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Interferon-gamma FlowSpot assay for the measurement of the T-cell response to cytomegalovirus

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ABSTRACT

Objectives: We describe a new method, FlowSpot, to assess CMV-specific T-cell response by quantification of interferon-gamma (IFN-γ). CMV-specific, T-cell-released IFN-γ was captured by flow beads and measured via flow cytometry. In the present study, we used FlowSpot to assess CMV-specific T-cell response in healthy individuals. The FlowSpot results were compared with those of serological analysis and enzyme-linked immunospot (ELISpot) assay.
Methods: Experimental results and parameter analysis were investigated by using serological, ELISpot, and FlowSpot assays.
Results: The levels of IFN-γ, which is released from CMV-specific T-cells, were measured, and the results and parameter analysis showed a good correlation between FlowSpot and ELISpot. However, FlowSpot was more sensitive and better reflected the strength of IFN-γ secretion than did ELISpot.
Conclusions: Compared to ELISpot, FlowSpot has a high sensitivity and is cost and time effective. Thus, this method can be used in wider clinical and scientific applications.

Abbreviations: CMV, Cytomegalovirus; IFN-γ, interferon-gamma; TNF, tumour necrosis factor; SFC, spot-forming cells; Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

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1. Introduction

Cytomegalovirus (CMV) infection rate has been reported to be as high as 45 %–100% worldwide [1]. As clinical symptoms are efficiently controlled by cell-mediated immunity, the characteristics of its immune responses and primary infection are poorly reported in healthy individuals [2,3]. Immunity against CMV is widespread in healthy people and is associated with age, as life-long infections frequently reactivate immune cells to enhance the body response [4,5]. Individual variability in terms of immune cell subpopulations is high among healthy individuals. In a healthy person, clinical symptoms are not obvious after the first infection. Nevertheless, CMV may be reactivated in organ transplantation recipients at an immunosuppressive state. CMV-specific functional T-cells have been extensively studied. Recent studies showed that CMV-specific $CD8^+$ T-cells are associated with a low risk of viral activation and good prognosis in diverse organ systems [6–9]. However, whether low-functional CMV-specific T-cell subsets indicate a high risk of CMV infection remains controversial [10]. CMV activates a particular memory and cytotoxic T-cell response, which secretes interferon-gamma (IFN-γ). Activated CD4 and CD8 T-cells are tumour necrosis factor (TNF) and IFN-γ-secreting cells that are the mainstay of CMV-specific immunity. IFN-γ is a type-*II* interferon, which promotes the activation of macrophages and T-cells, and finally defends against CMV infection.

CMV repeatedly attempts to reactivate during life-long carriage to have a huge impact on humoral and cellular immunology [11]. Quantification of CMV-specific humoral immunity, defined by *anti*-CMV immunoglobulin (Ig) G, is a recognised indicator of virus-neutralising ability. CMV seropositivity, as a part of the 'immune risk profile,' is the greatest non-genetic factor affecting differences in immune status among humans [12–14]. Moreover, CMV serological results are commonly recommended to stratify the risk of posttransplant infection [15]. However, quantitative and semi-quantitative methods for CMV serologic assays lack standardisation to directly compare experimental results.

Classic IFN-γ detection assays include enzyme-linked immunosorbent assay (ELISA), flow cytometry, and enzyme-linked immunospot (ELISpot) assays. Different laboratories, and even the same laboratory, often come to variable conclusions, owing to variations in cut-offs [16–18]. The combination of intracellular cytokine staining and flow cytometric analysis provides high sensitivity and clear phenotype definition of IFN-γ-producing T-cells [19]. From this perspective, ELISpot is the most sensitive method for detecting antigen specific T-cell activation [20–22]. Virus-specific peptides closely mimic the natural virus infection, specifically activating IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells [23,24]. However, compared to ELISA and intracellular cytokine staining, ELISpot is time-consuming, complexly performed, and requires large volumes of blood sample. Thus, a new assay that combines accuracy and convenience is needed to detect CMV-specific IFN-γ-producing T-cells.

