Reader-free ELISPOT assay for immuno-monitoring in peptide-based cancer vaccine immunotherapy

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Abstract. Cancer vaccine immunotherapy is a therapy that induces cellular immune responses against a target molecule to elicit clinical anti-tumor effects. These cellular immune responses against the target molecule are monitored to evaluate whether the antigen-specific cellular immune responses are induced and maintained during the vaccination period. Enzyme-linked immunospot (ELISPOT) assay is widely performed to analyze not only the frequency of immune cells, but also their effector functions as determined by their cytokine production/secretion. The present study aimed to develop a reader-free ELISPOT assay using a handy membrane-punching device termed ELI 8. With the assistance of particle analysis by ImageJ software, the results of spot counting were reproducible with high inter-assay and inter-examiner concordance. Immune cells that produce and secrete Th1 cytokines without antigen-peptide stimulation of peripheral blood mononuclear cells (PBMCs) were detected, and their frequencies in patients with cancer were significantly higher compared with those in healthy individuals. These frequencies varied between individuals, as well as between time points during the course of cancer vaccine immunotherapy in each patient. Due to the variability in spontaneous cytokine production/secretion by PBMCs, an antigen-specific immune response (IR) index is proposed, which is a ratio of the number of spot-forming cells (SFCs) subjected to antigen-stimulation to that of SFCs with spontaneous cytokine secretion without antigen-stimulation. This index may be used as a marker for antigen-specific cellular immune responses in patients treated with cancer immunotherapy. The IR index successfully detected the induction of Wilms' tumor 1-specific cellular immune responses in patients with cancer treated with cancer vaccine immunotherapy.

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Abbreviations: ELISPOT, enzyme-linked immunospot; WT1, Wilms' tumor 1; SFC, spot forming cell; PBMC, peripheral blood mononuclear cell; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α

Key words: immunotherapy, cancer vaccine, immuno-monitoring, ELISPOT

Introduction

Immunotherapy has been established as the fourth mode of cancer treatment with the advent of immune checkpoint inhibitors, which have become new therapeutic targets for various tumors such as malignant melanoma (1), non-small cell lung cancer (2,3), gastric cancer (4,5), malignant mesothelioma (6) and Hodgkin's lymphoma (7). Recent reports have suggested the clinical benefits of immune checkpoint inhibitors in combination with chemotherapy (8,9). Therapeutic cancer vaccine immunotherapy is a therapy that induces cellular immune responses against the target molecule to elicit clinical anti-tumor effects (10). Although cancer vaccine immunotherapy has not been established as a monotherapy, cancer vaccines may be efficiently combined with other modalities, including immune checkpoint inhibitors (10,11). It is essential to monitor cellular immune responses against the target molecule to evaluate the induction and maintenance of antigen-specific cellular immune responses during the vaccination period; analysis of antigen-specific cellular immune responses includes *in vivo* testing and DTH skin reaction test as well as *ex vivo* tests such as flow cytometric multimer, proliferation and enzyme-linked immunospot (ELISPOT) assays (12). The ELISPOT assay detects cytokine-producing cells in the antigen-stimulation conditions. Therefore, it is possible to analyze not only the frequency of antigen-specific immune cells, but also the effector functions of immune cells as determined by cytokine production/secretion (12). In addition, the ELISPOT assay is adaptable to human leukocyte antigen (HLA) class II binding helper T lymphocyte epitopes, for which qualified multimers for flow cytometric assay are not currently available to the best of our knowledge. This assay is also capable of multi-sample measurement since the procedures are simple and the assay is commonly performed in 96-well plates. Therefore, the ELISPOT

assay is widely used as a monitoring tool for cellular immune response in clinical trials for infectious diseases (13,14) and cancer immunotherapy (15-17).

The Wilms' tumor 1 (*WT1*) gene was originally isolated as a gene responsible for pediatric kidney neoplasm and had been regarded as a tumor suppressor gene (18). However, a number of researchers consider the *WT1* gene to serve an oncogenic role in leukemia (19-22) and a wide variety of solid tumors (23-25) based on the results reported by our group and other groups (26-28). The WT1 protein is highly immunogenic (29). Immunotherapies targeting WT1 have been developed in a number of countries as novel, promising therapeutic strategies for various types of cancer such as leukemia, glioblastoma and pancreatic cancer (30-39).

