Ex vivo screening for immunodominant viral epitopes by quantitative real time polymerase chain reaction (qRT-PCR)

Maurizio Provenzano*1, Simone Mocellin2, Paola Bonginelli1,4, Dirk Nagorsen1, Seog-Woon Kwon3 and David Stroncek1

Address: 1Molecular Immunology section, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, USA, 2Surgery Branch, Department of Oncological and Surgical Science, University of Padova, Italy, 3Blood Banks, Department of Medicine, Asan Medical Center, Seoul, Korea and 4Current address: Department of Oncology, Division of Medical Oncology, San Filippo Neri, Via Martinotti 20, 00135, Rome, Italy

Email: Maurizio Provenzano* - mprovenzano@mail.cc.nih.gov; Simone Mocellin - mocellins@hotmail.com; Paola Bonginelli - paolabonginelli@yahoo.com; Dirk Nagorsen - dnagorsen@mail.cc.nih.gov; Seog-Woon Kwon - swkwon@amc.seoul.kr; David Stroncek - dstroncek@dtm.cc.nih.gov

* Corresponding author

Abstract

The identification and characterization of viral epitopes across the Human Leukocyte Antigen (HLA) polymorphism is critical for the development of active-specific or adoptive immunotherapy of virally-mediated diseases. This work investigates whether cytokine mRNA transcripts could be used to identify epitope-specific HLA-restricted memory T lymphocytes reactivity directly in fresh peripheral blood mononuclear cells (PBMCs) from viral-seropositive individuals in response to ex vivo antigen recall. PBMCs from HLA-A*0201 healthy donors, seropositive for Cytomegalovirus (CMV) and Influenza (Flu), were exposed for different periods and at different cell concentrations to the HLA-A*0201-restricted viral FluM1 58–66 and CMVpp65 495–503 peptides. Quantitative real time PCR (qRT-PCR) was employed to evaluate memory T lymphocyte immune reactivation by measuring the production of mRNA encoding four cytokines: Interferon-γ (IFN-γ), Interleukin-2 (IL-2), Interleukin-4 (IL-4), and Interleukin-10 (IL-10). We could characterize cytokine expression kinetics that illustrated how cytokine mRNA levels could be used as ex vivo indicators of T cell reactivity. Particularly, IFN-γ mRNA transcripts could be consistently detected within 3 to 12 hours of short-term stimulation in levels sufficient to screen for HLA-restricted viral immune responses in seropositive subjects. This strategy will enhance the efficiency of the identification of viral epitopes independently of the individual HLA phenotype and could be used to follow the intensity of immune responses during disease progression or in response to in vivo antigen-specific immunization.

Introduction

T cell-directed active or adoptive immunotherapy is an emerging treatment option for chronic viral infections, virally-mediated diseases, and virally-induced cancers. Several virally-induced cancers are caused by relatively common latent viral infections. Epstein Barr Virus (EBV) can induce post-transplant lymphoma [1], Burkitt’s lymphoma, Hodgkin’s disease [2], and nasal pharyngeal...
carcinoma [3]. Simian virus 40 (SV40) has been associated with mesothelioma [4,5] and Human Papilloma Virus (HPV) with cervical cancer [6] and it appears that T cell reactivity may control their growth. Thus, we have been interested in tools that allow for a simplified and flexible screening of relevant anti-viral immune responses in seropositive subjects. Among such methods we found particularly suitable the detection of cytokine mRNA levels produced by immune cells in response to viral-epitope stimulation. This method involves the detection of cytokine mRNA levels after a short-term ex vivo sensitization of peripheral blood mononuclear cells (PBMCs) from seropositive individuals exposed to human leukocyte antigens (HLA)-associated viral-epitopes. Cytokine transcript levels were assessed by quantitative real-time PCR (qRT-PCR). Others have used a similar approach to monitor the kinetics of cytokine induction following polyclonal epitope activation [7,8]. Cytokine transcript measurement by qRT-PCR has also been used to determine the cytokine profile of tumor microenvironment [9] or to monitor cancer-specific immune responses [10]. Kamnula et al. pioneered cytokine transcript monitoring by investigating cytokine mRNA expression by melanoma antigen-specific CD8+ T cells in melanoma metastases from patients undergoing epitope-specific vaccination [11]. Recently, cytokine level assessment by qRT-PCR has been used to monitor immune response to other tumors such as soft tissue limb sarcomas after loco-regional therapy [12]. Although the assessment of cytokine production by lymphocytes following stimulation with viral peptides has been characterized after long term in vitro cell culture [13], the direct ex vivo reactivation of memory T lymphocytes from seropositive healthy donors exposed to HLA-associated viral epitopes has not been fully characterized [14,15]. Direct ex vivo sensitization has several advantages over in vitro assays; it is simpler and independent of biases introduced by exposing immune cells to arbitrary doses of growth factors routinely used in culture such as Interleukin-2. This assay is a direct quantitative estimate of the immune reactivity toward a given epitope. A subtle but more important advantage of ex vivo testing of immune reactivity consists in the identification of easily detectable immune specificities likely to be immunodominant in the context of a given disease and HLA phenotype. Thus, such strategy should be considered an easy screening tool for the identification and characterization of immunodominant epitopes that can be readily applied to any individual independently of HLA phenotype through the utilization of overlapping peptide libraries.