FlowSpot is a reliable, simple, and highly sensitive method to detect CMV-specific T-cells and CMV-specific T-cell immunity. Peripheral blood mononuclear cells (PBMCs) and IFN-γ capture beads were mixed and incubated together in the presence of stimulation with specific CMV peptides. After stimulation, data analysis using a flow cytometry software showed the percentage of positive beads and fluorescence intensity, reflecting the relative number of CMV-specific T-cells and cytokine release activity, respectively [25]. FlowSpot has a possible sensitivity advantage over the traditional flow cytometric method, experimental time advantage over the ELISpot assay, and stability advantage over the serological method. (Table 3) Here, we described this new method and compared the FlowSpot method with ELISpot.

2. Materials and methods

2.1. Study population and biological specimens

In the present study, we analysed 15 peripheral blood samples from healthy individuals by performing FlowSpot, ELISpot, and serologic analyses during the same time period. All volunteers were >18 years of age with no history of immune disease, viral infection, or other diseases. Additional information is presented in Table 1. FlowSpot, ELISpot and the serologic analysis of all samples are showed in Table 2. Informed consent was obtained from all individuals included in this study. The study was approved by the ethics committee of the First Affiliated Hospital, Sun Yet-sen University ([2021]535) and conducted in accordance with the tenets of the Declaration of Helsinki.

2.2. IFN-γ FlowSpot assay

Blood samples were collected in lithium heparin tubes from 15 healthy individuals with known CMV serotypes. PBMCs were

Table 1
Demographic characteristics of the study participants.

Characteristics	Number (%) or mean (SD)
Healthy individual number (n)	15
Age (years)	32 (2.098)
Gender, n (%)	
Male	10 (66.67%)
Female	5 (33.33%)

Abbreviations: SD, Standard deviation.

Table 2
FlowSpot, EliSpot and Serology types of 15 samples.

Patient Number	Elispot	Flow spot	Serology
1	Positive	Positive	Positive
2	Positive	Positive	Positive
3	Positive	Positive	Positive
4	Positive	Positive	Positive
5	Positive	Positive	Positive
6	Positive	Positive	Positive
7	Positive	Positive	Positive
8	Positive	Positive	Positive
9	Positive	Positive	Positive
10	Positive	Positive	Positive
11	Positive	Positive	Positive
12	Positive	Positive	Positive
13	Negative	Positive	Negative
14	Positive	Positive	Negative
15	Positive	Positive	Negative

Table 3
Advantages comparisons of 3 methods.

Points	Elispot	Flow spot	Serology
detection point	specific T cells	specific T cells and IFNr level	*anti*-CMV IgG
sensitivity	high	high	low
time	6–8h/sample	3–5h/sample	0.2–0.5 h/sample
operations	complex	simple	Most simple
cost	60-70$/sample	30-40$/sample	8-10$/sample

isolated using standard Ficoll-Paque density centrifugation according to the manufacturer's instructions (Lymphoprep, Stem Cell Technologies, USA). PBMCs were suspended in complete medium and counted automatically in a blood routine analyser. Next, PBMCs were stimulated with 19 CMV antigen peptides (μg/mL) (JPT peptide technologies, Germany) or mitogen phytohemagglutinin (PHA) (3 μg/mL) for T-cell activation. Unstimulated cells were used to detect the background cytokine production. Briefly, 0.1×10^6 freshly isolated PBMCs were co-cultured with 50 μl, mixture of IFN-γ-specific capture beads (BD technologies, USA) at 37 °C and 5% CO_2 for 16 h in three replicates (approximately 7000 beads per replicate). After incubation, the plate was centrifuged at 1500×*g* for 3 min, and the cell medium was discarded. PBMCs were lysed in NP-40 lysis buffer (Beyotime Biotechnology, China) for 10 min at room temperature. In this process, the plate was patted gently with dry absorbent paper to remove drops of the buffer from the well. The phycoerythrin secondary antibody detection reagent and detection reagent dilutions were added to each well and incubated for 2 h at room temperature. Thereafter, capture beads were analysed on a BD FACSCanto II flow cytometer after two rounds of washing with 150 μl BD wash buffer.