The aim of the present study was to test a simple Reader-free ELISPOT assay method for reproducibility and apply it to the analysis of cytokine production/secretion of PBMCs in healthy volunteers and patients with cancer, including those who were treated with WT1 peptide-based vaccine immunotherapy.

Materials and methods

Peripheral blood mononuclear cells (PBMCs). PBMCs were obtained with written informed consent from 17 patients with cancer (12 male and 5 female; median age, 45 years; age range, 21-72 years) and six healthy individuals (2 male and 4 female; median age, 24 years; age range, 23-52 years). The types of cancer included seven cases of glioblastoma, seven cases of anaplastic glioma, one case of lung cancer, one case of salivary gland cancer and one case of rhabdomyosarcoma. Of the 17 patients, one patient with lung cancer and two patients with salivary gland cancer and glioblastoma were enrolled in clinical trials of WT1 peptide vaccine cancer immunotherapy registered as UMIN#000002001 and UMIN#000023579, respectively. In the clinical trials, WT1 peptide vaccine was administered weekly (40) or biweekly for three months. Peripheral blood was collected before and one, two, and three months after the initiation of the treatment. PBMCs were isolated from heparinized whole blood using the Ficoll-Paque method (GE Healthcare) according to the manufacturer's instructions and cryopreserved in liquid nitrogen until use. The present study was performed under the approval of the Ethical Review Board of the Faculty of Medicine, Osaka University (Suita, Japan).

Peptide synthesis. Peptides for the ELISPOT assay were synthesized by PH Japan. The amino acid sequences were as follows: WT1-235 peptide, CMTWNQMNL; WT1-126 peptide, RMFPNAPYL.

ELISPOT assay. Following hydrophilization treatment with 35% ethanol for 1 min and three washes with PBS, a membrane of each well in a 96-well filtration plate (Merck KGaA) was incubated with capture antibodies, anti-human interferon-γ (IFN-γ) monoclonal antibody (cat. no. 3420-3-250; Mabtech AB; final concentration, 15 μg/ml in PBS) and anti-human tumor necrosis factor-α (TNF-α) monoclonal antibody (cat. no. 3510-3-250; Mabtech AB; final concentration, 7.5 μg/ml in PBS) at 4˚C overnight. Following four washes with PBS, the membrane was incubated with 200 μl 1X Blocking one (cat. no. 03953-95; Nacalai Tesque, Inc.) for 2 h and washed three times with PBS.

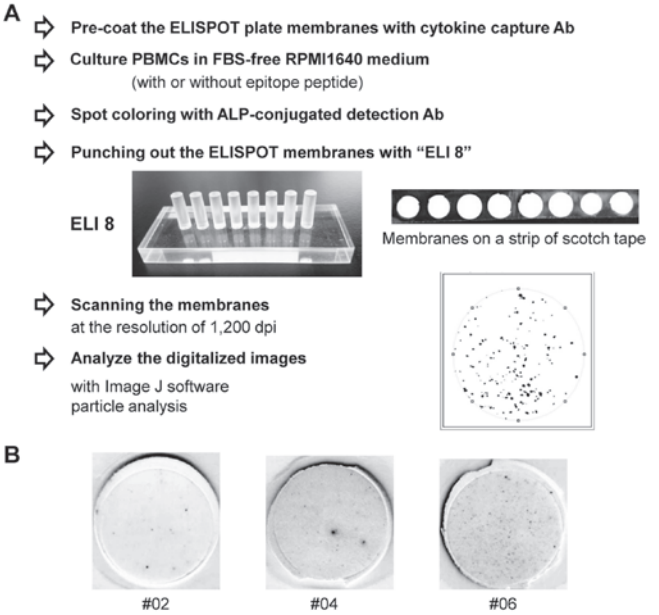

Figure 1. ELISPOT assay procedures. (A) Outline of the reader-free ELISPOT assay. The bottom right image represents a digitalized membrane image. (B) Representative scanned images of ELISPOT membranes. ELISPOT, enzyme-linked immunospot; PBMCs, peripheral blood mononuclear cells; ALP, alkaline phosphatase; Ab, antibody.

