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Sequence flexibility of the immunodominant HLA A*0201 restricted ppUL83 CD8 T-cell epitope of human cytomegalovirus

Jakub Kopycinski, Mohamad Osman, Paul Griffiths, Vincent Clive Emery

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**Sequence flexibility of the immunodominant HLA A*0201
restricted ppUL83 CD8 T-cell epitope of human
cytomegalovirus**

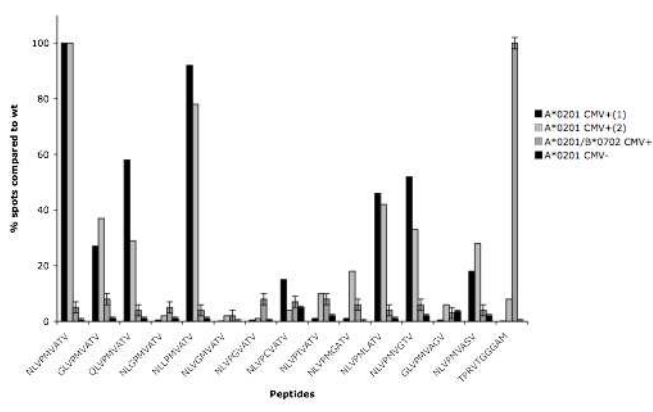
Journal:	<i>Journal of Medical Virology</i>
Manuscript ID:	JMV-08-1194.R2
Wiley - Manuscript type:	Research Article
Keywords:	CTL escape, mutations, T-cell receptor, class I HLA, peptides



Review

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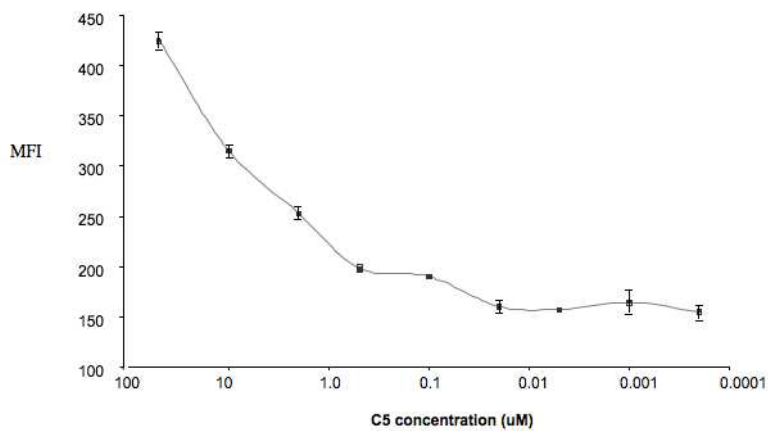
Figure 1 Kopycinski et al



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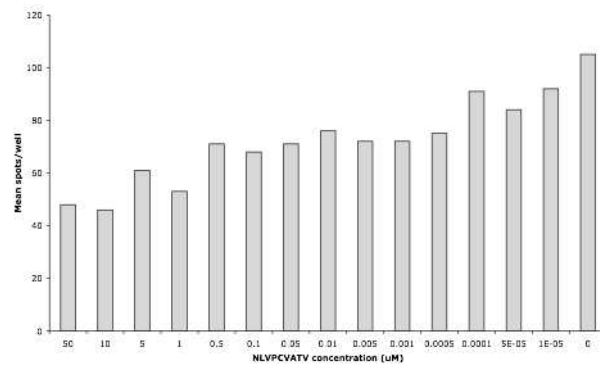
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Figure 3 Kopycinski et al



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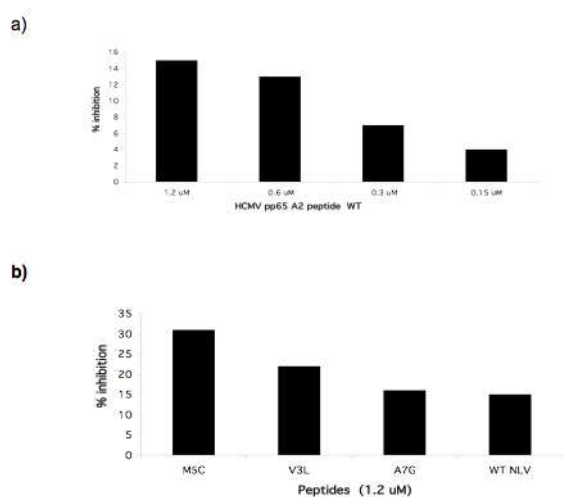
Figure 4 Kopycinski et al



