-treatment N/R ratio (<4) was associated with a good prognosis (OS>1 year) for pancreatic cancer patients in this study. Previously, an early reduction in the N/L ratio after effective treatment was reported to be associated with improved survival in cancer patients (12). Gemcitabine has been shown to up-regulate antigenic peptides on the HLA molecules of tumor cells (13), increase antigen cross-presentation (14) and decrease the immunosuppressive myeloid-derived suppressive cell (MDSC) (15) and regulatory T-cell (Treg) populations (16), resulting in the augmentation of antitumor immunity. The rapid decrease in the N/R ratio immediately following the initial gemcitabine course, as shown above, may have been induced by the reduced tumor-associated inflammatory and immunosuppressive responses. Moreover, the combination of DC/WT1-I/II vaccination and gemcitabine administration was also associated with additional reductions in the blood N/L ratio. A low N/L ratio was predictive of longer survival in patients with advanced pancreatic cancer who received gemcitabine (17).

The treatment of patients with advanced pancreatic cancer using DC/WT1-I/II vaccination plus gemcitabine-based chemotherapy has been associated with disease stability (8). In a clinical phase I trial, WT1-specific DTH-positive patients exhibited significant improvements in OS and PFS compared to negative controls. Moreover, all patients with strong DTH reactions were super-responders. In DC-based vaccines, autologous DCs are generated from GM-CSF- and IL-4-treated monocytes and subsequently mature through incubation with penicillin-killed and lyophilized preparations of a low-virulence *Streptococcus pyogenes* (OK-432) strain (Su) and prostaglandin E2 (PGE2). The expression levels of HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 on the DCs derived prior to treatment did not differ between patients, thus indicating a uniform DC quality. Interestingly, in the super-responders, the HLA-DR and CD83 expression levels increased significantly after treatment relative to pre-treatment levels. Vaccination with fusion products generated from DCs and whole tumor cells has been reported to result in DC maturation (18, 19). Our results were also consistent with reports in which the phenotypic features of DCs were found to differentiate *in vitro* following vaccination. These results suggested that the increased surface expression of DC markers after treatment indicates improved antigen-presenting function in these cells (18). Patients with advanced pancreatic cancer exhibited impaired DC function; however, gemcitabine improved DC function (20). The significantly increased levels of HLA-DR and CD83 expression on the DCs derived from patients who received DC/WT1-I/II and gemcitabine suggest that chemoimmunotherapy may restore DC function. In the super-responders, the improved DC phenotype (HLA-DR and CD83) may be associated with longer survival.

The plasma cytokine profile may be important when assessing the prognostic markers associated with chemoimmunotherapy. In this study, the pancreatic cancer patients received DC/WT1-I/II vaccines combined with chemotherapy. Therefore, we analyzed the Th1 and Th2 cytokine profiles after vaccinations. Our results revealed no differences between the cytokine profiles of super-responders and non-super-responders. The levels of the immunosuppressive cytokines IL-4, IL-10 and IL-6 were higher in non-super-responders than in super-responders, although this difference was not significant. Unexpectedly, the levels of the Th1 cytokines IFN-γ, TNF-α and IFN-α were also higher in non-super-responders; however, this difference was not significant. We also analyzed in greater detail the Th1 and Th2 responses of PBMCs upon WT1 peptide stimulation *in vitro*. Similarly, there were also no differences between the super-responders and non-super-responders in terms of IFN-γ or IL-10 production by PBMCs after 6-8 vaccinations. Interestingly, IL-6 plasma levels in non-super-responders were significantly increased after chemoimmunotherapy relative to the pretreatment levels. IL-6 is one of the major immunosuppressive cytokines, which can induce tumor progression by manipulating immune responses. Therefore, an increased IL-6 level in plasma may be associated with poor prognosis. The immune checkpoint blockade targeted-agents, such as programmed cell death protein 1(PD-1) and programmed death-ligand 1 (PD-L1) have been used to successfully treat patients with advanced melanoma (21). Therefore, it may be more important to inhibit immuno-suppressive responses than to stimulate immunity in patients with advanced pancreatic cancer. The primary limitation of our study is the relatively small size of the evaluated sample. Further studies are required to evaluate the prognostic markers of chemoimmunotherapy with DC/WT1-I/II.

Conflicts of Interest

The Authors declare that they have no competing interests.

Acknowledgements

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References

1 Steinman RM: The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9: 271-296, 1991.

2 Koido S, Homma S, Takahara A, Namiki Y, Tsukinaga S, Mitobe J, Odahara S, Yukawa T, Matsudaira H, Nagatsuma K, Uchiyama K, Satoh K, Ito M, Komita H, Arakawa H, Ohkusa T, Gong J and Tajiri H: Current immunotherapeutic approaches in pancreatic cancer. Clin Dev Immunol, 2011: 267539, 2011.

3 Tanaka Y, Koido S, Ohana M, Liu C and Gong J: Induction of impaired antitumor immunity by fusion of MHC class II-deficient dendritic cells with tumor cells. J Immunol *174*: 1270-1280, 2005.
4 Koido S, Enomoto Y, Apostolopoulos V and Gong J: Tumor regression by CD4 T-cells primed with dendritic/tumor fusion cell vaccines. Anticancer Res *34*: 3917-3924, 2014.
5 Oji Y, Nakamori S, Fujikawa M, Nakatsuka S, Yokota A, Tatsumi N, Abeno S, Ikeba A, Takashima S, Tsujie M, Yamamoto H, Sakon M, Nezu R, Kawano K, Nishida S, Ikegame K, Kawakami M, Tsuboi A, Oka Y, Yoshikawa K, Aozasa K, Monden M and Sugiyama H: Overexpression of the Wilms' tumor gene WT1 in pancreatic ductal adenocarcinoma. Cancer Sci *95*: 583-587, 2004.
6 Sugiyama H: WT1 (Wilms' Tumor Gene 1): biology and cancer immunotherapy. Jpn J Clin Oncol *40*: 377-387, 2010.
7 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM and Matrisian LM: The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res *15*: 5323-5337, 2009.
8 Koido S, Homma S, Okamoto M, Takakura K, Mori M, Yoshizaki S, Tsukinaga S, Odahara S, Koyama S, Imazu H, Uchiyama K, Kajihara M, Arakawa H, Misawa T, Toyama Y, Yanagisawa S, Ikegami M, Kan S, Hayashi K, Komita H, Kamata Y, Ito M, Ishidao T, Yusa S, Shimodaira S, Gong J, Sugiyama H, Ohkusa T and Tajiri H: Treatment with chemotherapy and dendritic cells pulsed with multiple Wilms' tumor gene 1 (WT1)-specific MHC class I/II-restricted epitopes for pancreatic cancer. Clin Cancer Res *20*: 4228-4239, 2014.
9 Kimura Y, Tsukada J, Tomoda T, Takahashi H, Imai K, Shimamura K, Sunamura M, Yonemitsu Y, Shimodaira S, Koido S, Homma S and Okamoto M: Clinical and immunologic evaluation of dendritic cell-based immunotherapy in combination with gemcitabine and/or S-1 in the patients with advanced pancreatic carcinoma. Pancreas *41*: 195-205, 2012.
10 Ueno H, Ioka T, Ikeda M, Ohkawa S, Yanagimoto H, Boku N, Fukutomi A, Sugimori K, Baba H, Yamao K, Shimamura T, Sho M, Kitano M, Cheng AL, Mizumoto K, Chen JS, Furuse J, Funakoshi A, Hatori T, Yamaguchi T, Egawa S, Sato A, Ohashi Y, Okusaka T and Tanaka M: Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. J Clin Oncol *31*: 1640-1648, 2013.
11 Kobayashi M, Shimodaira S, Nagai K, Ogasawara M, Takahashi H, Abe H, Tanii M, Okamoto M, Tsujitani S, Yusa S, Ishidao T, Kishimoto J, Shibamoto Y, Nagaya M and Yonemitsu Y: Prognostic factors related to add-on dendritic cell vaccines on patients with inoperable pancreatic cancer receiving chemotherapy: a multicenter analysis. Cancer Immunol Immunother *63*: 797-806, 2014.
12 Lee Y, Kim SH, Han JY, Kim HT, Yun T and Lee JS: Early neutrophil-to-lymphocyte ratio reduction as a surrogate marker of prognosis in never smokers with advanced lung adenocarcinoma receiving gefitinib or standard chemotherapy as first-line therapy. J Cancer Res Clin Oncol *138*: 2009-2016, 2012.
13 Takahara A, Koido S, Ito M, Nagasaki E, Sagawa Y, Iwamoto T, Komita H, Ochi T, Fujiwara H, Yasukawa M, Mineno J, Shiku H, Nishida S, Sugiyama H, Tajiri H and Homma S: Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response. Cancer Immunol Immunother *60*: 1289-1297, 2011.
14 Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, Collins EJ, Frelinger JA and Robinson BW: Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. J Immunol *170*: 4905-4913, 2003.
15 Suzuki E, Kapoor V, Jassar AS, Kaiser LR and Albelda SM: Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res *11*: 6713-6721, 2005.
16 Rettig L, Seidenberg S, Parvanova I, Samaras P, Curioni A, Knuth A and Pascolo S: Gemcitabine depletes regulatory T-cells in human and mice and enhances triggering of vaccine-specific cytotoxic T-cells. Int J Cancer *129*: 832-838, 2011.
17 An X, Ding PR, Li YH, Wang FH, Shi YX, Wang ZQ, He YJ, Xu RH and Jiang WQ: Elevated neutrophil to lymphocyte ratio predicts survival in advanced pancreatic cancer. Biomarkers *15*: 516-522, 2010.
18 Neves AR, Ensina LF, Anselmo LB, Leite KR, Buzaid AC, Camara-Lopes LH and Barbuto JA: Dendritic cells derived from metastatic cancer patients vaccinated with allogeneic dendritic cell-autologous tumor cell hybrids express more CD86 and induce higher levels of interferon-gamma in mixed lymphocyte reactions. Cancer Immunol Immunother *54*: 61-66, 2005.
19 Koido S, Homma S, Hara E, Mitsunaga M, Namiki Y, Takahara A, Nagasaki E, Komita H, Sagawa Y, Ohkusa T, Fujise K, Gong J and Tajiri H: *In vitro* generation of cytotoxic and regulatory T cells by fusions of human dendritic cells and hepatocellular carcinoma cells. J Transl Med *6*: 51, 2008.
20 Yanagimoto H, Takai S, Satoi S, Toyokawa H, Takahashi K, Terakawa N, Kwon AH and Kamiyama Y: Impaired function of circulating dendritic cells in patients with pancreatic cancer. Clin Immunol *114*: 52-60, 2005.
21 Zitvogel L and Kroemer G: Targeting PD-1/PD-L1 interactions for cancer immunotherapy. Oncoimmunology *1*: 1223-1225, 2012.