Thawed PBMCs were suspended in FBS-free RPMI-1640 medium (Nacalai Tesque, Inc.) and $5x10^4$ cells per 100 μl were seeded in each well in triplicate and incubated with 5% CO_2 in a humidified atmosphere at 37˚C for 48 h. To stimulate PBMCs, an antigen peptide was added to each well at a final concentration of 10 μg/ml. Following removal of the cell suspension, each membrane was washed with 200 μl PBS containing 0.05% Tween-20 for 10 min and treated with 100 μl ACCUMAX™ (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min with gentle agitation. After three washes with PBS containing 0.05% Tween-20, each membrane was incubated at 4˚C overnight with the corresponding detection antibodies in PBS containing 1% BSA and 0.05% Tween 20: Biotinylated anti-human IFN-γ monoclonal antibody (cat. no. 3420-6-250; Mabtech AB; final concentration, 3 μg/ml) and biotinylated-anti-human TNF-α monoclonal antibody (cat. no. 3510-6-250; Mabtech AB; final concentration, 1.5 μg/ml). Following four washes with PBS, each membrane was incubated with alkaline phosphatase-conjugated streptavidin (cat. no. 3310-8; Mabtech AB; diluted 1:500 with 0.05% Tween-20 in phosphate buffered saline without magnesium and calcium [PBS (-)]) at room temperature for 1 h. After washing both sides of the membranes with deionized water for 3 min, the spots were stained with BCIP/NBT solution (Nacalai Tesque, Inc.) for 3 min followed by washing with deionized water. Following drying at 4˚C overnight, a strip of clear adhesive tape was attached to the back of the membranes of 8 wells in a single row. The membranes were punched out with an acrylic device ELI 8 (Create Ltd.). The membranes were subsequently sandwiched a second strip of adhesive tape and scanned at the resolution of 1,200 dpi. The generated digital images were analyzed by spot counting using particle analysis by ImageJ 1.45 software (National Institutes of Health) (Fig. 1).

In concordance analysis, two different examiners with the experience of ELISPOT assay of >6 months performed spot counting with the assistance of ImageJ software. Scanned images of colored membranes from six wells of ELISPOT assay with variable numbers of spots were used. Examiner-1 performed spot counting of six images on two different days for analysis of inter-assay concordance.

Statistical analysis. Difference in IFN-γ and TNF-α secretion by PBMCs between patients with cancer and healthy individuals was analyzed by Welch's t-test using Statcel 3 software (OMS Publisher). Individual values are presented. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inter-assay and inter-examiner concordance of the ImageJ software-assisted spot counting step in the ELISPOT assay. First, a reader-free ELIPSOT assay was developed (Fig. 1). For the membrane preparation step, the present study developed a handy acrylic punching device ELI 8. With this device, the membranes of eight wells were easily punched out at once in an array on a strip of adhesive tape. Since the counting reproducibility of spot numbers is an important factor in the ELISPOT assay, the inter-assay and inter-examiner concordances in the spot-counting step of the ELISPOT assay were examined using six sample membranes with a variable number of spots. The number of spots detected on each sample membrane was scored as follows: -, no spot; 1+, 1-9 spots; 2+, 10-29 spots; 3+, 30-89 spots; 4+, ≥90 spots. Concordance was defined as follows: i) Scores for one sample judged by one examiner on different days or by two or more examiners are identical; ii) scores for one sample are different, but are in a range across the border number between two score categories. For example, 5-15 (10±5) spots, 1+ and 2+; 20-40 (30±10) spots, 2+ and 3+; 75-105 (90±15) spots, 3+ and 4+.

First, inter-assay concordance was examined by Examiner 1. As presented in Table I, the scores judged on two different days were identical in all six examined samples. Subsequently, inter-examiner concordance was examined. The scores judged by Examiner 2 were also identical in all six examined samples (Table I). These results indicated that ImageJ software-assisted spot counting was reproducible with good inter-assay and inter-examiner concordance.