2.3. IFN-γ ELISpot assay

Fresh isolated PBMCs were suspended in complete medium and counted automatically in a blood routine analyser. Ninety-six-well polyvinylidene fluoride plates (Multiscreen, Millipore, MA, USA) were coated overnight at 4 °C, as recommended by the manufacturer, with monoclonal antibody 1-D1K diluted in phosphate-buffered saline (PBS; Mabtech, Nacka Strand, Sweden) (15 μg/mL). After removing the liquid and washing five times with PBS, the plates were blocked with RPMI-1640 containing 10% foetal bovine serum for 1 h at room temperature. Next, 0.1×10^6 freshly isolated PBMCs were co-cultured in the plates with CMV peptide (experimental group, 5 μg/mL) (JPT peptide technologies, Germany) or PHA (positive contrast, 3 μg/mL) at 37 °C and 5% CO_2 for 16 h in three replicates for T-cell activation. After incubation, cells were removed and the plates were washed, added with biotinylated monoclonal antibody 7-B6-1 (1 μg/mL) diluted in PBS (contained 0.5% foetal bovine serum), and incubated for 2 h. After washing, streptavidin-horseradish peroxidase (dilution 1:1000) was added, and the plates were incubated for 1 h. Finally, 3,3′,5,5′-tetramethylbenzidine substrate (Mabtech, Nacka Strand, Sweden) was added and distinct spots emerged. Colour development was stopped by extensive washing with deionised water. Plates were air-dried, and spots were counted using an automated ImmunoSpot analyser (CTL ImmunoSpot S6 Ultra, C.T. L Technologies, USA). All procedures after incubation were performed at room temperature. Comparability between the two assays was increased by having the same incubation time and condition for the same number PBMC from the same individual.

2.4. Serology analysis

Serological testing of CMV in healthy donors was performed by the Guangzhou Jinyu Medical Inspection Office.

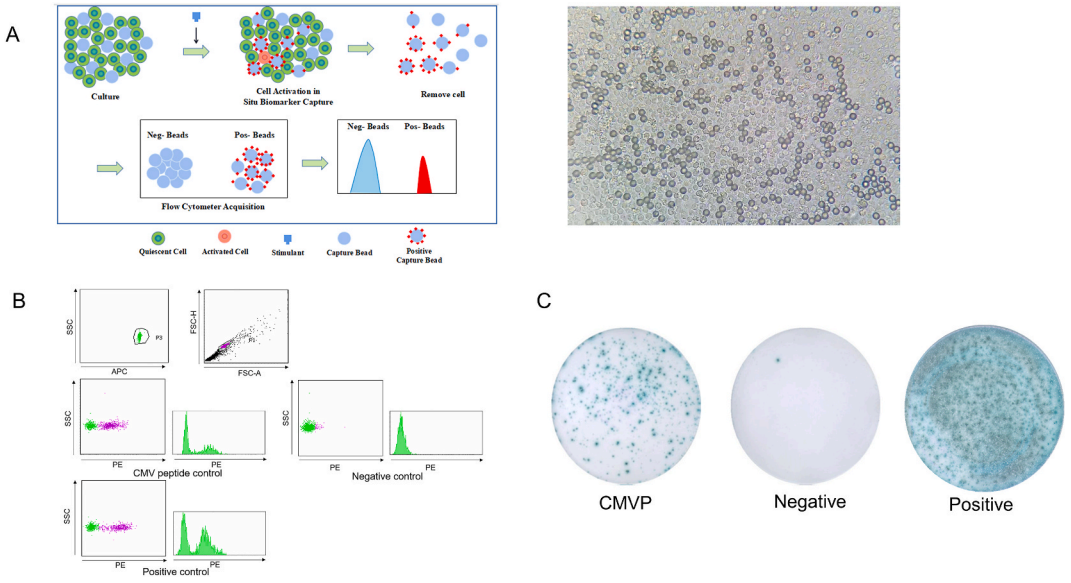

Fig. 1. FlowSpot principles and typical FlowSpot and enzyme-linked immunospot (ELISpot) results. **(A)** The principal diagram of interferon (IFN)-γ detection by using FlowSpot (left). Microscopic images of capture beads (dark) and peripheral blood mononuclear cells (PBMCs) (light) (right); **(B)** representative flow cytometric plot of FlowSpot (left) and determination of mean fluorescence intensity (MFI) values (right) for cytomegalovirus (CMV) peptide control, negative control, and positive control; **(C)** corresponding ELISpot images for CMV peptide control, negative control, and positive control.