The aim of this study was to characterize the usefulness of cytokine mRNA determination following exposure of PBMCs from human viral-seropositive to relevant epitopes in determining individual immune competence to known immunodominant determinants. Peptides from Influenza A virus matrix protein M1 (FluM158–66) [16], Cytomegalovirus matrix protein 65 (CMVpp65495–503) [17], and from the Tumor Associate Antigen (TAA) Mage-12 (Mage12170–178) [18] were used to analyze peptide-specific T cell memory reactivation in three HLA-A*0201 homozygous healthy donors. The short-term kinetics of Interferon-γ (IFN-γ), Interleukin-2 (IL-2), Interleukin-4 (IL-4), and Interleukin-10 (IL-10) were determined by measuring qRT-PCR amplification products. Monitoring of cytokine mRNA production was performed at different time points (from 3 to 48 hours), to select the cytokine with the consistently higher mRNA expression level within the shortest period and to compare this molecular response to short-term protein production evaluated by flow cytometry and ELISA.

The study found that mRNA cytokine analysis of seropositive subjects after ex vivo viral and/or tumor peptide exposure is a simple and valid alternative to other methods that test epitope-induced T lymphocyte reactivation. The simplicity and sensitivity of this method in identifying immune dominant epitopes makes it suitable for the investigation of clinically relevant T cell response.

**Material and Methods**

**Peptide synthesis, reconstitution and storage**

The HLA-A*0201-restricted Influenza A virus matrix protein M158–66 Peptide (FluM158–66, GILGFVFTL), the HLA-A*0201-restricted CMV phosphorylated matrix protein 65495–503 peptide (CMVpp65495–503, NLVPVMATV), and the HLA-Cw*0702-restricted irrelevant peptide Mage-12 (Mage12170–178, VRIGHLYIL), were synthesized with a purity from 90 to 100% as determined by High Pressure Liquid Chromatography (HPLC) analysis (Princeton Bio-molecules, Langhorne, PA, USA). Each peptide was dissolved in 25% dimethyl sulfoxide (DMSO) at the final concentration of 1 mg/mL. Up to 50% DMSO was added to those mixtures of peptide that did not dissolve immediately. The peptide solutions were stored at 4°C and replaced every month.

**Donor Selection**

After obtaining informed consent, a lymphapheresis was performed on four healthy subjects at the Department of Transfusion Medicine (DTM), Warren G. Magnuson Clinical Center (CC), NIH, Bethesda, Maryland using a CS3000 Plus blood cell separator (Fenwal, Baxter Healthcare Corporation, Deerfield, IL). All donors were HLA-A*0201 homozygous. High resolution HLA class I genotyping was performed by sequence-specific PCR using genomic DNA (HLA Laboratory, DTM, CC, NIH, Bethesda, MD). The presence of IgG and IgM CMV antibodies in each donor was analyzed by passive latex agglutination (CMVSCAN kit, Becton Dickinson Microbiology System, Cockeysville, MD). Two of the three donors were
seropositive for CMV. The donors were not tested for antibodies to Influenza A since almost all individuals are seropositive.