Increased spontaneous production/secretion of Th1 type cytokines by PBMCs in patients with cancer. Counts of spot-forming cells (SFCs) are defined as the number of SFCs in the respective antigen-stimulated test conditions minus the number of SFCs in antigen-free control conditions. Thus, spontaneous production and secretion of Th1 type cytokines IFN-γ and TNF-α by PBMCs in the absence of antigen peptides was analyzed by the ELISPOT assay in 17 patients with cancer and six healthy subjects. The numbers of cells that spontaneously produced IFN-γ and TNF-α were between 8 and 548 (median, 103) and between 23 and 756 (median, 100), respectively, per $1.5x10^5$ PBMCs in 17 patients. By contrast, the numbers of IFN-γ and TNF-α producing cells in healthy subjects were between 5 and 28 (median, 7) and between 5 and 26 (median, 11.5), respectively, per $1.5x10^5$ PBMCs (Fig. 2A).

Table I. Inter-assay and inter-examiner concordance of spot counting.

	Score (spot count)		
Sample no.	Examiner 1 Day 1	Examiner 1 Day 8	Examiner 2 Day 1
1	1+ (9)	1+ (8)	1+ (8)
2	2+ (12)	2+ (17)	2+ (13)
3	2+ (29)	2+ (27)	2+ (16)
4	3+ (36)	3+ (37)	3+ (33)
5	3+ (50)	3+ (50)	3+ (40)
6	4+ (176)	4+ (143)	4+ (152)

To investigate the spontaneous cytokine secretion by immune cells in cancer vaccine-treated patients, spontaneous production of IFN-γ by PBMCs was analyzed in patients with cancer treated with WT1 peptide vaccine cancer immunotherapy at different time points during three months of treatment. Pt-01 was a patient with lung cancer treated with the WT1-235 peptide vaccine 12 times. Pt-02 and Pt-03 were patients with salivary gland cancer and glioblastoma, respectively, who were treated with WT1 Trio peptide vaccine composed of three WT1 peptides including WT1-126 and WT1-235 seven times. WT1-126 and WT1-235 are HLA class I-binding CTL peptides specific for HLA-A*02:01 and HLA-A* 24:02, respectively. SFCs with spontaneous secretion of IFN-γ increased 40.7- and 4.1-fold in two patients, but decreased 0.2-fold in one patient after three months of WT1 peptide vaccine cancer immunotherapy (Fig. 2B).

These results indicated that the changes in the numbers of spontaneous cytokine-producing immune cells should be taken into consideration in the monitoring of antigen-specific cellular immune responses.

Antigen-specific immune response (IR) index as a marker for antigen-specific cellular immune response. Secretion of IFN-γ by PBMCs was analyzed by ELISPOT assay in three patients with cancer at the indicated time points during three months of WT1 peptide vaccine cancer immunotherapy. WT1-235 and WT1-126 peptides were used for antigen-stimulation of HLA-A*24:02 patients (Pt-01 and Pt-03) and HLA-A*02:01 patient (Pt-02), respectively. First, WT1 antigen-specific IFN-γ secretion by PBMCs was described as antigen-specific spot number: (Number of SFCs in antigen-stimulated test conditions)-(number of SFCs in antigen-free control conditions) (Fig. 3). As presented in Fig. 3A, masked by the number of spontaneous cytokine-producing cells, antigen-specific IFN-γ-secreting spot numbers became negative at multiple time points. In addition, the number of SFCs provided no information about the frequency of antigen-specific cytokine-secreting cells in the total pool of cytokine-producing cells. Therefore, when the number of antigen-specific SFCs increased after vaccine immunotherapy, it remained unclear whether this increase was due to antigen-non-specific effects or the induction of antigen-specific cellular immune responses. Therefore, WT1 antigen-specific IFN-γ secretion by PBMCs