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Transduction of a Novel HLA-DRB1*04:05-restricted, WT1-specific TCR Gene into Human CD4+ T Cells Confers Killing Activity Against Human Leukemia Cells

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Transduction of a Novel HLA-DRB1*04:05-restricted, WT1-specific TCR Gene into Human CD4+ T Cells Confers Killing Activity Against Human Leukemia Cells

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Abstract. *Background/Aim: Wilms' tumor gene 1 (WT1) product is a pan-tumor-associated antigen. We previously identified WT1 protein-derived promiscuous helper peptide, $WT1_{332}$. Therefore, isolation and characterization of the $WT1_{332}$-specific T-cell receptors (TCRs) are useful to develop broadly applicable TCR gene-based adoptive immunotherapy. Materials and Methods: A novel HLA-DRB1*04:05-restricted $WT1_{332}$-specific TCR gene was cloned and transduced into human $CD4^+$ T-cells by using a lentiviral vector. Results: The $WT1_{332}$-specific TCR-transduced $CD4^+$ T-cells showed strong proliferation and Th1-cytokine production in an HLA-DRB1*04:05-restricted, $WT1_{332}$-specific manner. Furthermore, the $WT1_{332}$-specific TCR-transduced $CD4^+$ T-cells could lyse HLA-DRB1*04:05-positive, WT1-expressing leukemia cells in vitro. Conclusion: The novel TCR gene cloned here should be a promising tool to develop adoptive immunotherapy of $WT1_{332}$-specific TCR-transduced $CD4^+$ T-cells for the treatment of WT1-expressing cancer, such as leukemia.*

Wilms' tumor gene 1 (*WT1*) was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor, and encodes a zinc finger transcription factor involved in the regulation of cell differentiation, proliferation and apoptosis (1-3). Abundant expression of the wild-type *WT1* gene has been detected in various kinds of solid tumors and hematological malignancies (4). In particular, the expression levels of *WT1* clearly correlated with disease aggressiveness and prognosis in leukemia (5, 6). Importantly, a recent study has demonstrated that chemotherapy-resistant human leukemia stem cells abundantly expressed *WT1* (7). The accumulating evidence indicated that WT1 should be a good target for treatment of leukemia. In fact, WT1-targeting immunotherapy was effective in leukemia (4, 8-10). Based on accumulated evidence, WT1 has been rated as the most promising tumor-associated antigen (TAA) among 76 TAAs (11).

A number of cancer immunotherapy approaches, such as a TAA-targeting vaccine and adoptive transfer of TAA-specific T-cells and TAA-specific T-cell receptor (TCR)-transduced T-cells, have been devoloped and conducting. However, most of the studies have been focusing on $CD8^+$ T-cells as effector cells, while the studies on $CD4^+$ T-cell as effector cells are limited. We previously identified a WT1-derived HLA class II-restricted peptide, $WT1_{332}$, which could bind to multiple HLA class II molecules and induce strong Th1 response (12-14), and reported that HLA class I(A*24:02)-restricted WT1 peptide vaccination induced $WT1_{332}$-specific $CD4^+$ T-cell responses and that the higher responses were correlated with better clinical outcome indicating the important roles of $CD4^+$ T-cell responses in anticancer immunity (15). Furthermore, we reported that HLA-DPB1*05:01-restricted, $WT1_{332}$-specfic TCR-transduced $CD4^+$ T-cells not only enhanced the induction of WT1-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) but also directly killed leukemic cells

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in vitro (16). These finding raised the concept that adoptive immunotherapy using HLA class II-restricted $WT1_{332}$-specfic TCR-transduced $CD4^+$ T cells should be a promising strategy for cancer treatment, especially for leukemia treatment because leukemia usually expresses HLA class II.

Major obstacles for application of the adoptive immunotherapy to broad-range population are the HLA-restriction and limited expression of tumor-associated antigens (TAAs). A way to solve these problems is cloning of various TCR repertoires that are specific for the complexes consisted of broadly-expressing TAAs (epitopes) and corresponding HLA class II molecules. Since the $WT1_{332}$ helper peptide is derived from a typical pan-TAA, WT1, and since it has the capacity to bind to multiple HLA class II molecules that are frequent in not only Asian but also Caucasian populations, cloning of a $WT1_{332}$-specific TCR gene is useful to the preparation of TCR gene sets for the adoptive immunotherapy. Herein, we cloned a novel HLA-DRB1*04:05-restricted, $WT1_{332}$-specific TCR gene and demonstrated that transduction of this TCR gene into human $CD4^+$ T-cells confers $WT1_{332}$-specific proliferative response, Th1 cytokine production and killing activity against WT1-expressing human leukemia cells. Thus, this novel $WT1_{332}$-specific TCR gene should be a promising tool to develop broadly-applicable TCR gene-based adoptive immunotherapy.

Materials and Methods

Cell lines. The TCRαβ-deficient T-cell lines Jurkat 76 cell line (J76) was obtained from Dr. Hans Stauss (University College London, UK). Endogenously WT1-expressing and HLA-DRB1*04:05-positive MEG-01 (megakaryoblastic leukemia cell line) was obtained from Dr. Masaki Yasukawa (Ehime University, Ehime, Japan). Endogenously WT1-expressing and HLA-DPB1*05:01-positive C2F8 (early erythroblastic leukemia cell line) was kindly provided from Dr. Tatsuo Furukawa (Niigata University, Niigata, Japan) (17). Endogenously WT1-expressing and HLA-DRB1*04:05-negative K562 was obtained from Dr. Yoshiki Akatsuka (Fujita Health University, Aichi, Japan). Epstein-Barr virus (EBV)-transformed B cell line, B-LCL(–) and WT1-expressing B-LCL(+) were previously established from an HLA-DRB1*04:05-positive donor (13). All cell lines were cultured in RPMI1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated FBS (Euro-Ione, Milano, Italia) and 1% penicillin/streptomycin (Nacalai Tesque). X-VIVO™ 15 (Lonza, City, MD, USA) supplemented with 10% AB serum (Gemini, City, NC, USA) and 20 IU/ml interleukin-2 (IL-2) (kindly donated by SHIONOGI & Co., Ltd., Osaka, Japan) was used for culture of peripheral blood mononuclear cells (PBMCs) and T cells.

