Cytomegalovirus Protein Spanning PepMix™ Peptide Pools to Discover Changes in T-Cell Immunity in the Aging Population

F. Kern

Brighton and Sussex Medical School, Medical School Research Building, Rm 109, Brighton BN1 9PS, East Sussex, UK

The use of overlapping peptide pools is extremely efficient for immunostimulation of CD4 and/or CD8 T-lymphocytes and diagnostic applications. In this Note, we describe the use of a panel of 19 overlapping peptide pools (PepMixes™) from HCMV to determine the influence of age on T-cell immunity to HCMV in older people.

Background

The T-cell response to Human Cytomegalovirus (HCMV) is very broad in terms of protein recognition. Currently, there is an increasing interest in HCMV-specific T-cell responses in older people as a number of studies have reported large expansions of HCMV specific T-cells in the older population. However, no attempt has been made to substantiate this as a general phenomenon by examining a large population cross-section and including a representative range of HCMV proteins[1]. A study published in 2005 identified > 150 different HCMV protein targets recognised by CD4 and/or CD8 T-cells[2]. The summated response frequency for each protein taking into account 33 tested individuals (average age around 35 years) was found to be a useful compound measure of target relevance. Following the ranking of both CD4 and CD8 T-cell responses according to *summated response frequencies*, statistical evaluation determined that the sum of responses to the top ranked 6 CD4 and 15 CD8 target proteins correlated very closely (>0.9) to the respective sums of the T-cell responses to all investigated proteins. The slope of the regression line was 0.42 for CD4 and 0.67 for CD8 T-cell responses, indicating that the summated response to all proteins in each individual was approximately 2.5 times and 1.5 times the response measured with the reduced peptide pool set, respectively. There was an overlap of 2 proteins between the top ranked CD4 and CD8 T-cell targets (UL83 and UL99).

Generation of Peptide Pools

In order to apply this finding to a study comparing the overall size and functionality of T-cell responses in young and older people by flow-cytometry following ex-vivo stimulation, the previously identified proteins were synthesized as overlapping peptides. Peptides were assessed to have at least 70% purity by HPLC-MS before they were combined into 'PepMixes™' (JPT Peptide Technologies, Berlin). Initially, subpools of 10-25 peptides were generated. The presence of all subpool peptides was tracked by HPLC-MS. Several peptides of each subpool were defined as "marker peptides" based on their unique molecular weight and HPLC retention time. After combining the subpools, the final pool was analysed by HPLC-MS confirming the presence of each "marker peptide" to guarantee the presence of all peptides within the final PepMix™.

Downscaling the Standard Protocol

Our standard over night stimulation protocol for the detection of activated T-cells by intracellular cytokine staining is described in detail elsewhere[3]. Because the amount of blood that could be taken from older people was limited, sometimes yielding merely 15 million PBMC (peripheral blood mononuclear cells), we were unable to use the usual amount of 2 million cells per stimulated tube in 1 ml stimulation end-volume. The stimulation and subsequent staining volume was, therefore, reduced to 0.5 ml stimulation end-volume to include 1 million PBMC. PepMixes™ for each protein, containing 25 'tests'/vial (1 test corresponding to 1 µg of each peptide to be used in 1 ml stimulation end-volume) were dissolved in 100 µl of DMSO (Pierce) and aliquoted for short-term storage at -20°C. For each tube to be stimulated, 2 µl of this PepMix™ working solution (i.e. 0.5 µg of each peptide) or the appropriate amount of SEB, HCMV-lysate, or PPD working solution, or DMSO alone, and 1.5 µl of anti-CD107a antibody (BD Oxford) as well as 0.5 µl Monensin ('Golgistop' BD) was added to 46 - 48 µl supplemented RPMI 1640 (100 U/ml Penicillin, 100 U/ml Streptomycin, 2mM L-glutamine, 10% FCS) to give a final volume of 50 µl of 'stimulation media'. For stimulating 20 tubes, 40 µl of peptide solution were added to 960 µl of supplemented RPMI containing Monensin and anti-CD107. Then, 50 µl of this stimulation media were pipetted into each stimulation tube. Next, 1 million cells (PBMC) in 200 µl of supplemented RPMI were added to each tube for a final volume of 250 µl. After 2 hours of incubation at 37°C (standard incubator, 5% CO_2, humidified atmosphere), 250 µl of Brefeldin A (BFA, Sigma) solution (1 µl of BFA/DMSO stock solution at 5 mg/ml in 249 µl of supplemented RPMI) was added to each tube. Incubation was stopped after an additional 14 hours.