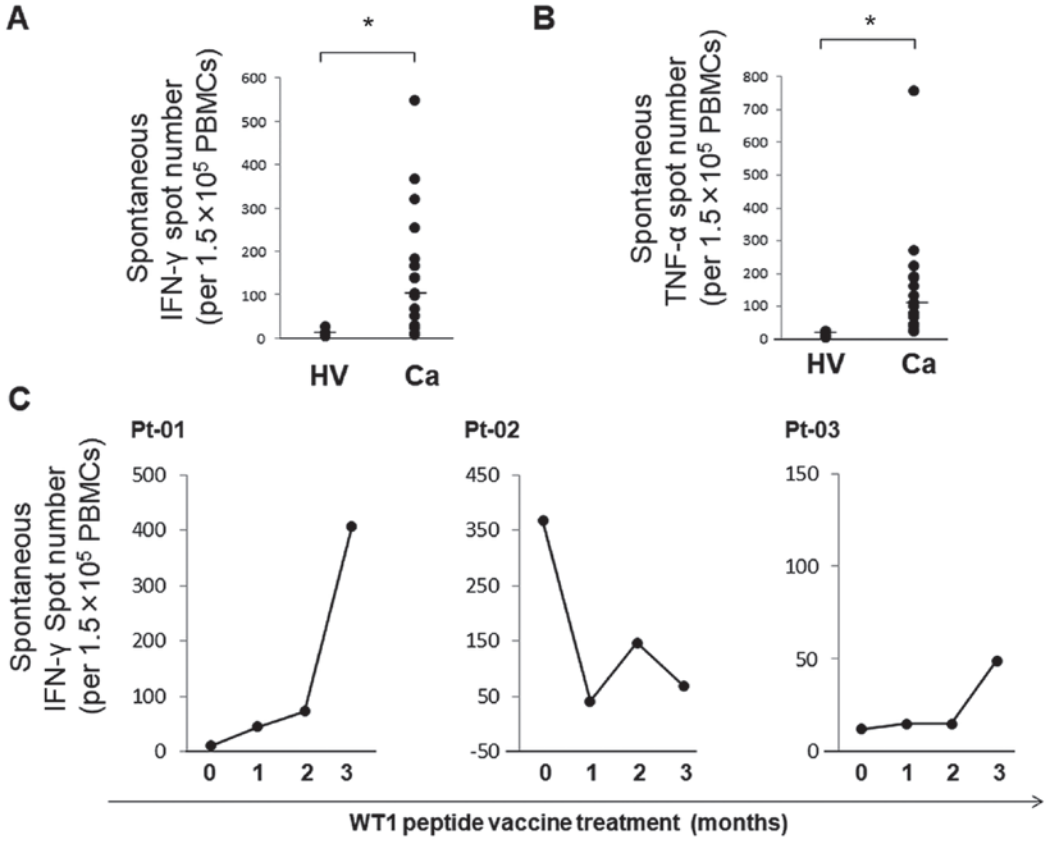

Figure 2. Spontaneous secretion of Th1 cytokines in patients with cancer. (A and B) Spontaneous secretion of (A) IFN-γ and (B) TNF-α by PBMCs was analyzed using ELISPOT assay in 17 patients with cancer and six healthy individuals. (C) Spontaneous secretion of IFN-γ by PBMCs was analyzed by ELISPOT assay in three patients with cancer at the indicated time points during three months of WT1 peptide vaccine cancer immunotherapy. (A-C) Spot numbers are the sum of numbers of PBMC spot forming cells in three wells under antigen-free control conditions. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; PBMCs, peripheral blood mononuclear cells; ELISPOT, enzyme-linked immunospot; Ca, patients with cancer; HV, healthy individuals; WT1, Wilms' tumor 1; Pt, patient; HLA, human leukocyte antigen. (A and B) Horizontal lines indicate median values. *P<0.05.

was also described as antigen-specific IR index: (Number of SFCs in antigen-stimulated test conditions)/(number of SFCs in antigen-free, control conditions). With this index, the direction of the cellular immune responses to the targeted antigen WT1 was successfully detected (Fig. 3B).

Discussion

In the present study, a reader-free ELISPOT assay was developed using a membrane-punching device ELI 8. Using particle analysis by ImageJ, the results of spot counting were reproducible with good inter-assay and inter-examiner concordance. ELISPOT analysis demonstrated that immune cells that produced and secreted Th1 cytokines without antigen-peptide stimulation were present in PBMCs, and that their frequencies in patients with cancer were significantly higher compared with those in healthy individuals. These frequencies varied between individuals or time points during the course of cancer vaccine immunotherapy. Due to the variability in spontaneous cytokine production/secretion by PBMCs, the present study proposed an antigen-specific IR index rather than the number of spot-forming cells as a marker for the cellular immune responses in patients treated with cancer vaccine immunotherapy. This index successfully detected the induction of WT1-specific cellular immune responses in patients with cancer treated with WT1 peptide vaccine immunotherapy.