2.5. Statistical analysis

Differences between variables were analysed using GraphPad Prism version 8.0 (GraphPad, Inc., San Diego, CA, USA). Correlations between two continuous variables were analysed using Spearman's rank correlation.

3. Results

3.1. Principles and process of FlowSpot and examples of typical CMV peptide-stimulated, negative, and positive results in FlowSpot and ELISpot assays

Ex vivo FlowSpot is a new method for detecting CMV-specific T-cells. In this study, the first step was to isolate PBMCs from the blood samples. To obtain sufficient CMV-specific T-cells for research, we co-cultured PBMCs with CMV peptides and fluorescence-tagged capture beads, which fully obtained IFN-γ-secreting T-cells. Then, we removed the cells to obtain the positive and negative magnetic beads for subsequent flow detection (Fig. 1A). Using microscopy, we observed a mixture of cells and capture beads and found that they showed a relatively uniform distribution (Fig. 1A).

We compared CMV-specific T-cell responses under different conditions using FlowSpot and ELISpot assays. As a control, we included a strongly positive individual, with high serum CMV IgG level, and considered the sample as strongly positive. In the FlowSpot assay, we first detected cell activity using 7-aminoactinomycin D reagents. An active cell proportion of >85% was suitable for FlowSpot detection. CMV-specific T-cells represent the antiviral potential of the cell population and were activated by CMV peptide stimulation and were positive for IFN-γ (purple). A comparable number of IFN-γ^+ PBMCs were detected in this study. The negative control showed the number of IFN-γ-secreting T-cells in the current stage, and a small number of IFN-γ^+ cells was detected. In the positive control, we used a low concentration (25 ng/mL) of PHA-stimulating T-cells to secrete IFN-γ, and a large number of IFN-γ^+ cells were detected (Fig. 1B). A typical example of ELISpot assay results is shown in Fig. 1C. Corresponding IFN-γ^+ spot-forming cells (SFC) were presented in the peptide-stimulated, negative, and positive groups. We observed a comparable positive fraction in the experimental group, very few positive fractions in the negative control group, and massive positive fractions in the positive control group in both the FlowSpot and ELISpot assays.

3.2. Comparisons of the FlowSpot, ELISpot, and serologic assay parameters

We further compared the FlowSpot, ELISpot, and serologic assay parameters following the CMV peptide treatment in 15 healthy individuals (>18 years old). The PI values are the product of MFI and the average percentage of positive magnetic beads, which precisely reflects the secretion of IFN-γ, is shown in Fig. 2A. For better comparison of CMV-mediated immunity, we analysed multiples of the CMV peptide stimulation group and no stimulation group of FlowSpot and ELISpot and found similar trends for these two