Review

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Figure 5 Kopycinski et al



Review

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4 1 **Sequence flexibility of the immunodominant HLA A*0201 restricted ppUL83 CD8 T-**
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6 2 **cell epitope of human cytomegalovirus**
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12 4 Jakub Kopycinski, Mohamed Osman, Paul D Griffiths, Vincent C Emery
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15 5
16
17
18 6 Department of Infection

19
20 7 Centre for Virology

21
22 8 University College London (Hampstead Campus)

23
24
25 9 Rowland Hill Street

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27 10 Hampstead

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29
30 11 London NW3 2QG
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32 12
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34 13 Correspondence to: Professor Vincent C Emery

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36 14 Email: v.emery@ucl.ac.uk

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44 17
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47 18 Keywords: CTL escape, mutations, T-cell receptor, class I HLA, peptides
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53 20 Running title: HCMV CTL escape
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Abstract

The cytomegalovirus ppUL83 protein contains an immunodominant A*0201 restricted epitope between residues 495-503. We investigated the tolerance of this epitope to sequence variation in the context of peptide binding to HLA A*0201 and the ability to induce an Interferon (IFN) gamma response through engagement with the T-cell receptor (TCR). The majority of mutations investigated resulted in a decrease in the production of IFN gamma indicating that if such variants occurred in vivo they would not be recognised by CD8 T-cell clones specific for the wild type epitope. The mechanistic basis for the majority of the mutant peptides was their failure to bind and stabilise class I HLA cell surface expression. However, one peptide with a mutation at the P5 position (methionine to cysteine) resulted in a significant enhanced binding to HLA A*0201 and also an increase in cell surface expression over the wild type peptide but was unable to engage with the CD8 TCR and trigger interferon gamma production. This peptide acted as a competitive inhibitor of the wild type peptide but could not fully inhibit IFN gamma production by the latter. We subsequently investigated whether mutations of the HLA A*0201 epitope were evident in immunocompromised patients experiencing either rapid exponential or persistent cytomegalovirus replication.

1
2
3 **1 Abbreviations**
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5
6 2 DMSO: Dimethylsulphoxide
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9 3 HCMV: Human cytomegalovirus
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11 4 ge: genomes
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13 5 HLA: human leukocyte antigen
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16 6 HIV: human immunodeficiency virus
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18 7 IFN γ : Interferon gamma
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20 8 PCR: Polymerase chain reaction
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23 9 SIV: simian immunodeficiency virus
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25 10 TCR: T-cell receptor
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1 Introduction

Human cytomegalovirus (HCMV), a betaherpesvirus, infects between 60 and 100% of the human population. The virus has evolved a complex range of immune manipulation strategies that enable its persistence in the human host. These include a variety of gene products that down regulate class I HLA presentation and overcome natural killer cells [Mocarski, 2004; Lilley and Ploegh, 2005]. The extent of the total T-cell response against HCMV is still being unravelled but data suggest that the human host devotes a substantial proportion of both the CD8 and CD4 compartments to responses against HCMV and that the proportion increases with age [Appay et al., 2002; Northfield et al., 2005; Pourgheysari et al., 2007; Gratama et al., 2001; Raeiszadeh et al., 2006; Weekes et al., 1999; Engstrand et al., 2000]. Historically, the CD8 immune response has been investigated by focusing on two immunodominant proteins, pp65 (ppUL83) and pIE1 (pUL123), although a more recent proteomic analysis indicates that many other HCMV proteins elicit T-cell responses [Sylwester et al., 2005]. Nevertheless, the HLA A*0201 restricted peptide in ppUL83 (amino acids 495-503) is particularly immunodominant eliciting responses of between 1-5% in normal healthy individuals and in some patients with active replication, responses can reach 50% of the total CD8 T-cell population [Raeiszadeh et al., 2006; Sylwester et al., 2005]. Given the immune pressure that individual epitopes must be sustaining and the rapid, dynamic replication of HCMV in vivo [Emery et al., 1999; Emery and Griffiths, 2000], it may be expected that this environment should favour the generation and persistence of CD8 T-cell escape mutants. CTL escape mutants have previously been described in several other viral infections including HIV [Borrow et al., 1997; Karlsson et al., 2007; Geels et al., 2006; Pillay et al., 2005; Goulder et al., 2001; Jones et al., 2004], SIV [Allen et al., 2000; Nacsa et al., 2003; Loffredo et al., 2004; Loh et al., 2007] and during primary HBV infection [Bertoletti et al., 1994; Whalley et al., 2004]. However, to