**Preparation of PBMCs for qRT-PCR kinetic by ex vivo sensitization (EVS)**

PBMCs from apheresis products were separated from red blood cells and platelets by Ficoll (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. To eliminate residual erythrocytes, an ACK Lysis Buffer (Bio Whittaker, Walkersville, MD) at 1:10 dilution was used while platelets were removed by washing cells twice in HBSS (Biofluid, Rockville, MD) at 800 RPM for 10 min. For the short-term qRT-PCR assays, immediately after PBMCs were isolated, cells were plated in a 96 U-bottom well plate with RPMI (Biofluid, Rockville, MD) complete medium (10% human serum, Gemini Bio-Products, Woodland, CA; supplemented with 1% HEPES, Biofluid) at concentrations of 1 × 10^5, 2 × 10^5, 1 × 10^6, 2 × 10^6 cells/well. Amplified cDNA was then purified and quantitated by spectrophotometry (OD260). The number of cDNA copies was calculated using the molecular weight of each gene amplicon. Serial dilutions of the amplified gene at known concentrations were tested by qRT-PCR. Quantitative RT-PCR reactions of cDNA specimens and cDNA standards were conducted in a total volume of 50 μl with 1× TaqMan Master Mix (Applied Biosystem, Foster City, CA) and primers and probes at optimized concentrations (primer 400 nmol and probe 200 nmol) in a 96-well optical reaction plate ( Applied Biosystem, Foster City, CA). Thermal cycler parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min; final volume 50 μl. Real time monitoring of fluorescent emission from the cleavage of sequence specific probes by the nuclease activity of Taq polymerase allowed definition of the threshold cycle during the exponential phase of amplification. Standard curves generated for IFN-γ IL-2, IL-4, IL-10, and β-actin genes were found to have excellent PCR amplification efficiency (90–100%; 100% indicates that after each cycle the amount of template is doubled) as determined by the slope of the standard curves. Linear regression analysis of all standard curves was > 0.99. Standard curve
extrapolation of gene copy number was performed for all genes studied. Normalization of values was performed by dividing the copies of the genes of interest (IFN-γ, IL-2, IL-4 and IL-10) by the copies of the reference gene (β-actin). All standard and sample PCR assays were performed in triplicate and reported as the average.

**Intracellular staining (ICS) assay**

Freshly isolated PBMCs from seropositive donors at a concentration of 1.5 × 10^6 cells/ml of RPMI complete medium (without HEPES, Biofluid, Rockville, MD) were rested overnight in a 14 mL polystyrene tube (Becton Dickinson, Franklin Lakes, NJ) and then stimulated with peptide at a final concentration of 10 µg/mL each tube. One hour after cell activation, Brefeldin A (Sigma, Saint Louis, MI) at a final concentration of 10 µg/mL was added to each tube culture. After 5 more hours (6 hours total) cells were transferred to 5 mL polystyrene round bottom tubes (Becton Dickinson, Franklin Lakes, NJ) and cell incubation was stopped washing cells in 2 mL cold PBS for 5 min (1500, 4 ºC). Pellets were re-suspended in 1 mL PBS containing 1 mmol EDTA and tubes were incubated at 37 ºC for 10 min to detach adherent cells. After washing cells with 2 mL cold FACS buffer (PBS plus 0.5%BSA) by centrifugation (5 min, 1500, 4 ºC), cells were stained with either 20 µl of human anti IFN-γ-FITC or mouse IgG1 isotype-FITC (BD, Bioscience, San Jose, CA) and incubated for 30 minutes at 4 ºC (on ice) in the dark. After staining, the cells were washed once (2 mL cold FACS Buffer, 10 min, 1500, 4 ºC) and immediately analyzed on a flow cytometer or fixed in 200 µl 4% paraformaldehyde in PBS solution, kept at 4 ºC in the dark and analyzed later.

**Peptide-specific ex vivo (EVS) and in vitro sensitization (IVS) for protein release kinetics**

Cell supernatants from ex vivo stimulated PBMCs sensitized at different concentrations and times of exposure were used for the measurement of cytokine protein release with an enzyme-linked immunoabsorbent assay (ELISA) kit (Endogen, Woburn, MA). Briefly, after each time point harvested cells were used for mRNA cytokine transcription while supernatant were used for protein release detection.