Antibodies, peptides and reagents. $WT1_{332}$ peptide (KRYFKLS HLQMHSRKH), 14 truncated $WT1_{332}$ peptides (as described in Figure 1), tumor cell lysates, Ac-IETD-Cho (granzyme B inhibitor) and blocking monoclonal antibodies (mAbs) were prepared as described previously (16). For flow cytometry analysis, the following mAbs were used: anti-CD107a-allophycocyanin (APC), anti-CD3-Pacific Blue, anti-CD4-APC-H7, anti-granzyme B-phycoerythrin (PE), anti-IL-5-PE, anti-IL-10-PE, anti-interferon (IFN) γ-PE-Cy7, anti-IFNγ-PE, anti-tumor necrosis factor (TNF) α-APC, anti-granulocyte-macrophage colony-stimulating factor (GM-CSF)-PE, anti-perforin-APC, anti-IL-17A-PE, (eBioscience, San Diego, CA, USA) and anti-IL-2-APC (BioLegend, San Diego, CA, USA).

Generation of $WT1_{332}$-specific $CD4^+$ T cell clones. $WT1_{332}$-specific $CD4^+$ T-cell clones were generated by using the CD154 expression assay as described previously (16, 18-20). Briefly, $WT1_{332}$-primed PBMCs were re-stimulated with the $WT1_{332}$ peptide for 6h and then $CD154^+$ $CD4^+$ T cells were single-cell sorted in 96-well plate. The sorted cells were expanded with phytohemagglutinin (PHA), IL-2 and irradiated allogeneic PBMCs. Then, established single-cell-derived $CD4^+$ T-cell clones were screened for $WT1_{332}$-specificity and used for further experiments.

Cloning of TCR α- and β-genes and preparation of recombinant lentivirus. HLA-DRB1*04:05-restricted, $WT1_{332}$-specific TCR α- and β-genes were amplified from a $WT1_{332}$-specific $CD4^+$ T cell clone (clone K) by using 5`-RACE (rapid amplification of cDNA ends) and separately inserted into a cloning vector as described previously (16). A TCR α-p2A-TCR β cassette was constructed and cloned into CSII-EF-MCS-IRES2-Venus lentiviral vector (kindly provided from Drs. Hiroyuki Miyoshi and Atsushi Miyawaki, RIKEN BioResource Center, Tsukuba, Japan). KOD FX DNA polymerase (Toyobo, Osaka, Japan) and the primers (as listed in Table I) were used to amplify TCRs. Recombinant lentivirus were generated by co-transfecting 293T cells with CSII-EF-MCS-IRES2-Venus encoding $WT1_{332}$-specific TCR gene or empty plasmid, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev (kindly provided by Dr. H Miyoshi) as described previously (16).

*Establishment of HLA-DRB1*04:05-positive K562 cells.* cDNA was synthesized from total RNA of HLA-DRB1*04:05+ PBMCs by Super Script III (Invitrogen Life technologies, Carlsbad, CA, USA). HLA-DRA1-p2A-HLA-DRB1 cassette was constructed with primers as listed in Table I and inserted into the *Not I* and *BamH I* site of pcDNA3.1 (+) expression vector (Invitrogen Life technologies). The establishment of HLA-DRB1*04:05-positive K562 was accomplished by electroporation of the vector.

Transduction of $WT1_{332}$-specific TCR gene into J76 cells. Three hundred thousand J76 cells were added to a 48-well plate and incubated with $WT1_{332}$-specific TCR genes-encoding ($WT1_{332}$-TCR) or control lentivirus (mock=empty vector) in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO, USA). After 12 h of incubation, the medium was changed and transduced cells were further cultured and analyzed for the expression of CD3 molecules on their cell surface.

Generation of $WT1_{332}$-specific TCR gene-transduced $CD4^+$ T-cells. Freshly-isolated $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) mAbs in the presence of 40 IU/ml IL-2 for 2 days. Three hundred thousand of the activated cells were incubated in the presence of recombinant lentivirus and 8 μg/ml polybrene in a RetroNectin (TaKaRa Bio Co., Shiga, Japan)-coated 48-well plate. The plate containing the cells was centrifuged at 1,000 ×g at 33°C for 1 h. After 12 h of incubation, medium change was carried out and the cells were further incubated for 48-72 h. Then, $Venus^+$ $CD4^+$ T cells were sorted as transduced cells by FACSAria (BD Bioscience, San

Table I. *Primers employed.*

Primers	Sequences 5 to 3'
For cloning of TCRs	
Cα 3'UTR-primer	CACAGGCTGTCTTACAATCTTGCAGATC
Cβ1 3'UTR-primer	CTCCACTTCCAGGGCTGCCTTCA
Cβ2 3'UTR-primer	TGACCTGGGATGGTTTTGGAGCTA
For construction of TCR α-p2A-TCR β cassette	
primer 1*(for TCR α 21.2)	CGCTCTGCGGCCGCGCCACCATGGAGACCCTCTTGGGCCTGCTTA
primer 1*(for TCR α 26-1.2)	CGCTCTGCGGCCGCGCCACCATGAGGCTGG
primer 2*	GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGA AAACCCCGGTCCCATGAGCAACC
primer 3*(for TCR α 21.2)	GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGA AGTTCGTGGCTCCGGAACCGCTGGACCAC
primer 3*(for TCR α 26-1.2)	GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGA AGTTCGTGGCTCCGGAACCGCTGGACCAC
primer 4*	CCGGGATCCTCAGAAATCCTTTCTCTTGACCATGGCCAT
For cloning of HLA-DRA1/DRB1*04:05	
DRA1 primer Forward	CGCTCTGGATCCGCCACCATGGCCATAAGTGGAGTCCCTGTGC
DRA1 primer Reverse	GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGA AGTTCGTGGCTCCGGAACCCAGAGGCCCCCTGCGTTCTGCTGCA
DRB1 primer Forward	GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAA ACCCCGGTCCCATGGTGTGTCTGAAGTTCCCTGGAG
DRB1 primer Reverse	CCGGCGGCCGCTCAGCTCAGGAATCCTGTTGGCTGA

*Primer positions are indicated in Figure 1c.

Jose, CA) and re-stimulated with irradiated, $WT1_{332}$ peptide-pulsed autologous PBMCs. Mock-transduced $CD4^+$ T-cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) mAbs in the presence of 40 IU/ml IL-2. One week later, the established $CD4^+$ T-cells were used for various experiments as described below. In order to maintain stably the established $CD4^+$ T-cells, they were re-stimulated with irradiated, $WT1_{332}$-pulsed autologous PBMCs every 10 days.

Intracellular cytokine staining assay and CD107a mobilization assay. For intracellular cytokine staining assays, 1×10^5 $CD4^+$ T-cells were incubated with the respective peptides in the presence of 2 μg/ml CD28/CD49d Costimulatory Reagent and 10 μg/ml Brefeldin A (Sigma) for 4 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 and CD4 molecules. The cells were analyzed with FACSAria. The data were analyzed with the FlowJo software (TreeStar, San Carlos, CA, USA).

For the CD107a mobilization assay, 1×10^5 $CD4^+$ T-cells were incubated with 1×10^5 $WT1_{332}$ peptide-pulsed or -unpulsed HLA-DRB1*04:05-positive K562 in the presence of 2 μM BD GolgiStop™ and anti-CD107a-APC mAb for 5 h. Then, the cells were harvested and intracellular cytokine staining was performed as described above.

Proliferation assay. The proliferative capacity was assessed using a standard [^{3}H]-thymidine incorporation assay, as described previously. In brief, 3×10^4 $CD4^+$ T cells were cultured with 2×10^5 irradiated autologous PBMCs pulsed or unpulsed with tumor lysate and WT1 peptide (20 μg/ml) for 2 days. Subsequently, [^{3}H]-thymidine (Amersham Biosciences, City, NJ, USA) was added to the cell culture and the cells were cultured for further 18 h. For the blocking assays, L243, SPVL3 and B7/21 mAbs were added to the proliferation assays at their optimal concentrations for blocking HLA-DR, -DQ and -DP, respectively.

^{51}Cr release assay. ^{51}Cr release assays were performed as described previously (16). Briefly, target cells (1×10^4 cells) labeled with ^{51}Cr were added to wells containing varying numbers of effector cells in 96-well plates. After 16 h of incubation at 37°C, the supernatant was collected and measured for radioactivity. For granzyme B inhibition, target cells were pre-treated with 100 μM Ac-IETD-Cho, or DMSO as a control, at 37°C for 2 h and used for the experiment.

Statistical analysis. The paired *t*-test was used to assess differences between groups. A p-value <0.05 was considered significant.

