Urea-modified protein antigens to simultaneously assess CMV-specific CD4 and CD8 T-cell responses using a new CMV-ELISPOT assay and flow-cytometry

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Introduction

CMV-specific T-cell monitoring is currently explored as a valuable tool to identify transplant recipients at risk for viral complications. Both CMV-specific CD4 and CD8 T cells are critically involved in efficient CMV control, but currently used assays are only optimised to either detect CD4 or CD8 T cells. Thus, the aim of the study was to compare the flow-cytometric analysis and ELISPOT assay with focus on the use of urea-modified CMV proteins (IE-1* and pp65*) for their potential to simultaneously stimulate CD4 and CD8 T cells of healthy controls and immunocompromised patients. In addition, a non-modified CMV-lysate was used as a stimulus.

Study Design and Methods

- **Study population**

  40 healthy controls (43.3±13.7 years): 30 seropositive, 10 seronegative

  40 hemodialysis patients (hd, 65.9±13.3 years): 29 seropositive, 11 seronegative

  40 renal transplant recipients (tx, 54.7±14.3 years): 29 seropositive, 11 seronegative

- **Methods**

  Stimulation of antigen-specific CD4 and CD8 T cells (short time stimulation assay; **Fig. 1A**)

  - 1.5ml heparinized whole blood
  - 6h stimulation with control antigen (Co-ag, negative control), urea-modified (*) immediate early protein 1 (IE-1), urea-modified pp65, non-modified CMV antigen (CMV-ag) or *Staphylococcus aureus* enterotoxin B (SEB, positive control)
  - Addition of Brefeldin A after 2h
  - Analysis of antigen-specific CD4 T cells by flow-cytometry
    - Functional markers (IFNγ, IL-2, TNFα) and phenotypical markers (CD4, CD69)
    - Detection limit (CMV-lysate): >0.05% IFNγ⁺CD69⁺ CD4 T cells

  ELISPOT assay (**Fig. 1B**)

  - Isolation of PBMCs from 9ml heparinized whole blood
  - 19h stimulation with different antigens (see above)
  - Detection of IFNγ-secreting cells (Spots)

Results

Short time stimulation assay

- Flow-cytometry revealed that median frequencies (% IFNγ⁺CD69⁺) of CMV-specific CD4 T cells in CMV IgG positive individuals were lower after stimulation with urea-modified pp65 (median (IQR) 0.09% (0.30%)) compared to non-modified CMV-lysate (1.93% (3.57%)). In contrast, induction of CMV-specific CD8 T cells was stronger after stimulation with urea-modified pp65 (0.21% (0.94%)) as compared to the CMV-lysate (0.14% (0.46%)). Median IE-1-specific CD4 and CD8 T-cell frequencies were rather low (0.02% (0.03%) and 0.04% (0.17%); **Fig. 2**).
- Independent of the amount of CD4 and CD8 T-cell frequencies, no significant differences were observed between patients and controls (**Fig. 3**).

ELISPOT assay

- Median ELISPOT counts in CMV IgG positive individuals were low for IE-1 (3.8 (10.5)), medium for pp65 (65 (179.5)) and highest for CMV-lysate (120 (160)), and no significant differences were observed between patients and controls. IgG negative individuals had almost no spots regardless of the antigens used (0-0.5 (0-0.5); **Fig. 4**).

Correlation

- Correlation between ELISPOT and flow-cytometry (a combination of CD4 and CD8 T-cell frequencies) was significant for all three antigens (p<0.0001) and highest with pp65 (r=0.83) and CMV-lysate (r=0.86) (**Fig. 5**).

Conclusions

- The non-modified CMV-lysate is well-suited for detection of CD4 T cells, whereas urea-modification of protein antigens represents a viable approach to improve simultaneous detection of CMV-specific CD8 T cells.
- As compared to peptide stimulation, this approach does not require knowledge of antigenic epitopes or HLA type.
- Both ELISPOT and flow-cytometry are equally reliable readouts and allow monitoring of CMV-specific cellular immunity in both healthy controls and immunocompromised patients.

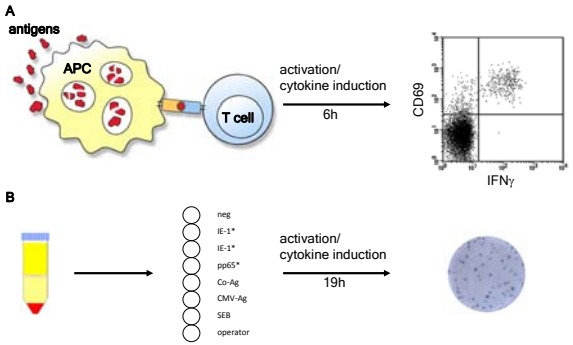

Fig. 1: A) Antigen-specific stimulation of T cells in whole blood samples (short time stimulation assay). **B)** Detection of IFNγ positive cells after antigen-specific stimulation of PBMCs and 19h inkubation (ELISPOT assay).