In parallel, a 2-week in vitro sensitization was performed using PBMCs from CMV-seropositive donors. Cells were plated at a concentration of 3 × 10^6/2 ml of medium per well in a anti-human CD3 T-cell activation 24-well plate (Becton Dickinson, Bedford, MD) and directly stimulated with 3 µl/mL of peptide (day 0). One day later (day 1), recombinant human interleukin-2 (rhIL-2, 100 U/ml) and recombinant human interleukin-7 (rhIL-7, 10 ng/ml) (PeproTech, Rocky Hill, NJ) were added to the cell culture. rhIL-2 only (100 U/ml) was continuously administered

| Genes | Primers and Probes |
|-------|-------------------|
| β-actin | (f) 5'-GGCACCCACCAATGAAG-3'  
|        | (r) 5'-GCCGATACCAAGGATCT-3'  
|        | (p) 6FAM-TCAAGATCTTGCTCTCTGAGA-3'  
| IFN-γ | (f) 5'-AGCTGCTCATGTTGTTGT-3'  
|        | (r) 5'-GGTCCATATCCGCTACATCTTGGA-A-3'  
|        | (p) 6FAM-TCTTGGCTTTACTGCCAGGACCA-3'  
| IL-2  | (f) 5'-ACCCAGAGCTCACTTCACTTAAATGTTT-3'  
|        | (r) 5'-GGATTGGTACGTTCTTTAGACTCGT-3'  
|        | (p) 6FAM-CATCGCGGAGAAGGCGCCAGA-3'  
| IL-4  | (f) 5'-GCCGATATCACCTACAGGATCAT-3'  
|        | (r) 5'-GGTACCGTGCACTGCGTGGA-3'  
|        | (p) 6FAM-TGCGGCGAAGCCTCCTC-3'  
| IL-10 | (f) 5'-GCCGTTGGACAGTTCA-3'  
|        | (r) 5'-GAAGATGTCGAAATCCTGACATG-3'  
|        | (p) 6FAM-TGCCCTTTAATAGCTCCAGAAGGCACTACA-3'  

(f) forward primer; (r) reverse primer; (p) probe * These primers and probes have been previously published [9,19]
every other day. At day 15, each group of cells was washed and directly re-stimulated in 2 mL of fresh medium with 3 µL/mL of peptide or was not re-stimulated. Eighteen hours after peptide boosting, the in vitro sensitized PBMCs were harvested. Supernatants were collected to measure IFN-γ protein release using the ELISA assay.

Data analysis
Quantitative RT-PCR results were reported as the number of IFN-γ, IL-2, IL-4, and IL-10 gene copies normalized by 10^5 β-actin gene copies and plotted against the different times of exposure. The cytokine production, as shown, represents the mRNA copy number of the gene of interest from stimulated cells relative to the copy number of the gene of interest from unstimulated cells, in both cases after normalization by β-actin. Figure 3 shows the results as mRNA copy numbers corrected by β-actin. Student's t test was used to compare cytokine mRNA release as well as cytokine protein expression by PBMCs stimulated under different conditions. ELISA results were extrapolated from a standard curve generated by linear regression. Three-color flow cytometry was performed using a FACS-Calibur flow cytometer and data were analyzed using CellQuest software. For each analysis, 250,000 events were acquired. A light scatter region was designed to include only viable lymphocytes. FACS analysis was performed on gated CD3^bright expression allowing the exclusion of residual contaminating cells.

Results
Quantification of human IFN-γ, IL-2, IL-4, and IL-10 mRNAs after ex vivo epitope stimulation of PBMCs
To determine the best target cytokine for assessing epitope-specific memory T lymphocytes reactivation, gene amplification of four cytokine, IFN-γ, IL-2, IL-4, and IL-10 was estimated by qRT-PCR. Cytokine mRNA levels were measured over 48 hours following ex vivo peptide exposure of PBMCs from three HLA-A^*0201 subjects. Since approximately 90% of the population has been exposed to Influenza, we initially analyzed the response to the peptide FluM158–66 (GILGFVFLT). After 3 hours large quantities of IFN-γ transcripts were detected, but little or no IL-2,
IL-2, IL-4, and IL-10. Maximum levels of IL-2 transcripts were detected at hour 24. IL-4 and IL-10 transcripts levels increased above baseline level after 24 hours and continued to rise up to 48 hours from stimulation (Fig 1). Although IFN-γ transcript rapidly decreased during the 48 period, significant levels were still detectable at hour 12 in all three donors. These results show that following ex vivo peptide stimulation of memory T cells, IFN-γ precedes IL-2, IL-4, and IL-10 transcription.