Results

*Cloning of TCR genes from HLA-DRB1*04:05-restricted, $WT1_{332}$-specific $CD4^+$ T-cell clone.* In order to establish HLA-DRB1*04:05-restricted, $WT1_{332}$ helper peptide-specific $CD4^+$ T-cell clones, PBMCs from an HLA-DRB1*04:05-positive (HLA-DRB1*04:05/08:03) donor were cultured in the presence of the $WT1_{332}$ peptide for one week and $WT1_{332}$ peptide-specifically activated $CD4^+$ T-cells were single-cell sorted from the PBMCs by using the CD154 assay as described previously (16). Consequently, 32 $WT1_{332}$-specific $CD4^+$ T-cell clones were established. Clone K showed $WT1_{332}$-specific proliferative response, which was remarkably inhibited by addition of anti-

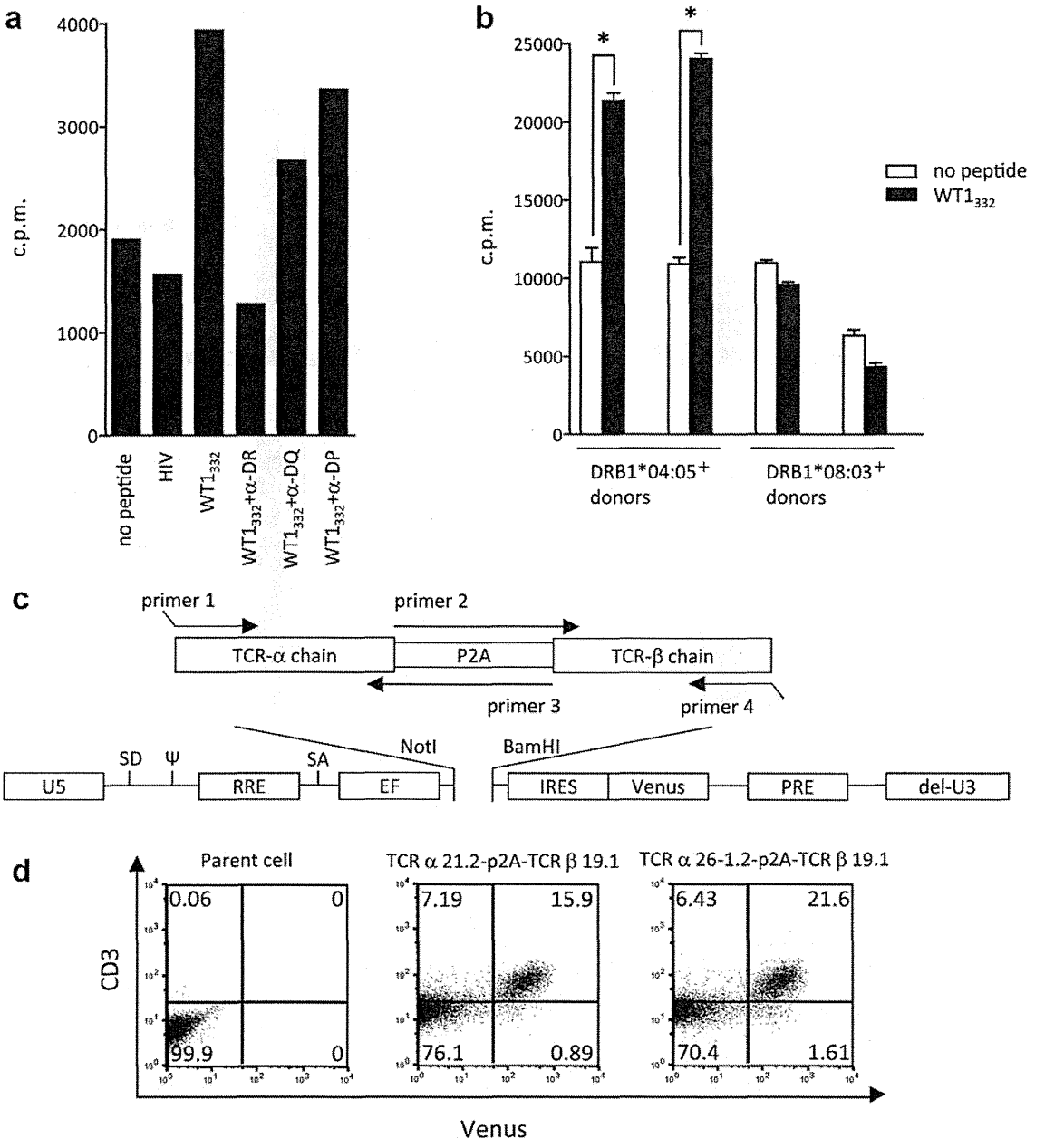


Figure 1. *Continued*

HLA-DR-blocking mAb (partial inhibition, which was frequently observed, by an anti-HLA-DQ-blocking mAb was a non-specific reaction) (Figure 1a). Furthermore, clone K strongly proliferated in response to $WT1_{332}$ peptide-pulsed allogeneic HLA-DRB1*04:05-positve PBMCs but not to $WT1_{332}$ peptide-pulsed allogeneic HLA-DRB1*08:03-positve PBMCs (Figure 1b). These results indicated that clone K was a $WT1_{332}$-specific, HLA-DRB1*04:05-restricted $CD4^+$ T cell clone.

Next, full-length TCR α-chain and β-chain cDNA derived from clone K were isolated and two α-chains (TCR α 21.2 and 26-1.2) and one β-chain (TCR β 19.1) were identified, indicating that allelic exclusion of α-chain in this clone was incomplete. Then, each α-chain was linked with the β-chain *via* the p2A peptide to definitely express both α- and β-chains and TCR α 21.2-p2A TCR β 19.1 and TCR α 26-1.2-p2A TCR β 19.1 cassettes were inserted into the lentiviral