1
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3 1 date there have been relatively few studies of CD8 T-cell escape in HCMV infection. We
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5
6 2 therefore investigated the sequence flexibility of the ppUL83₄₉₅₋₅₉₃ epitope using a
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8 3 synthetic peptide approach and complemented these studies with an analysis of HCMV
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10 4 sequence variants emerging during rapid dynamic infection post transplantation or
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13 5 persistent active replication associated with a high viral turnover where a significant
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15 6 selective pressure for mutation should be present.
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For Peer Review

1 **Materials and Methods**

2 **Isolation and storage of PBMCs from Whole Blood**

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4 The part of the study was approved by the local research ethics committee of the Royal
5 Free Hospital NHS Trust. 20ml of fresh whole blood was taken from patients and
6 volunteers obtained using sodium-heparin syringes (Sarstedt, Leicester, UK). The blood
7 was then overlaid onto Ficoll-Paque Plus (Amersham Biosciences) at a 1:1 volume ratio in
8 50ml sterile Falcon® universal tubes and centrifuged at 400g for 20min with slow
9 acceleration and without braking. The tubes were removed from the centrifuge and the
10 interphase, created between the plasma layer and the lymphocyte separation medium ,
11 was carefully removed, placed into a fresh universal tube and diluted to 30ml with un-
12 supplemented RPMI 1640; this the tube then centrifuged at 400g for 10 minutes to remove
13 any residual Ficoll. The supernatant was discarded and the resultant pellet was
14 resuspended in an 20ml of RPMI 1640. Cells were washed twice by centrifugation at 300g
15 for 5 minutes and after the second wash the cells were resuspended in 10 ml of
16 supplemented RPMI 1640. Viable cells were then stained with 4% trypan-blue and
17 counted using a haemocytometer. Isolated PBMCs were centrifuged at 1400g for 5
18 minutes and the pellets were resuspended at a density of $>5 \times 10^6$ cells/ml with 90%
19 FCS/10% DMSO that had been pre-chilled to 4°C. The suspensions were transferred to
20 1.5ml cryovials and placed in a 4°C isopropanol-containing freezing chamber, then
21 transferred to a -80°C freezer, which allowed cells to cool at a rate of 1°C/minute, thereby
22 preserving cell viability. After 48 hours cells were transferred to a liquid nitrogen storage
23 unit until use.

24 **ppUL83₄₉₅₋₅₀₃ peptides**

25 A number of variants of the pp65 HLA-A*0201 epitope NLVPMVATV were synthesised
26 commercially (Altabiosciences, University of Birmingham, UK) with a purity >90%. Peptide

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3 1 variants were designed using either individual glycine or like-for-like substitutions based on
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6 2 charge and shape. Lyophilised peptides were resuspended in DMSO to 5mg/ml. The
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8 3 peptide variants synthesised are shown in Table 1.
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10 4