**Human IFN-γ transcript quantification following stimulation with CMVpp65<sub>495-503</sub>, FluM1<sub>58-66</sub> or Mage1<sub>2,170-178</sub> peptide stimulation.** In the period of 3 to 12 hours, IFN-γ cytokine production was inversely proportional to the time of exposure during FluM1<sub>58-66</sub> stimulation (panel A), maintained an average level during CMVpp65<sub>495-503</sub> stimulation (panel B) or was not produced during Mage1<sub>2,170-178</sub> exposure (panel C). The results are expressed in mRNA copy numbers calculated relative to unstimulated cells after normalization by β-actin and represent the expressed values from each donor tested. Panel D specifically shows the kinetics of a representative donor (donor B) after the different viral peptide exposures relative to the negative value (unstimulated cells).

The three HLA-A*0201 donors were stimulated with CMVpp65<sub>495-503</sub> (NLVPMTAV), FluM1<sub>58-66</sub> (GILGFVFTL), and the irrelevant Mage1<sub>2,170-178</sub> (VRIHGLYIL) peptides. Analysis of the response curves showed that CMV stimulation did not result in the same transcription kinetics of Flu (Fig 2, donor A and B in panel B). IFN-γ transcription levels upon Flu stimulation were greatest after 3 hours and showed a linear reduction (Fig 2, panel A). In contrast, IFN-γ transcription levels after CMV stimulation varied but did not differ significantly between 3, 6, and 12 hours before falling drastically after 24 hours (Fig 2, panel B). Stimulations of all three donors with the irrelevant Mage1<sub>2,170-178</sub> and stimulation of the CMV-seronegative donor with CMVpp65<sub>495-503</sub> failed to induce IFN-γ transcription at any time point (Fig 2, panel C and donor C in panel B).
Correlation between IFN-\(\gamma\) transcription and cell concentration following ex vivo CMVpp65,495–503 stimulation of PBMCs from HLA-A*0201 CMV-seropositive and seronegative donors

To investigate the reasons for differences observed in the kinetics of IFN-\(\gamma\) transcription between CMVpp65,495–503 and FluM1,58–66 peptide stimulation, we tested whether concentration of responded cells could affect such kinetics. Thus, the correlation between IFN-\(\gamma\) mRNA production and number of lymphocytes exposed to CMVpp65,495–503 was explored. PBMCs from all three donors were stimulated ex vivo at various concentrations of \(1 \times 10^5, 2 \times 10^5, 1 \times 10^6, 2 \times 10^6\) cells/200\(\mu\)l with CMVpp65,495–503 and evaluated at 3, 12 and 24 hours. We found that at the 3-hour time point IFN-\(\gamma\) transcription increased directly with cell concentration (Fig 3, panel A), but after 12 hours IFN-\(\gamma\) transcription reached a peak at intermediate concentrations \((2 \times 10^5\) cells/200\(\mu\)l\) (Fig 3, panel B). At hour 24, IFN-\(\gamma\) transcription was low and similar at all cell concentrations (Fig 3, panel C). Interestingly
in this experiment, the kinetics of IFN-γ transcription were similar to the results observed previously with CMVpp65_{495–503} stimulation (Fig 2, panel B) only when cells were tested at a concentration of 1 × 10^5 (data not shown) and 2 × 10^5/200 µl (Fig 3, panel D). Conversely, the slopes of both curves obtained at concentrations of 1 × 10^6 and 2 × 10^6 cells/200 µl (Fig 3, panel E and F) followed a decreasing IFN-γ transcription kinetics similar to that displayed by flu stimulation (Figure 2, panel A).

**Extra-cellular and intra-cellular release of IFN-γ protein following ex vivo peptide-stimulated PBMCs**

To determine if other measures of IFN-γ could be used to effectively evaluate T cell activation following ex vivo stimulation, the kinetics of IFN-γ protein release was assessed. Following various durations of exposure of PBMCs to either FluM1_{58–66} or CMVpp65_{495–503}, cell supernatants were analyzed for IFN-γ protein release. From 3 through 48 hours after sensitization, IFN-γ protein release by PBMCs from the seropositive subjects was not or only slightly different from the protein release by PBMCs from the seronegative subject (Fig 4, panels A and B). In contrast, when the cells where sensitized for 2 weeks and boosted with the same peptides, IFN-γ protein production was 10-fold higher, confirming that it is possible to use protein production to estimate the T cell response when in vitro cell sensitization is carried out (Fig 4, panel C). Conversely, the IFN-γ intra-cellular staining was consistently positive upon ex vivo stimulation with CMVpp65_{495–503} peptide and moderately positive with FluM1_{58–66} peptide (Fig 5). No evidence of intra-cellular protein accumulation was observed at different time points tested.
before the standard 6 hour sensitization (data not shown).