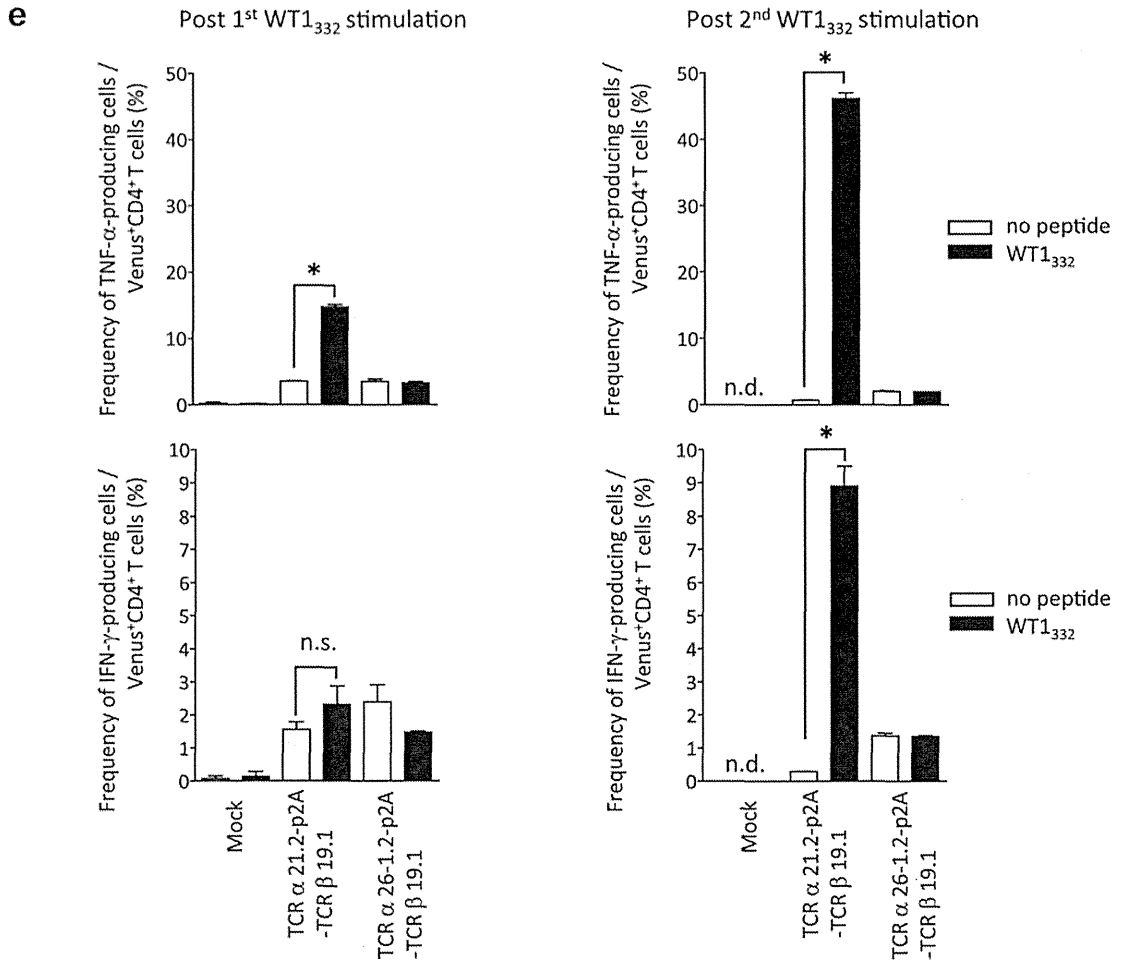
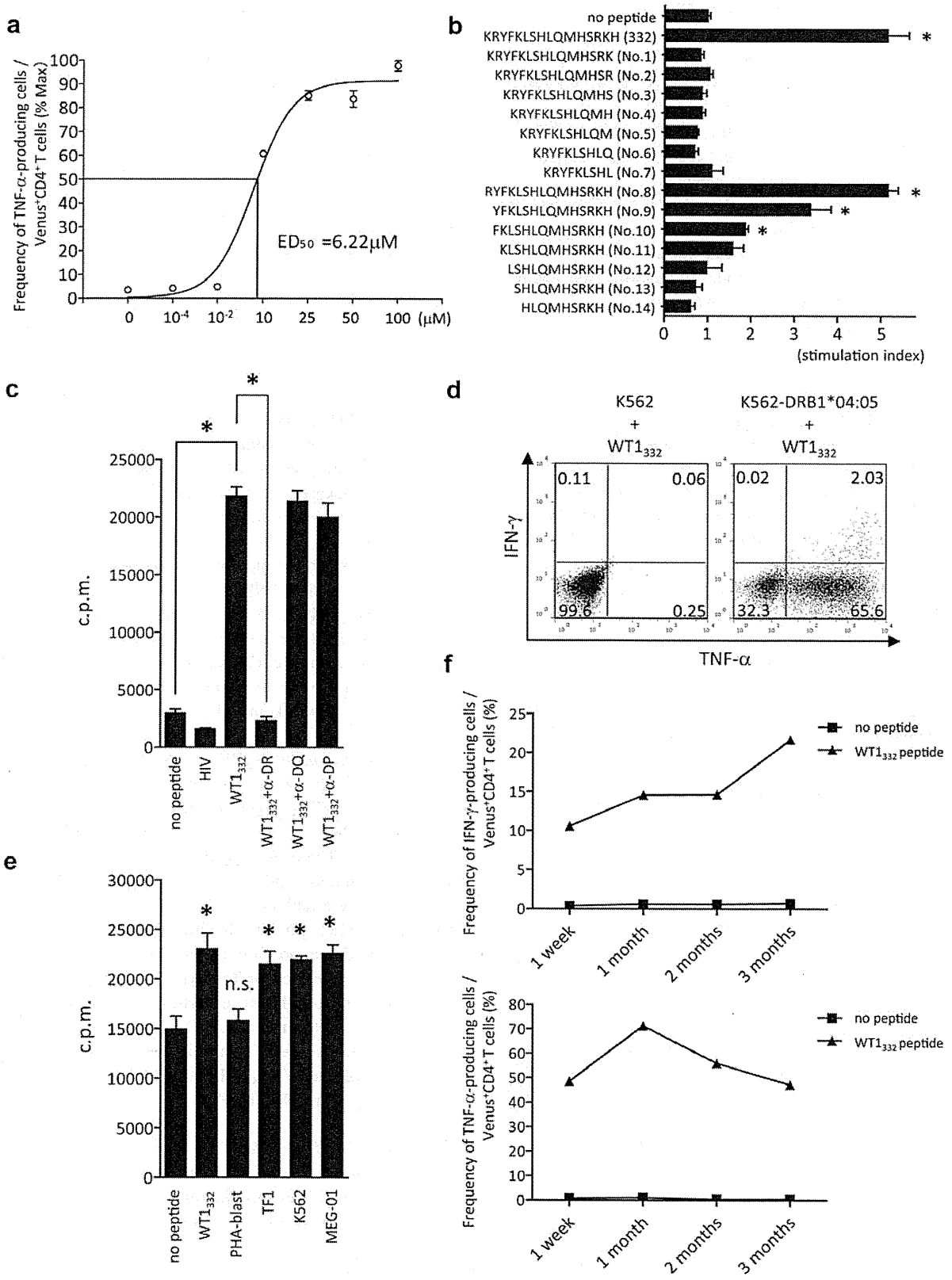


Figure 1. *Cloning and expression of TCR genes isolated from an HLA-DRB1*04:05-restricted $WT1_{332}$-specific $CD4^+$ T-cell clone. (a) Clone K was co-cultured with irradiated autologous peripheral blood mononuclear cells (PBMCs) pulsed with $WT1_{332}$ or irrelevant peptide in the presence or absence of HLA-DR-, HLA-DQ- or HLA-DP-blocking monoclonal antibody (mAb) and tested for proliferative responses (c.p.m., counts per minute). (b) Clone K was co-cultured with HLA-DRB1*04:05- or HLA-DRB1*08:03-positive PBMCs pulsed with or without $WT1_{332}$ peptide and tested for proliferative responses (*p<0.01). (c) Construction of a lentiviral vector encoding full-length TCR α and β genes and primer positions for cloning of TCR (SD, splicing donor site; Ψ, packaging signal; RRE, rev responsive element; SA, splicing acceptor site; EF, human elongation factor 1 α subunit promoter; IRES, encephalomyocarditis virus internal ribosomal entry site; Venus, a variant of yellow fluorescent protein (YFP) gene; PRE, Woodchuck hepatitis virus post-transcriptional regulatory element; del-U3', deletion of enhancer and promoter sequences in the U3 region). (d) CD3 expression in J76 cells after the transduction of lentiviral vector. (e) TNF-α (Upper) and IFN-γ (Lower) expression in each TCR-transduced $CD4^+$ T-cell were analyzed after $WT1_{332}$-restimulation at the indicated time points Data represent mean±SEM from duplicate (b) or triplicate wells (e). (*p<0.01 (b) or 0.05 (e); n.s., not significant; n.d., not done).*

vector (Figure 1c). Since the J76 cell line does not originally express TCR α/β that is required for expression of CD3 molecules on the cell surface, we transfected J76 cells with TCR α 21.2-p2A-TCR β 19.1- or TCR α 26-1.2-p2A-TCR β 19.1-expressing lentivirus and investigated CD3 expression on the cell as a measure of accurate formation of TCR. Transduction of both TCRs yielded comparable frequency of $CD3^+$ cells in $Venus^+$ (a marker for transduction) cells (Figure 1d) indicating that both TCRs could be correctly formed on the J76 cells. To determine whether TCR α 21.2-p2A-TCR β 19.1 or TCR α 26-1.2-p2A-TCR β 19.1 responded to $WT1_{332}$ peptide, $CD4^+$ T-cells obtained from an HLA-DRB1*04:05-positive healthy donor were transfected with TCR α 21.2-p2A-TCR β 19.1-, TCR α 26-1.2-p2A-TCR β 19.1- or mock (empty vector)-encoding lentiviral vector. After 3 days of transfection, the $Venus^+$ $CD4^+$ T-cells were sorted and stimulated every 10 days with irradiated, $WT1_{332}$ peptide-pulsed autologous PBMCs.


a
Frequency of TNF-α-producing cells / Venus+CD4+ T cells (% Max)
ED50 =6.22μM
0 10-4 10-2 10 25 50 100 (μM)
b
no peptide
KRYFKLSHLQMHSRKH (332)
KRYFKLSHLQMHSRK (No.1)
KRYFKLSHLQMHSR (No.2)
KRYFKLSHLQMHS (No.3)
KRYFKLSHLQMH (No.4)
KRYFKLSHLQM (No.5)
KRYFKLSHLQ (No.6)
KRYFKLSHL (No.7)
RYFKLSHLQMHSRKH (No.8)
YFKLSHLQMHSRKH (No.9)
FKLSHLQMHSRKH (No.10)
KLSHLQMHSRKH (No.11)
LSHLQMHSRKH (No.12)
SHLQMHSRKH (No.13)
HLQMHSRKH (No.14)
(stimulation index)
c
c.p.m.
no peptide
HIV
WT1332
WT1332+α-DR
WT1332+α-DQ
WT1332+α-DP
d
K562 + WT1332
K562-DRB1*04:05 + WT1332
IFN-γ
TNF-α
0.11
0.06
99.6
0.25
0.02
2.03
32.3
65.6
e
c.p.m.
n.s.
no peptide
WT1332
PHA-blast
TF1
K562
MEG-01
f
Frequency of IFN-γ-producing cells / Venus+CD4+ T cells (%)
no peptide
WT1332 peptide
1 week
1 month
2 months
3 months
Frequency of TNF-α-producing cells / Venus+CD4+ T cells (%)
no peptide
WT1332 peptide
1 week
1 month
2 months
3 months

Response of each TCR-transduced CD4$^+$ T-cells to $WT1_{332}$ peptide was examined by an intracellular cytokine assay. As shown in Figure 1e, TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T cells showed TNF-α production in response to $WT1_{332}$ peptide after the 1st $WT1_{332}$ stimulation. Furthermore, the response of the CD4$^+$ T cells to $WT1_{332}$ peptide was enhanced after the 2nd $WT1_{332}$ stimulation, leading to an increase in the frequencies of TNF-α, as well as IFN-γ-producing cells. On the other hand, no expression of cytokine in response to $WT1_{332}$ was observed in TCR α 26-1.2-p2A-TCR β 19.1- or mock-transduced CD4$^+$ T-cells, even after the 2nd $WT1_{332}$ peptide stimulation. Thus, the pair of TCR α 21.2 and TCR β 19.1 was identified as a $WT1_{332}$ peptide-responsive TCR.