5 **Interferon gamma Enzyme-Linked Immuno Spot (ELISpot)**

16 6 Mouse anti-human-IFN γ mAb (1-D1K, MabTech, Sweden) was diluted in sterile-filtered
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18 7 PBS (Sigma) and used to block 96-well nitrocellulose plates (MabTech, Sweden) overnight
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21 8 at 4⁰C. Plates were subsequently washed six times in sterile, filtered PBS and blocked
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23 9 with RPMI 1640 (supplemented with 10% FCS). PBMCs isolated from HCMV seropositive
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26 10 and control (seronegative) volunteers were re-suspended to 5 x 10⁴ cells/well in
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28 11 supplemented RPMI 1640. 20 μ M of the wild-type NLVPMVATV and variant peptides were
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31 12 added in triplicate to each well along with 1 μ g/ml of co-stimulatory anti-CD28
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33 13 (BDBiosciences, Oxford) and diluted in RPMI to make volumes up to 200 μ l/well.
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36 14 Cell/peptide mixes were incubated overnight at 37⁰C in an atmosphere of 5% CO₂. Cell
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38 15 suspensions were then discarded from the plate and wells were washed six times with
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40 16 sterile, filtered PBS/Tween-20 (0.05% v/v). Plates were then coated with biotinylated
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43 17 mouse anti-human-IFN γ mAb (7-B6-1, MabTech, Sweden), diluted 1:1000 in PBS/1%
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45 18 FCS, for 3 hours at room temperature. Plates were washed again, as above, and
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48 19 incubated with streptavidin-alkaline phosphatase diluted 1:1000 using PBS 1% FCS for 2
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50 20 hours at room temperature. Plates were washed as before, substrate was then added and
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52 21 spots were allowed to develop for approximately 15 minutes. Reactions were quenched
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55 22 using tap water and dried overnight prior to counting using a light microscope.
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60 24 **Peptide stabilization assay using T2 cells**

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3 1 TAP-deficient T2 cells were grown in suspension using upright vented T75 flasks
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5 2 (Sarstedt, Leicester, UK) with RPMI 1640 medium supplemented with 10% FCS and
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7 3 penicillin streptomycin (Gibco, Paisley Scotland) in a humidified incubator at 37⁰C in 5%
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9 4 CO₂. Cells were split 1:5 with fresh supplemented RPMI 1640 medium every three to five
10
11 5 days. Cell density and viabilities were ascertained by staining with 4% trypan blue in PBS
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13 6 (Sigma, Dorset UK) and counting under a disposable haemocytometer. T2 cells were
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15 7 seeded in 48 well plates at a density of 5 x 10⁵ cells/ ml in fresh RPMI 1640 and were left
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17 8 at room temperature for 1 hour to equilibrate. They were subsequently incubated with the
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19 9 peptides for 16h at 37⁰C 5% CO₂, after which the suspensions were centrifuged at 1200
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21 10 rpm 5 minutes and washed three times in 5ml PBS (4⁰C) to remove any unbound
22
23 11 peptides. Cell pellets were resuspended and incubated with mouse anti-human IgG2b
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25 12 HLA-A*0201 FITC conjugate (BB7.2 SeroTec), diluted 1/10 in PBS/1% FCS, for 30 min at
26
27 13 4⁰C. The cells were centrifuged and washed, as before, and were finally resuspended in
28
29 14 PBS 0.01% sodium azide (Sigma-Aldrich, Poole UK). Isotype control (IgG2b) antibodies
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31 15 were also used to stain cells using the same conditions. Increase in mean fluorescence
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33 16 intensity was analysed using CellQuestTM software on a FACSCANTM (Becton Dickinson
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35 17 Biosciences). The percentage increase in fluorescence intensity was measured calculated
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37 18 as: (MFI (peptide)/MFI (control)) x 100 = % increase in MFI.
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48 20 **Peptide competition assay**

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50 21 The competition assay was performed as described in van der Burg (human Immun
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52 22 1995). Briefly; 25ul of Fluorescein labelled reference peptide (an HLA A*0201 –binding
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54 23 peptide derived from HBV core antigen amino acids 18-27; FLPSDC(F1)FPSV) at a
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56 24 concentration of 150 nM was incubated with the wild type or mutant ppUL83 peptides in a
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58 25 volume of 25 ul. A total of 4x 10⁴/well in 100 ul of mild acid (0.1M HCl) treated B-LCLs
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60 26 (HLA A*0201 positive) were added to these wells. After incubation for 24 h at 4⁰C, cells