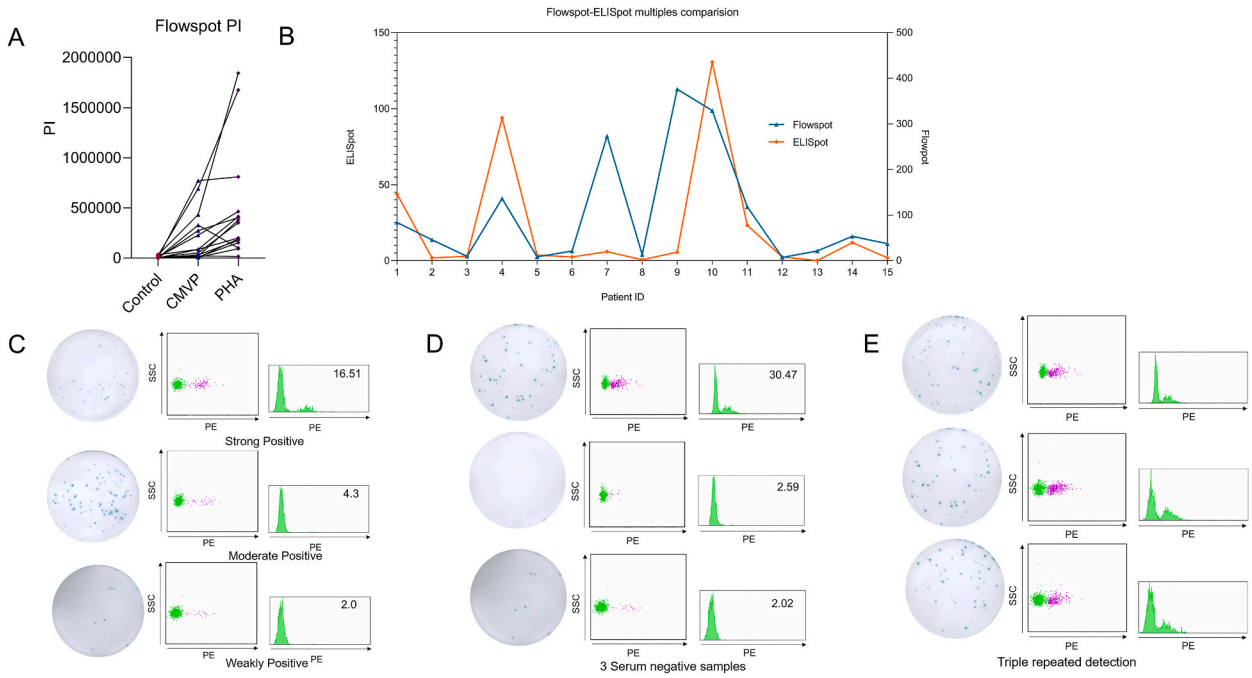

Fig. 2. FlowSpot, enzyme-linked immunospot (ELISpot), and serological assay parameter comparisons. **(A)** Dot plot of total mean PI values for cytomegalovirus (CMV) peptide control, negative control, and positive control in the FlowSpot assay (N = 15); **(B)** line diagram of PI values in FlowSpot and spot number in ELISpot for the CMV peptide control group; **(C)** representative strong positive, medium positive, and weak positive flow cytometric PMB% and MFI analysis (right) and corresponding ELISpot images from the same individual for CMV peptide control; **(D)** flow cytometric PMB% and MFI analysis (right) and corresponding ELISpot images of three serum negative samples from the same individual for CMV peptide control; **(E)** representative flow cytometric PMB% and MFI analysis (right) and corresponding ELISpot images for three repeated detections from the same individual for CMV peptide control.

methods (Fig. 2B). We defined the positive CMV-specific T-cell response as PI ratio (CMV peptide stimulation group/no stimulation group) > 2, and PHA treatment caused IFN-γ production. Notably, the experimental results were not entirely consistent in CMV-positive samples. For example, the FlowSpot moderately positive sample showed more IFN-γ^+ SFC than that of the strong positive sample in the ELISpot assay (Fig. 2C). Interestingly, among the three seronegative of the 15 samples, one was positive in both the FlowSpot and ELISpot assay (Fig. 2D). Three replicates were set for each condition in each group, and good repeatability further illustrated the stability and reliability of our results (Fig. 2E). These findings indicate that consistency and differences exist in the three methods.