Discussion

Memory T lymphocytes represent a population of active immune effectors that retain a prior stimulus and evoke a stronger and more rapid immune response after a secondary immunization. Among the cytokines produced after immune reactivation, IFN-γ is released within a few hours after stimulation. IFN-γ is produced in response to antigen-specific stimulation by cytotoxic T lymphocytes (CTLs) and inflammatory CD4+ T cells (T1,1) and their precursor (T1,0) or nonspecifically by natural killer (NK) cells [20]. Its levels are an indicator of the immune activity of armed effector T cells.

The evaluation of memory T cell reactivation can be performed between 3 to 12 hours irregardless of the source of peptides. Initially, cytokine mRNA transcription was tested against 2 × 10^5 PBMCs by using FluM158–66. Flu sensitization displayed the greatest rates of IFN-γ release 3 hours after stimulation. In spite of the inverse correlation of the transcript levels to the time of exposure, FluM158–66 maintained significant amounts of IFN-γ transcription during the 3 to 12 hour period. In contrast, peak IL-2, IL-4 and IL-10 mRNA did not occur until 24 to 48 hours after peptide sensitization. Conversely, CVMpp65495–503 stimulation displayed different IFN-γ mRNA transcription kinetics regardless of cell concentration and periods of exposure. However, studies of the kinetics of expression of the four cytokines revealed that IFN-γ mRNA can be detected either after both FluM158–66 and CVMpp65495–503 peptide stimulation between 3 and 12 hours at the standard concentration of 2 × 10^5 cells.

These results suggest that it is possible to vary the time of cell analysis from 3 to 12 hours after peptide stimulation. In some cases it may be necessary to analyze cells more than 3 hours after stimulation due to variations in the interactions between HLA antigens and peptides. Our group has already found that analysis of cells 5 hours after peptide exposure enhanced the ability to detect HLA-A*0301, A*3301, and A*3303 interactions with CMV pp65 peptides [21,22].
Different types of assays have been used to identify CTL specificity by measuring IFN-γ protein levels including ELISA, ELISpot and intracellular staining (ICS). While both ELISA and ELISpot assays are effective, they have the major disadvantage of requiring that cells are stimulated and cultured for several days or even weeks before monitoring immune T cell activity [23]. Moreover, prolonged cultures of sensitized cells may lead to the artificial expansion of irrelevant cell populations. Consistent with the fact that a 3-hour sensitization is a short time for mRNA translation and protein production or for the proliferation of a sub-population of responding memory T cells [24,25], we found that protein detection assays failed to detect activated cells when carried out on unexpanded PBMCs demonstrating the higher sensitivity of the technique we used to induce and detect a T cell response after few hours [26-29].

The ancillary use of ICS or IVS protein detection assays to investigate the specific immune activity of potential immunodominant peptides is helpful in completing the understanding of how those peptides work in the context of specific immune reactivation.

In conclusion, we confirmed that molecular analysis by qRT-PCR was effective in identifying immune activity of viral peptides by reactivation of stimulated memory T cells from seropositive HLA-restricted donors after only 3 hours. The secondary immune response resulted in a strong T<sub>H4</sub> cytokines production (IFN-γ and IL-2) within few hours. Negative results obtained following tumor epitope and to study their immune activity in a short period of time, with high specificity and without handling large quantities of cells. The strong response after viral restimulation and the ability to use seropositive donors make this method valuable for any disease-related viral peptide investigation.

By using this method we were able to confirm the immunogenicity of the peptides FluM1 58–66 and CMVpp65 495–501 [30-32]. The major practical advantage is that it allows the rapid evaluation of immune memory T cells reactivation by the detection of specific cytokine gene profiles. It makes it possible to screen a large number of viral and/or tumor epitopes and to study their immune activity in a short period of time, with high specificity and without handling large quantities of cells. The strong response after viral restimulation and the ability to use seropositive donors make this method valuable for any disease-related viral peptide investigation.

**Abbreviations**

APC, antigen presenting cells; CMV, Cytomegalovirus; Flu, Influenza virus; TAA, tumor associated antigens; IVS, in vitro sensitization; EVS, ex vivo sensitization; ICS, intracellular staining; HLA, human leukocyte antigens; PBMC, peripheral blood mononuclear cells

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