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3 1 were washed three times in PBS containing 1% BSA, fixed with 0.5% paraformaldehyde,
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6 2 and analysed with FACScan flow cytometry to measure the mean fluorescence (MF). The
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8 3 percentage inhibition of the FI-labeled reference peptide binding was calculated using the
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10 4 following formula: $(1 - (MF_{\text{reference + competitor peptide}} - MF_{\text{background}}) / (MF_{\text{reference peptide}} - MF_{\text{background}})) \times 100\%$. A titration of the wild type ppUL83 peptide was used to determine the
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12 5
13 6 optimal concentration resulting in >80% competition and this concentration (1.5 μ M) used
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PCR amplification of sequences encoding HCMV CD8 T-cell epitopes

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12 Primers for the amplification of pp65 HLA-A*0201 and -*B3501 epitopes by nested
13 polymerase chain reaction (PCR) were designed using Primer3 software available at:

14 http://www.genome.wi.mit.edu/genome_software/other/primer3.html

15 All primers were synthesised and purified commercially by MWG-Biotech (Germany). The
16 primers used are shown in Table 2. PCRs were performed using a Bio-X-act Short high-
17 fidelity polymerase (Bioline, London UK) to minimise the potential emergence of random
18 sequence changes during the amplification process. DNA was isolated from PCR-positive
19 solid organ transplant patients with viral loads >3 log genome copies/ml of whole blood.
20 DNA sequences were initially amplified using the external primers and 2 μ l of resultant
21 DNA was amplified using inner sense and antisense primers. The amplification conditions
22 comprised, 10X OptiBuffer (Bioline, London UK) 5 μ l, 50mM MgCl₂ Solution (2.5 μ l), 100mM
23 dNTP (1 μ l), template DNA (0.5 μ l), external or internal primers (100 μ M;1 μ l) and BIO-X-ACT
24 Short 4Units/ μ l (Bioline, London, UK)(1 μ l) in a total reaction volume of 50 μ l. PCR cycling
25 conditions for the first and second rounds were 95 $^{\circ}$ C for 5 minutes followed by 20 cycles of
26 55 $^{\circ}$ C for 30s, 70 $^{\circ}$ C for 30s and 95 $^{\circ}$ C for 30s with a final extension stage at 72 $^{\circ}$ C for 10

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3 1 min. Following amplification the DNA was mixed with loading dye and visualised on 1.5%
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6 2 agarose gel. Bands of interest were purified from 1.5% w/v low melting-point agarose gel
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8 3 using the QIAquick gel extraction protocol (Qiagen, Crawley).
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13 5 Purified PCR amplified DNA products were ligated into pGem-T[®]-easy vector (Promega,
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16 6 Southampton UK) according to the manufacturers instructions and then used to transform
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18 7 competent *E.coli* strain DH5-alpha cells (Gibco, BRL). Clones containing inserts were
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20 8 identified and cultured overnight and plasmid DNA extracted using standard protocols
21
22 9 (QIAprep MiniPrep Kit, Qiagen, London UK). The presence of the desired fragment was
23
24
25 10 confirmed by restriction endonuclease digestion and gel electrophoresis. Individual clones
26
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28 11 were then subjected to DNA sequence analysis on an ABI Prism[®] capillary-based
29
30 12 sequence analyser using the BigDye[®] terminator v3.1 cycle sequencing kit (Applied
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32 13 Biosystems, UK). Sequences were analysed and graphics were generated utilising
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34 14 Chromas 2.23 software available at: <http://www.technelysium.com.au>
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1 Results

2 3 Effect of amino acid substitutions in the immunodominant ppUL83 HLA-A2 epitope 4 on IFN γ production