3.3. FlowSpot and ELISpot correlation analysis

We then analysed the relationship between the FlowSpot and ELISpot assay parameters. The results of the positive capture bead ratio (PBM%) of FlowSpot showed a high correlation with the ELISpot parameter spot count, which commonly reflects the CMV-specific T-cell number (Fig. 3A). The PI value showed a high positive correlation with the spot number of the ELISpot total (Fig. 3B). The CMV peptide stimulation group and the no stimulation group for the FlowSpot MFI and ELISpot spot number showed a positive correlation (Fig. 3C). Relatively weak correlations were observed between the PI and total intensity (Fig. 3D). Additionally, the mean intensity of the ELISpot was highly negatively correlated with the PMB% of the FlowSpot (Fig. 3E). Moreover, PI of the FlowSpot showed a weak correlation with the MI of ELISpot (Fig. S1A). In the previous correlation analysis, ELISpot aberrant activation samples and one FlowSpot aberrant activation sample were excluded. For multiple analyses, we found that the ELISpot spot number was highly correlated with the FlowSpot PBM ratio (Fig. S1B). These data suggest that the result of the FlowSpot is related to the spot number parameter of the ELISpot rather than the intensity parameters.

4. Discussion

Here, we described a newly developed method and experimental validation of a standardised and optimised IFN-γ FlowSpot protocol for detecting CMV-reactive cell-mediated immunity. The application of FlowSpot to monitor the CMV-specific T-cell response can predict the risk of CMV infection or disease in transplant recipients and guide clinical antiviral therapy and the usage of immunosuppressive drugs. FlowSpot can be used for the (i) prediction and monitoring of vaccine efficacy, (ii) diagnosis and treatment of allergic diseases, (iii) monitoring of immune efficacy and diagnosis in patients with autoimmune diseases, and (iv) evaluation of