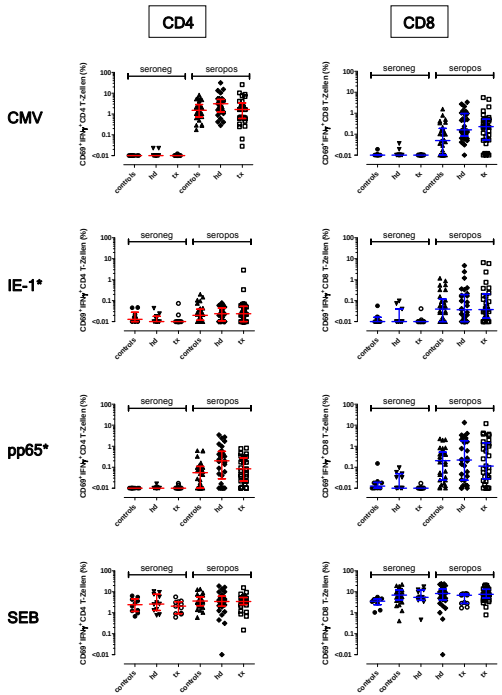

Fig. 3: Comparison of antigen-specific CD4 and CD8 T-cell frequencies according to serostatus and control/patient groups.

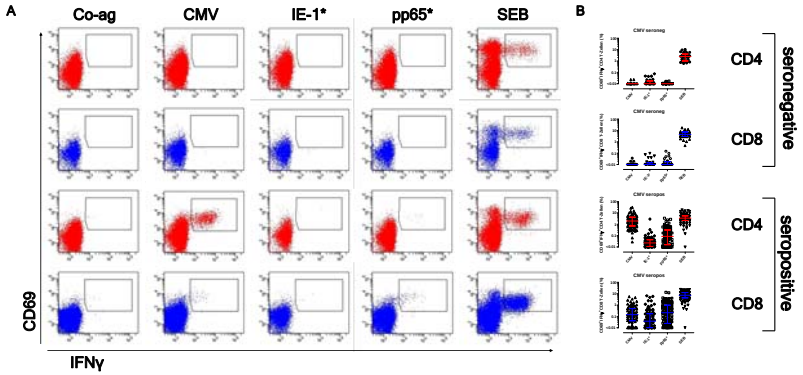

Fig. 2: Flow-cytometric analysis of CD69⁺IFNγ⁺ CD4 and CD8 T cells after antigen-specific stimulation. **A)** Representative results of one CMV-seronegative and one CMV-seropositive individual. **B)** Antigen-specific CD4 and CD8 T-cell frequencies of all seronegative and seropositive persons.

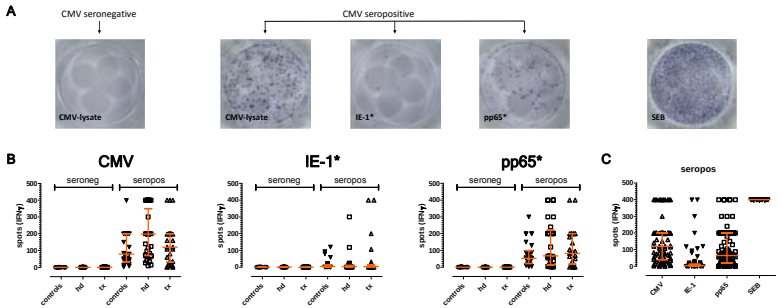

Fig. 4: A) Representative results of ELISPOT counts of one CMV-seronegative and one CMV-seropositive individual. **B)** Comparison of serostatus-dependent and antigen-specific amount of ELISPOT counts of healthy controls, hemodialysis patients and transplant recipients. **C)** Antigen-specific ELISPOT counts of all CMV seropositive persons.

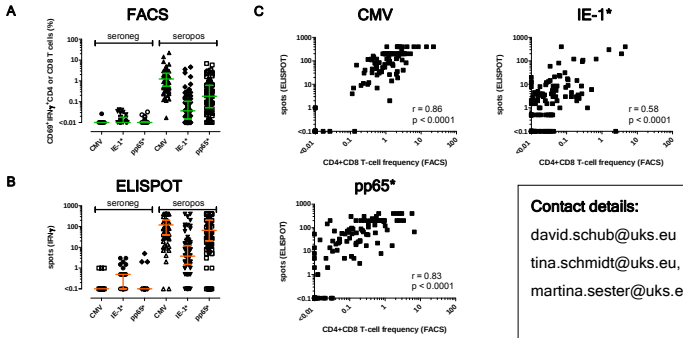

Fig. 5: A) Combination of antigen-specific CD4 and CD8 T-cell frequencies of all probands. **B)** Antigen-specific ELISPOT counts of all tested probands. **C)** Antigen-specific correlation between ELISPOT counts and CD4+CD8 T-cell frequencies, obtained by flow-cytometric analysis.

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