Functional expression of TCR α 21.2-p2A-TCR β 19.1 in human CD4$^+$ T cells. Next, whether or not the pair of TCR α 21.2 and TCR β 19.1 was specific for the $WT1_{332}$/HLA-DRB1*04:05 complex was investigated. As expected, TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T-cells showed TNF-α production dependently upon the concentration of $WT1_{332}$ peptide (Figure 2a). In addition, the cytokine production was not observed when the TCR-transduced CD4$^+$ T cells were stimulated with truncated WT1 peptides lacking one or more amino acids at the carboxyl terminus or 4 or more amino acids at the amino terminus (Figure 2b). These results showed that core amino acid sequence and minimal epitope for binding of $WT1_{332}$ peptide to HLA-DRB1*04:05 were RYFKLSHLQMHSRKH (amino acids 333-347) and FKLSHLQMHSRKH (amino acids 335-347), respectively. Furthermore, the $WT1_{332}$-specific proliferative response of the TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T cells was markedly inhibited by addition of an anti-HLA-DR-blocking mAb but not anti-HLA-DQ- or DP-blocking mAb (Figure 2c). In order to confirm HLA-DRB1*04:05-restriction of the CD4$^+$ T-cells, the HLA-DRB1*04:05-transduced K562 cell line was established and used as a stimulator. Consistent with Figure 1b, the TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T cells produced a large amount of cytokine only when they were stimulated with $WT1_{332}$ peptide-pulsed HLA-DRB1*04:05-positive K562 cells (Figure 2d). These results clearly demonstrated that TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T cells could specifically recognize $WT1_{332}$ peptide and produce cytokines in an HLA-DRB1*04:05-restricted manner. Thus, "$WT1_{332}$ TCR" was used hereafter instead of "TCR α 21.2-p2A-TCR β 19.1".

To confirm the response of $WT1_{332}$ TCR-transduced CD4$^+$ T-cells ($WT1_{332}$ TCR-Td T cells) to naturally processed cognate epitope, cells were stimulated with WT1-non-expressing or WT1-expressing cell lysate-pulsed autologous PBMCs and then their proliferative responses were examined. Consequently, the $WT1_{332}$ TCR-Td T-cells showed proliferative responses to the PBMCs pulsed with the lysate of WT1-expressing leukemia cell lines (TF-1, K562 and MEG-01) but not to those pulsed with the lysate of PHA blast cells (Figure 2e). $WT1_{332}$-specific IFN-γ production gradually increased during long-term culture (Figure 2f), while $WT1_{332}$-specific TNF-α production reached a peak at one month and gradually decreased; nevertheless, it remained at high levels (approximately 50 %) even after 3 months of culture. These results demonstrated that $WT1_{332}$ TCR-Td T-cells could respond to a natural epitope of WT1 protein and their function was kept stable for long-term culture.

Th1 type-cytokine profile of $WT1_{332}$ TCR-Td T-cells. In our previous study, it had been demonstrated that stimulation of PBMCs by the $WT1_{332}$ peptide could usually induce Th1-type helper CD4$^+$ T-cells with an HLA-DRB1*04:05-restriction (13). Therefore, whether or not $WT1_{332}$ TCR-Td T cells dominantly produced Th1 type-cytokines was investigated. We established $WT1_{332}$ TCR-Td T cells from 3 HLA-DRB1*04:05-positive healthy donors and examined them for cytokine expression by flow cytometry (Figure 3). As expected, expression of Th1-type cytokines (IL-2, IFN-γ, TNF-α and GM-CSF) but not Th2-type (IL-5 and IL-10) or Th17-type cytokines (IL-17) was observed in all established CD4$^+$ T-cells. Thus, it was demonstrated that transduction of the $WT1_{332}$ TCR could confer Th1 type-cytokine profile on CD4$^+$ T cells.

*Cytotoxicity of $WT1_{332}$ TCR-Td T cells against leukemia cells expressing both WT1 and HLA-DRB1*04:05.* Whether the $WT1_{332}$ TCR-Td T-cells could directly recognize and kill WT1-expressing leukemia cells in an HLA-DRB1*04:05-restriction manner was investigated. As expected, the $WT1_{332}$ TCR-Td T-cells effectively lysed HLA-DRB1*04:05-positive,

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Figure 2. *TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T-cells respond to $WT1_{332}$ peptide. TNF-α expression in TCR α 21.2-p2A-TCR β 19.1-transduced CD4$^+$ T cells after the stimulation with various concentrations of $WT1_{332}$ peptide (a) or the indicated WT1 peptides (b): (a) A half maximum effective dose (ED_{50}) is shown. (b) Data are shown as stimulation index (the indicated peptide stimulation/ no peptide stimulation). (c) Proliferative responses in the TCR-transduced CD4$^+$ T-cells were investigated as described in Figure 1a. (d) Cytokine expression in the TCR-transduced CD4$^+$ T cells after the stimulation with K562 or K562-DRB1*04:05 cells pulsed with $WT1_{332}$ peptide. Representative dot plots from duplicate wells are shown. (e) Proliferative responses of $WT1_{332}$ TCR-Td T cells after the stimulation with $WT1_{332}$ peptide, PHA-induced lymphoblast, WT1-expressing TF-1, K562 or MEG-01 leukemia cell lysate-pulsed autologous peripheral blood mononuclear cells (PBMCs). (f) $WT1_{332}$ TCR-Td T cells were tested for $WT1_{332}$-specific IFN-γ (Upper) and TNF-α (Lower) production at the indicated time points. Data represent mean±SEM from triplicate well (a, c and e), four experiments (b) or triplicate assays (f). *$p<0.05$ (b and e) or 0.01 (c); c.p.m., counts per minute.*

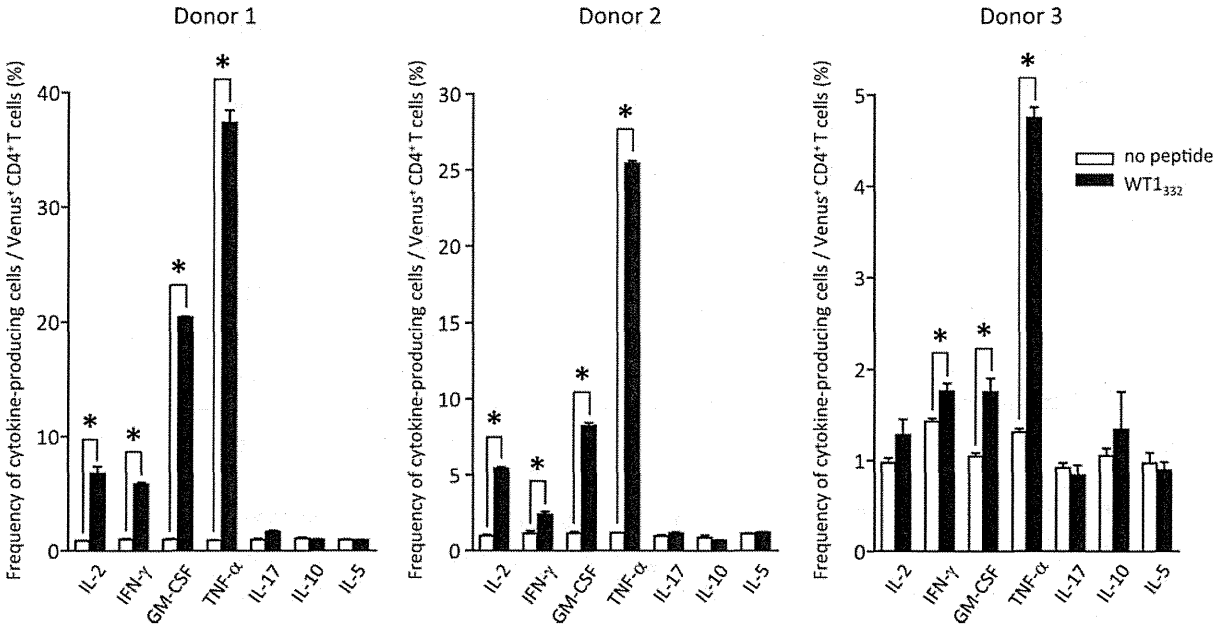

Figure 3. *Th1 type-cytokine profile of $WT1_{332}$ TCR-Td T cells. $WT1_{332}$ TCR-Td T-cells were established from 3 different HLA-DRB1*04:05-positive healthy donors and analyzed cytokine profile. Columns represent mean±SEM of results from triplicate wells. *p<0.05.*

WT1-expressing cells compared to HLA-DRB1*04:05-negative, WT1-expressing or HLA-DRB1*04:05-positive, WT1-non-expressing cells (Figures 4a and b). The $WT1_{332}$ TCR-Td T-cells could also kill endogenously the HLA-DRB1*04:05- and WT1-expressing leukemia cell line MEG-01 originated from human leukemia (Figure 4c). Cytotoxicity of mock-transduced $CD4^+$ T-cells (mock-Td T-cells) was weak or undetectable against these target cells (data not shown).