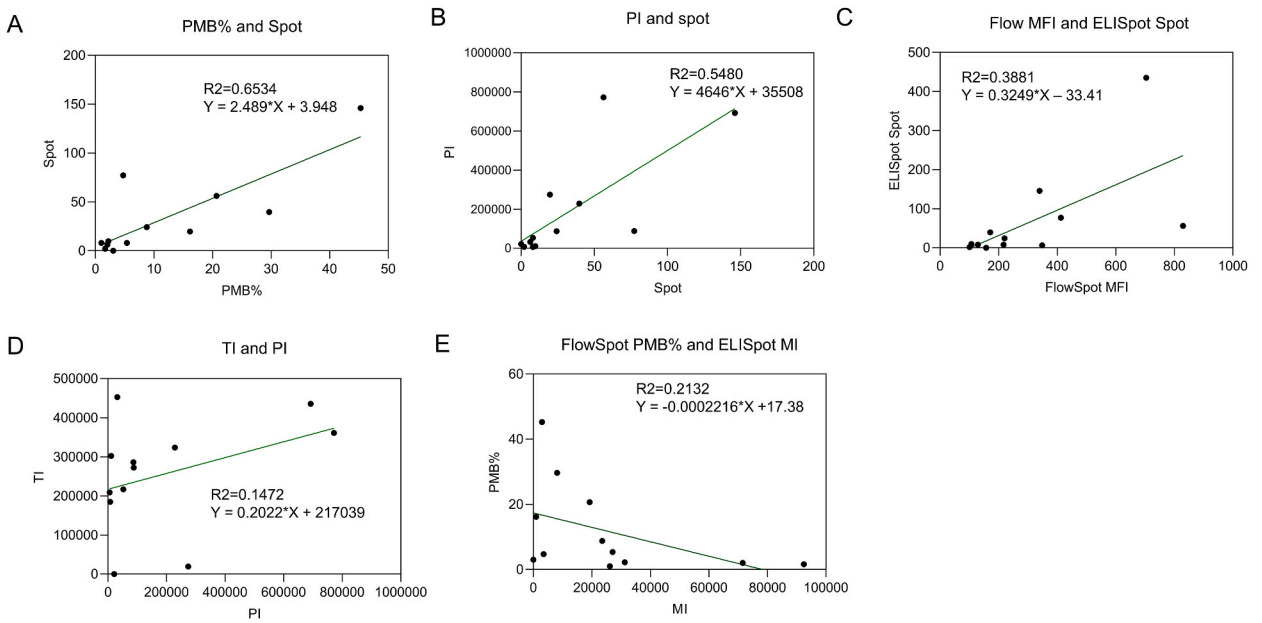

Fig. 3. FlowSpot, enzyme-linked immunospot (ELISpot), and serologic assay parameter correlation analysis. **(A)** Scatterplot diagram and linear regression line for the correlation between FlowSpot positive magnetic beads ratio (PMB%) and ELISpot spot number of the CMV peptide stimulation group; **(B)** scatter plot diagram and linear regression line for the correlation between FlowSpot PI value and ELISpot spot number of the CMV peptide stimulation group; (C) Scatterplot diagram and linear regression line showing correlation between FlowSpot mean fluorescence intensity (MFI) and ELISpot spot number of the cytomegalovirus (CMV) peptide stimulation group; **(D)** scatterplot diagram and linear regression line for the correlation between FlowSpot PI value and ELISpot total intensity (TI) of the CMV peptide stimulation group; **(E)** scatterplot diagram and linear regression line for the correlation between FlowSpot PMB% value and ELISpot mean intensity (MI) of the CMV peptide stimulation group.

specific immunity in graft donors and recipients, including the assessment of the risk of transplant rejection with a given donor, monitoring of graft rejection, and guidance for immunotherapy.

The CMV-specific FlowSpot assay combines the advantages of flow cytometry and spot immunity. Conversely, the ELISpot assay is an effective and highly sensitive tool for studying the function of specific T-cells and assessing the immunological response. However, it is not widely used in clinical diagnosis and scientific research owing to extensive time consummation and high cost. For the FlowSpot assay, settled T-cells are surrounded by microspheres coated with cytokine-specific antibodies; when activated T-cells release cytokines into the culture media, those cytokines are mostly captured by the cytokine capture beads lying nearby. The captured cytokines can be readily quantified by flow cytometry. This new assay allows highly effective detection of CMV-activated T-cells and shortens assay time significantly compared to ELISpot [26,27]. Furthermore, FlowSpot performs better than ELISA. Co-incubation with FlowSpot captured IFN-γ release of CMV specific T cells, and it was convenient for single sample detection with no waste of experimental materials and standards. FlowSpot is possibly highly sensitive to cytokine release, as it relies on the direct detection of cytokine-bound beads by flow cytometry.

The presence of CMV IgG indicates past CMV infection, and if the latent CMV is reactivated, severe complications may develop, particularly in immunodeficient patients [28,29]. The serological response reflects the CMV-IgG antibody-secreting ability of CMV-specific B cells and the intensity of humoral immunity [30,31]. Virus-specific cell-mediated immunity is essential for controlling viral replication [32,33]. CMV-specific T-cells not only were report as highly functional but also appeared in the early stage of infection when antibodies were not present in the blood [34–36]. The standardised IFN-γ ELISpot assay exhibited excellent properties in terms of reproducibility and precision [37]. The ELISpot analysis parameter SFC corresponds to the numbers of CMV-specific T-cells and intensities of cellular immunity; FlowSpot is a new method for detecting the existence of CMV-specific T-cells and their immune competence. The application of 19 different peptides in our FlowSpot assay achieved comprehensive detection of the CMV virus similar to that in the ELISpot assay. The measured positive bead percentage and MFI value are key parameters reflecting the CMV-specific T-cell number and average level of IFN-γ secreted by CMV-specific T-cells, respectively. FlowSpot and ELISpot assay results of some patients are different because FlowSpot reflects levels of secreting IFN-γ while ELISpot detect number of CMV-specific T-cells. In some instances, the secreting IFN-γ level is relatively high but CMV-specific T-cells are still low *in vivo*, so FlowSpot can be favourable for immunocompromised patients. The property differences of the three assays (serologic analysis, ELISpot, and FlowSpot) are shown in Fig. 4A. As shown in our results, the parameters of the FlowSpot assay provide comprehensive and accurate information on CMV-related immunity. Our findings suggest that the FlowSpot assay is more sensitive than the ELISpot assay for detecting CMV cellular immunity. This observation may provide support for developing the FlowSpot method as a potential clinical validated method for CMV immunity detection.