The ELISPOT assay is performed for various immuno-monitoring purposes including clinical trials for infectious diseases (13,14) and cancer immunotherapy (15-17). For reader-free ELISPOT assay, the preparation of ELISPOT membrane for spot counting can be a time-consuming process. A single-well punch kit, ELIPUNCH (EMD Millipore) is not currently available. To the best of our knowledge, Eli.Punch (A.EL.VIS GmbH) is the only available punching tool for the ELISPOT assay. In addition, since Eli.Punch is a device specifically designed for 96-well punching, it does not allow flexibility in well numbers. In the present study, a handy acrylic punching device ELI 8 was developed. With this device, membranes of eight wells may be easily punched out at once in an array on a strip of adhesive tape, allowing increased flexibility in the scale of the assay compared with commercially available methods. In addition, punching with ELI 8 is economical due to minimal requirements such as adhesive tape.

In the present study, digital images of scanned membranes were converted to binary images and analyzed using free ImageJ particle analysis software provided by the National Institutes of Health. In addition to saving labor by semi-automation, analysis using ImageJ demonstrated that spot counting in the ELISPOT assay achieved high inter-assay and inter-examiner concordance. In the spot counting process, the setting of the threshold for determining a spot is a crucial step; as an examiner typically sets the threshold value, recording this value makes the analysis