Since it is known that the perforin/granzyme B pathway is associated with cytotoxic activity in not only $CD8^+$ CTLs but also $CD4^+$ CTLs, expression of perforin and granzyme B was examined in the $WT1_{332}$ TCR-Td T cells. As shown in Figure 4d, the cells showed high expression of both perforin and granzyme B. Furthermore, when the $WT1_{332}$ TCR-Td T-cells were stimulated with $WT1_{332}$ peptide-pulsed HLA-DRB1*04:05-positive K562 cells, they produced IFN-γ and expressed CD107a, a marker of degranulation, indicating the activation of the perforin/granzyme B pathway (Figure 4e). Finally, we confirmed whether the cytotoxicity of the $WT1_{332}$ TCR-Td T-cells was dependent on the perforin/granzyme B pathway. Cytotoxicity significantly decreased against granzyme B inhibitor-treated target cells compared to control DMSO-pretreated target cells (Figure 4f). These results clearly demonstrated that $WT1_{332}$ TCR-Td T-cells exerted a cytotoxic activity against WT1-expressing leukemia cells through a perforin/granzyme B pathway in an HLA-DRB1*04:05-restricted manner.

Discussion

We have successfully cloned an HLA-DPB1*05:01-restricted, $WT1_{332}$-specific TCR gene. As demonstrated previously, $WT1_{332}$ could bind to multiple HLA class II molecules, including HLA-DRB1*04:05, 15:01, 15:02, DPB1*09:01 and 05:01, which are frequent in Asian populations. In addition, it has been recently reported that $WT1_{332}$ also binds to HLA-DRB1*07:01 and DRB3*02:02 molecules that are highly prevalent among Caucasians. This promiscuous binding property of $WT1_{332}$ has the primary advantage to overcome the limited application, caused by HLA restriction, of TCR gene-based adoptive immunotherapy. Accordingly, preparation and cloning of a $WT1_{332}$-specific TCR gene should be useful to develop broadly applicable TCR gene-based adoptive immunotherapy approaches. In the present study, based on this concept, we cloned a novel HLA-DRB1*04:05-restricted, $WT1_{332}$-specific TCR gene and showed that the TCR-transduced $CD4^+$ T-cells could proliferate and produce Th1 cytokine in response to $WT1_{332}$/HLA-DRB1*04:05 complex and exert direct killing activity against HLA-DRB1*04:05-positive, WT1-expressing human leukemia cells. The TCR gene cloned here broadens the application of adoptive immunotherapy targeting WT1.

There exists accumulating evidence that adoptive T-cell immunotherapy of human tumor associated antigen (TAA)-specific TCR-transduced $CD8^+$ T cell is effective and feasible

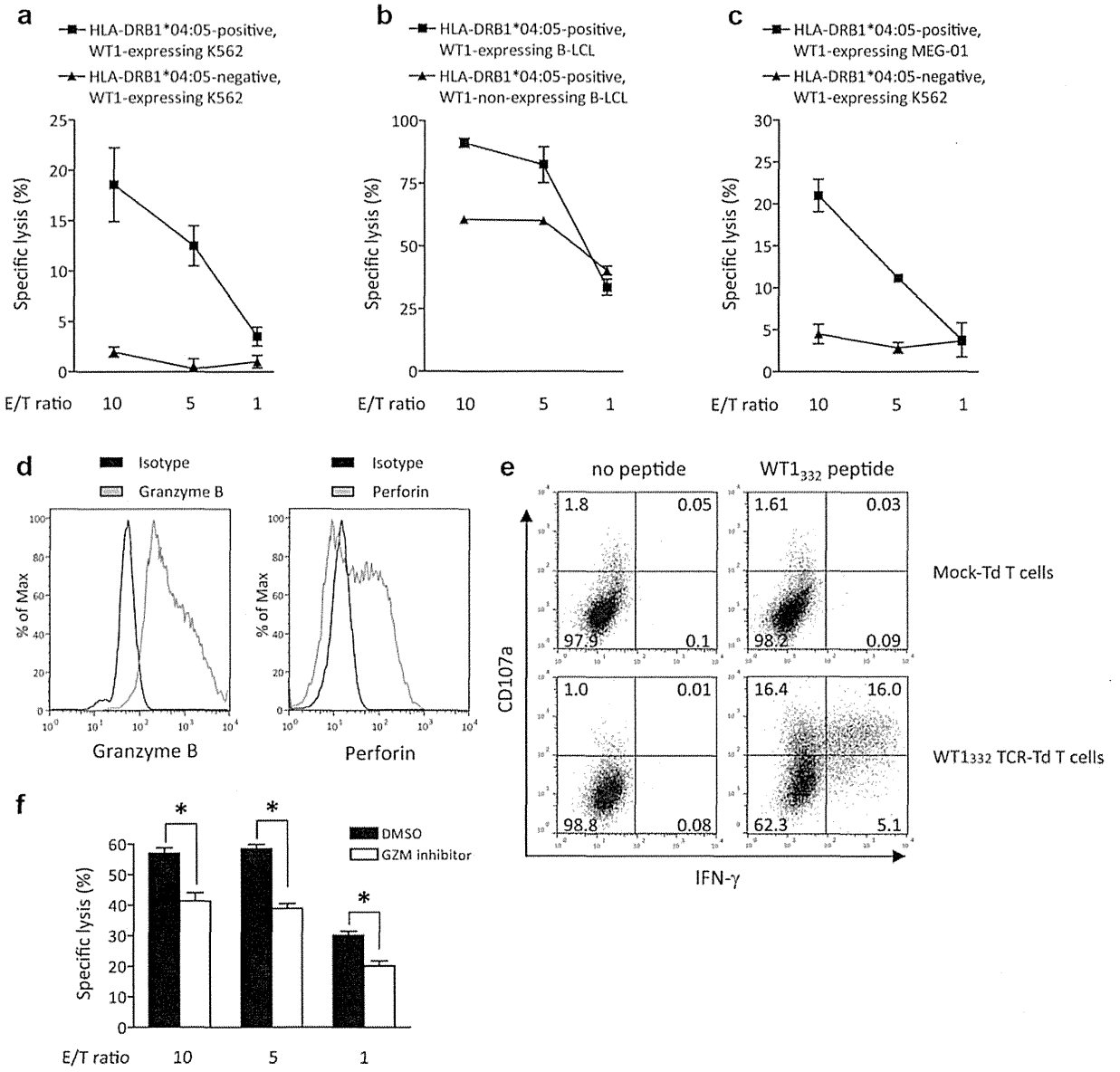

Figure 4. *Direct killing of leukemia cells by $WT1_{332}$ TCR-Td T-cells in an HLA-DRB1*04:05-restricted, $WT1_{332}$-specific manner. (a-c) $WT1_{332}$ TCR-Td T-cells were tested for cytotoxicity against the indicated target cells. These experiments were repeated several times and similar results were obtained (E/T ratio, ratio of effector:target cells). (d) Expression of perforin and granzyme B in $WT1_{332}$ TCR-Td T-cells. Representative histograms are shown. (e) CD107a mobilization and IFN-γ expression in $WT1_{332}$ TCR- and mock-Td T-cells were measured after the incubation with $WT1_{332}$ peptide-pulsed or -unpulsed HLA-DRB1*04:05-positive K562. The plots are gated on Venus$^+$ CD4$^+$ T-cells. (f) Killing activity of $WT1_{332}$ TCR-Td T-cells against granzyme B inhibitor-pretreated HLA-DRB1*04:05-positive K562 cells. These experiments were repeated several times and similar results were obtained. Data represent mean±SEM from triplicate wells (a-c and f). *p<0.05. GZM, granzyme; DMSO, dimethyl sulfoxide; E/T ratio, ratio of effector:target cells.*