The ELISpot assay is widely used for viral immune detection, cytokine secretion-related immune cell detection, and vaccine

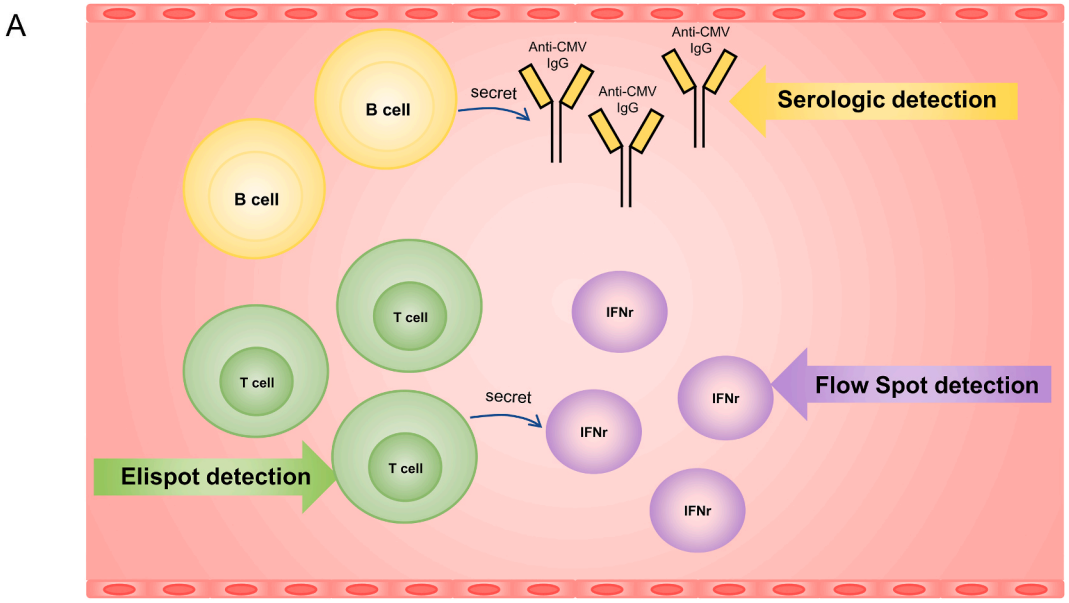

Fig. 4. FlowSpot, enzyme-linked immunospot (ELISpot), and serologic assay detection point comparison. **(A)** Schematic diagram of the FlowSpot assay (purple), ELISpot assay (green), and serological analysis assay detection (yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

research [38–40]. Our FlowSpot assay may also be applied to these areas. Particularly for transplantation CMV immune detection, the FlowSpot assay can be more suitable because its sensitivity may be higher than that of the ELISpot assay, making it possible for better diagnoses in immunosuppressed populations. Besides, the FlowSpot assay with relatively short execution time may provide timely results needed by the transplant physicians. The same incubation time was spent for FlowSpot and ELISpot assays, but the flow method speeds up detection. The FlowSpot assay may provide an assessment of CMV-specific immunity in transplant patients, a relevant prediction of CMV infection progression, and even a possible guide for immunosuppressive drug adjustment and treatment evaluation for patients with CMV infection.

In summary, compared to the ELISpot assay, the FlowSpot assay is a sensitive and accurate method to detect CMV-specific T-cell activation; it is estimated that nearly half the time and expenses were saved under the same conditions, with the same sensitivity and greater ease of performance (Table ~~S2~~3). The limitations of our study include small sample size and lack of testing samples from patient populations. However, with further research and development, the FlowSpot assay is expected to have wider applications in clinical testing and scientific research.

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Author contribution statement

Qianyu Ye: Performed the experiments; Analysed and interpreted the data; Wrote the paper.
Jiali Wang, Meijing Chen: Performed the experiments.
Weijian Nie, Huanxi Zhang: Analysed and interpreted the data.
Xiaojun Su, Liuting Ling: Contributed reagents, materials, analysis tools or data.
Xiangjun Liu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Longshan Liu: Conceived and designed the experiments.
Changxi Wang, Yifang Gao: Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16792.

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