Stimulant(s)	Nr. of Peptides	Stimulant(s)	Nr. of Peptides
SEB[1]	n.a.	UL153[5]	67
HCMV lysate[2]	n.a.	UL32[5]	260
PPD[3]	n.a.	UL28[5]	92
DMSO[4]	n.a.	UL48A[5]	281
UL55[5]	224	UL48B[5]	281
UL83[5]	138	US3[5]	44
UL86[5]	340	UL151[5], UL82[5]	82, 137
UL122[5]	120	UL94[5], US29[5]	84, 113
UL123[5]	143	UL103[5], US32[5]	60, 43
UL99[5]	45	US24[5], UL36[5]	123, 117

Table1: Antigen preparations used
1 Staphylococcus Enterotoxin B (Sigma, USA)
2 Purified Cytomegalovirus Lysate (ABI, USA)
3 Purified Protein Derivative, (human tuberculin) (SSI, Denmark)
4 Dimethylsulfoxide (Pierce, UK)
5 Peptide Pool (PepMix™, JPT, Germany)

The goal of this study was to measure the overall summated T-cell response to HCMV. The risk of missing some very small responses as a result of using fewer cells in the assay to begin with was considered acceptable since it would only marginally affect the final sums. It was estimated that given about 100.000 to 150.000 remaining CD4 or CD8 T-cells in each tube upon acquisition, even very small responses of 0.01 % (i.e. 1/10.000) would still yield 10 – 15 positive events, which can be identified as an activated cell cluster. In order to limit the number of tubes, PepMixes™ that were either small (number of peptides) or less frequently recognised were used in combination. The final list of stimulants is given in **Table 1**.

Because of the considerable amount of individual tubes to be prepared manually (20 tubes including positive controls and negative control per patient) we limited the number of parallel samples to three per technician per experimental run (60 tubes to be handled in each step, including staining).

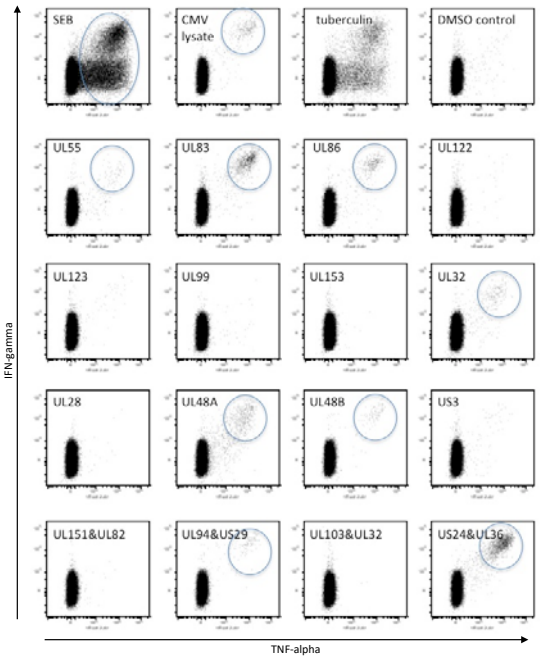

Figure1: CD4 T-cell responses (IFN-gamma and TNF-alpha) to 19 different antigen preparations and negative control (DMSO). PBMC were stimulated over night and then stained with surface (T-cell lineage) and intracellular staining antibodies (cytokines). This donor exhibits CD4 T-cell responses to 8 different PepMixes™. Responses are classified as positive when sufficiently different from the unstimulated control. Usually the response to DMSO alone is subtracted from the response to specific antigens to account for 'background' activation (this is not a staining control). In the example, the positive response threshold was set as 0.01% above control.

Results

The results of our study encompassing more than 120 old and 50 young individuals will provide new insight
into the differences in HCMV-specific T-cell immunity as the result of aging, in particular with respect to vaccine protein target selection and response functionality. Results from one donor are shown in **Figure 1**. The complete results of this study will be published in detail in due course.

References

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The Author

Prof Florian Kern
f.kern@bsms.ac.uk
Chair in Immunology
Brighton and Sussex Medical School
Brighton, East Sussex, UK
Area of Expertise:
Immunology of Infection, Antigen-specific Immunity
Research Areas:
Infection & Immunology, Neuroscience

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