for treatment of cancer patients. However, the evidence that CD4$^+$ T-cells can play direct cytotoxic roles in tumor eradication is limited. Previous investigations have indicated that perforin/granzyme B-dependent CD4$^+$ CTLs should be effector cells for cancer immunotherapy (21, 22). However, the direct anti-tumor effect of CD4$^+$ CTLs remained obscure *in vivo*, especially in humans. Using a non-obese diabetic/severe combined immunodeficient (NOD/SCID) murine model, Stevanovic *et al.* showed that HLA class II-mismatched CD4$^+$ T-cell infusion induced complete remission

in NOD/SCID mice that were implanted with primary leukemia cells from patients and that the infused $CD4^+$ T-cells acquired the mismatched HLA class II-restricted cytotoxicity against leukemic cells *in vivo*, thus suggesting a direct anti-tumor effect of human $CD4^+$ CTLs (23). However, the kind of $CD4^+$ T-cells, for example, TAA-specific $CD4^+$ T-cells, exerted anti-tumor effect remained obscure. To solve this issue, adoptive transfer of TCR gene-transduced $CD4^+$ T-cell was thought to be a good experimental model. A recent study reported that HLA-DRB1*04:05-transgenic NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mouse was generated and assumed to be useful to evaluate human $CD4^+$ T-cell function *in vivo*. These experimental tools, including the $WT1_{332}$-specific TCR gene cloned here, allow us to address accurate anti-tumor (leukemia) effect of TAA-specific $CD4^+$ CTLs.

In conclusion, a novel HLA-DRB1*04:05-restricted, $WT1_{332}$-specific TCR gene was successfully cloned and the transduction of the TCR gene into human $CD4^+$ T-cells conferred killing activity against WT1-expressing leukemia cells. Thus, this novel $WT1_{332}$-specific TCR gene should be a promising tool to develop broadly applicable TCR gene-based adoptive immunotherapy. Whether the TCR-transduced $CD4^+$ T-cells can exert *in vivo* anti-tumor activity is now under study.

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Conflicts of Interest/Financial Disclosure

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References

1 Hohenstein P and Hastie ND: The many facets of the Wilms' tumour gene, WT1. Hum Mol Genet 15 Spec No 2: R196-201, 2006.

2 Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H and Lewis WH: Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell *60*: 509-520, 1990.

3 Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH and Bruns GA: Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature *343*: 774-778, 1990.

4 Sugiyama H: WT1 (Wilms' tumor gene 1): biology and cancer immunotherapy. Jpn J Clin Oncol *40*: 377-387, 2010.

5 Inoue K, Ogawa H, Yamagami T, Soma T, Tani Y, Tatekawa T, Oji Y, Tamaki H, Kyo T, Dohy H, Hiraoka A, Masaoka T, Kishimoto T and Sugiyama H: Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. Blood *88*: 2267-2278, 1996.

6 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T and Nasu K: WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood *84*: 3071-3079, 1994.

7 Saito Y, Kitamura H, Hijikata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, Uchida N, Suzuki N, Sone A, Najima Y, Ozawa H, Wake A, Taniguchi S, Shultz LD, Ohara O and Ishikawa F: Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. Sci Transl Med *2*: 17ra19, 2010.

8 Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, Wu F, Fujiki F, Murakami M, Masuda T, Nishida S, Shirakata T, Nakatsuka S, Sasaki A, Udaka K, Dohy H, Aozasa K, Noguchi S, Kawase I and Sugiyama H: Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc Natl Acad Sci USA *101*: 13885-13890, 2004.

9 Tsuboi A, Oka Y, Kyo T, Katayama Y, Elisseeva OA, Kawakami M, Nishida S, Morimoto S, Murao A, Nakajima H, Hosen N, Oji Y and Sugiyama H: Long-term WT1 peptide vaccination for patients with acute myeloid leukemia with minimal residual disease. Leukemia *26*: 1410-1413, 2012.

10 Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, Duerkopp N, Roberts IM, Pogosov GL, Ho WY, Ochsenreither S, Wölfl M, Bar M, Radich JP, Yee C and Greenberg PD: Transferred WT1-reactive $CD8^+$ T cells can mediate antileukemic activity and persist in post-transplant patients. Sci Transl Med *5*: 174ra127, 2013.

11 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM and Matrisian LM: The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res *15*: 5323-5337, 2009.

12 Anguille S, Fujiki F, Smits EL, Oji Y, Lion E, Oka Y, Berneman ZN and Sugiyama H: Identification of a Wilms' tumor 1-derived immunogenic CD4(+) T-cell epitope that is recognized in the context of common Caucasian HLA-DR haplotypes. Leukemia *27*: 748-750, 2013.

13 Fujiki F, Oka Y, Tsuboi A, Kawakami M, Kawakatsu M, Nakajima H, Elisseeva OA, Harada Y, Ito K, Li Z, Tatsumi N, Sakaguchi N, Fujioka T, Masuda T, Yasukawa M, Udaka K, Kawase I, Oji Y and Sugiyama H: Identification and characterization of a WT1 (Wilms Tumor Gene) protein-derived HLA-DRB1*0405-restricted 16-mer helper peptide that promotes the induction and activation of WT1-specific cytotoxic T lymphocytes. J Immunother *30*: 282-293, 2007.

14 Fujiki F, Oka Y, Kawakatsu M, Tsuboi A, Nakajima H, Elisseeva OA, Harada Y, Li Z, Tatsumi N, Kamino E, Shirakata T, Nishida S, Taniguchi Y, Kawase I, Oji Y and Sugiyama H: A WT1 protein-derived, naturally processed 16-mer peptide, WT1(332), is a promiscuous helper peptide for induction of WT1-specific Th1-type CD4(+) T cells. Microbiol Immunol *52*: 591-600, 2008.

15 Fujiki F, Oka Y, Kawakatsu M, Tsuboi A, Tanaka-Harada Y, Hosen N, Nishida S, Shirakata T, Nakajima H, Tatsumi N, Hashimoto N, Taguchi T, Ueda S, Nonomura N, Takeda Y, Ito T, Myoui A, Izumoto S, Maruno M, Yoshimine T, Noguchi S, Okuyama A, Kawase I, Oji Y and Sugiyama H: A clear correlation between WT1-specific Th response and clinical response in WT1 CTL epitope vaccination. Anticancer Res *30*: 2247-2254, 2010.

16 Lin Y, Fujiki F, Katsuhara A, Oka Y, Tsuboi A, Aoyama N, Tanii S, Nakajima H, Tatsumi N, Morimoto S, Tamanaka T, Tachino S, Hosen N, Nishida S, Oji Y, Kumanogoh A and Sugiyama H: HLA-DPB1*05: 01-restricted $WT1_{332}$-specific TCR-transduced $CD4^+$ T lymphocytes display a helper activity for WT1-specific CTL induction and a cytotoxicity against leukemia cells. J Immunother *36*: 159-170, 2013.

17 Furukawa T, Koike T, Ying W, Kishi K, Aoki S, Gotoh T, Hashimoto S, Saitoh H, Hanano M and Shinada S: Establishment of a new cell line with the characteristics of a multipotential progenitor from a patient with chronic myelogenous leukemia in early erythroblastic crisis. Leukemia *8*: 171-180, 1994.

18 Chattopadhyay PK, Yu J and Roederer M: A live-cell assay to detect antigen-specific $CD4^+$ T cells with diverse cytokine profiles. Nat Med *11*: 1113-1117, 2005.

19 Chattopadhyay PK, Yu J and Roederer M: Live-cell assay to detect antigen-specific $CD4^+$ T-cell responses by CD154 expression. Nat Protoc *1*: 1-6, 2006.

20 Tachino S, Fujiki F, Oka Y, Tsuboi A, Morimoto S, Lin YH, Tamanaka T, Kondo K, Nakajima H, Nishida S, Hosen N, Oji Y, Kumanogoh A and Sugiyama H: Functional human Th17 clones with WT1-specific helper activity. Cancer Immunol Immunother *62*: 801-810, 2013.

21 Brown DM: Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy. Cell Immunol *262*: 89-95, 2010.

22 Hidalgo LG, Einecke G, Allanach K and Halloran PF: The transcriptome of human cytotoxic T cells: similarities and disparities among allostimulated CD4(+) CTL, CD8(+) CTL and NK cells. Am J Transplant *8*: 627-636, 2008.

23 Stevanović S, Griffioen M, Nijmeijer BA, van Schie ML, Stumpf AN, Rutten CE, Willemze R and Falkenburg JH: Human allo-reactive $CD4^+$ T cells as strong mediators of anti-tumor immunity in NOD/scid mice engrafted with human acute lymphoblastic leukemia. Leukemia *26*: 312-322, 2012.

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