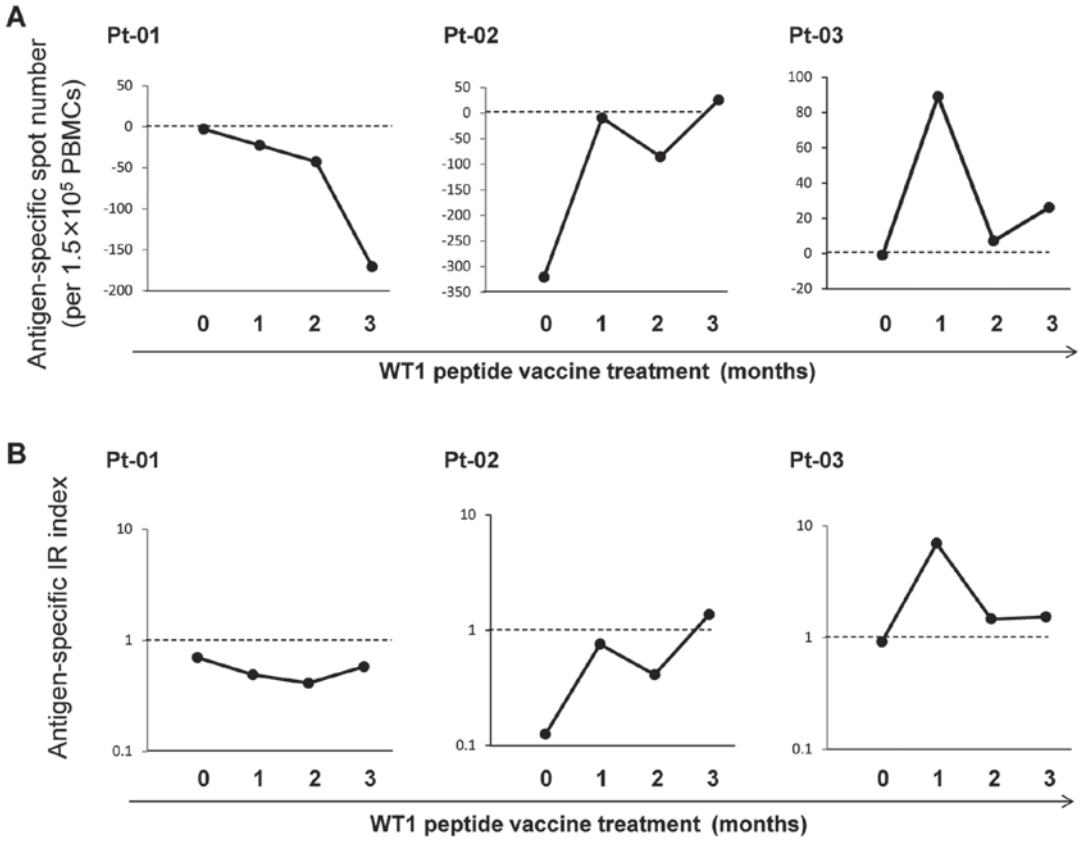

Figure 3. Induction of antigen-specific cellular immune response reported by different indicators of the ELISPOT assay. (A and B) Secretion of interferon-γ by PBMCs was analyzed by the ELISPOT assay in three patients with cancer at the indicated time points during three months of WT1 peptide vaccine cancer immunotherapy. The results are presented as (A) antigen-specific spot number and (B) antigen-specific IR index. Dotted lines indicate the level where the number of SFCs in the respective antigen-stimulated test conditions equals the number of SFCs in antigen-free control conditions. HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells; ELISPOT, enzyme-linked immunospot; WT1, Wilms' tumor 1; Pt, patient; SFCs, spot-forming cells.

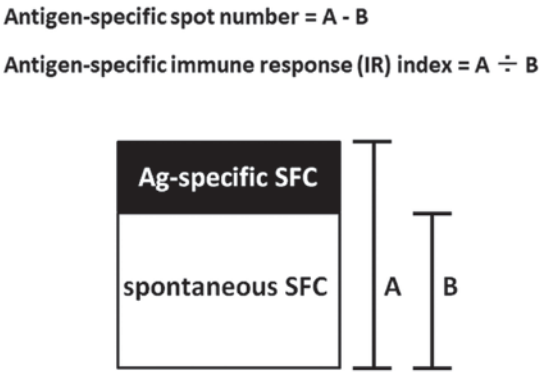

Figure 4. Schematic presentation of antigen-specific spot number and antigen-specific immune response index. A, the number of cytokine-secreting cells upon antigenic stimulation; B, the number of cytokine-secreting cells in antigen-free control conditions.

process traceable. Therefore, the threshold setting data may be useful for education purposes to match the criteria of threshold setting among multiple examiners, including beginners.

In the ELISPOT assay, antigen-specific cytokine secretion of immune cells is often reported as the number of SFCs in the respective antigen-stimulated test conditions minus the number of SFCs in antigen-free control conditions (41,42). The results of the present clearly demonstrated that there is a statistically significant difference between spontaneous cytokine production/secretion in patients with cancer and healthy individuals, and that spontaneous cytokine production/secretion changed over time in the three patients treated with WT1 peptide vaccine. Despite a small sample size, these results demonstrated that spontaneous production/secretion of cytokines in immune cells varied between individuals and over time during the course of cancer vaccine immunotherapy. Since the number of antigen-specific SFCs does not provide information about the frequency of antigen-specific cytokine-secreting cells in the total pool of cytokine-producing cells, changes in the number of spontaneous cytokine-producing immune cells should be taken into consideration in monitoring antigen-specific cellular immune responses. In addition, detection of antigen-specific cytokine secretion by immune cells in an ELISPOT assay may be better reported not only in terms of antigen-specific SFC numbers, but additionally with regard to a supplementary antigen-specific IR index (Fig. 4). One advantage of the antigen-specific IR index is its robustness in measuring error as an indicator of antigen-specific cytokine secretion. The number of spontaneous IFN-γ-secreting PBMCs changes widely even within the same patient. As demonstrated by the concordance analysis in the present study, as the number of spots increases, it is expected that the measurement error also increases. Assuming that the true numbers of SFCs for antigen-stimulation and antigen-free control conditions are 160 and 150, respectively, with a measurement error of 10%,

SFCs for the two conditions would be counted as 154-176 and 135-165, respectively. Thus, antigen-specific cytokine secretion would be reported as between -11 and 41 with respect to antigen-specific SFC number, which is a wide range of variation; however, it would be reported as between 0.93 and 1.303 with respect to antigen-specific IR index. This simulation indicates that the antigen-specific IR index may be more resistant to measurement error compared with the antigen-specific SFC number as an indicator of antigen-specific cytokine secretion.

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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

YuO, SH, SM, FF and HS contributed to the study conception and design. SH, RI, MA and SI performed the ELISPOT analysis. YuO, JN, SN, AT, NH, HN, KH and YoO contributed to the acquisition of the patient samples and conception of the study. YuO and SH drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Blood samples were obtained with written informed consent. The present study was approved by the Ethics Committee of Osaka University Hospital (approval nos. 13110 and 11293).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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