5 To assess the impact that mutations in the immunodominant HLA-A*0201 CD8 T-cell
6 epitope NLVPMVATV of the ppUL83 HCMV protein would have on CD8 T-cell recognition,
7 peptide variants of this sequence were designed based on substitutions of all residues
8 (except anchor residues at P2 and P9) with either a glycine residue or, where possible, a
9 conservative amino acid substitution (Table I). The peptides were used to stimulate
10 freshly prepared lymphocytes isolated from four healthy volunteers (three HCMV-
11 seropositives, two with haplotype HLA A*0201 and one with HLA B*0701, and one HCMV
12 seronegative HLA*A0201 individual) and CD8 T-cells secreting IFN γ enumerated. The
13 results showed that key amino acid substitutions abrogated IFN γ production by epitope-
14 specific CD8+ lymphocytes (Figure 1). Mutations in the central region of the peptide
15 (positions P4 and P5) had the most profound effect on IFN γ production and consistently
16 resulted in the production of less than 20% of the IFN γ response elicited by the wild-type
17 peptide. Interestingly, different substitutions at the same peptide positions had varying
18 effects on IFN γ production. For example, the V6G mutant abolished IFN γ production
19 whereas V6L still elicited a response, albeit at a substantially lower level than the wild-type
20 peptide. This phenomenon was also observed with the V3L, V3G and T8G, T8S
21 substitutions. Surprisingly, substitutions around the anchor residues impacted less on
22 IFN γ production than substitutions nearer the peptide core.

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24 Although these data demonstrated that certain substitutions affected IFN γ production by
25 epitope-specific CD8+ cells, it was unclear whether these effects were manifest at the level
26 of HLA class I binding or TCR recognition. Thus, we investigated the class I HLA binding

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3 1 efficiency of the variant peptides and the quantitative presentation of the class I HLA
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6 2 peptide complex on the cell surface.
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11 4 **Effects of mutations of the ppUL83 HLA A*0201 peptide on cell surface HLA A*0201**
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13 5 **expression and HLA binding**
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17 7 In order to determine whether amino acid substitutions in the HLA-A*0201 epitope
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20 8 influenced binding to HLA-A*0201, a T2 cell HLA-stabilisation assay was performed. As
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22 9 expected, the wild-type epitope, NLVPMVATV, increased the surface density of HLA-
23
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25 10 A*0201 by 180% compared to background levels (data not shown). A time course of
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27 11 stabilisation using FACS analysis of the wild type epitope showed that surface expression
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29 12 of HLA-A*0201 remained constant over the first 8 hours of the assay and remained
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31
32 13 unchanged at 16 hours. Many, but not all, of the peptide variants examined resulted in
33
34 14 only moderate stabilisation of expression cell surface class I HLA molecules (mean =
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36 15 120%). These results were consistent with aforementioned results showing that the
37
38
39 16 peptides were unable to elicit a substantial IFN γ response. However, exceptions were
40
41 17 apparent with the G6 and S8 substitutions, which weakly stabilised the HLA class I
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44 18 complex, but were able to elicit IFN γ production which was 80% of the wild type levels.
45
46 19 Conversely, the M5C mutation strongly upregulated HLA A*0201 cell surface expression
47
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49 20 (Figure 2) but failed to elicit IFN γ production. Titration of the C5M mutant peptide on T2
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51 21 cells showed that the lowest concentration required to stabilise class I HLA surface
52
53 22 expression to the same extent as the wild type peptide was 0.5 μ M which was 125 times
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56 23 lower than the optimal concentration of the wild type peptide (Figure 3).
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60 25 We next investigated whether the C5M variant could out-compete the wild-type peptide for
26 class I HLA binding. Initially an ELISPOT titration was performed to determine the optimal

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3 1 concentration of wild-type peptide required to elicit 50% maximal IFN γ production to
4 prevent saturation of all epitope-specific TCRs. The optimal concentration of the
5
6 2 NLVPMVATV peptide that satisfied this criterion was 0.75 μ M. The M5C variant peptide
7
8 3 was then titrated with the wild-type peptide and the number of IFN γ spots produced
9
10 4 enumerated. The results showed that the M5C peptide was able to reduce the amount of
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12 5 IFN γ elicited by the wild-type NLVPMVATV peptide by approximately 25% when co -
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14 6 incubated at low concentrations (10nM) although even at high concentrations it was
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16 7 unable to completely abrogate recognition of wildtype peptide by the ppUL83 A*0201
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18 8 specific CD8 T-cells (Figure 4).
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11 In order to determine the binding affinity of peptides which resulted in high level of
12 expression of HLA A 0201 on T2 cells, we used a fluorescent based competition assay. A
13 titration of the wild type ppUL83 peptide against the fluorescein labelled HBV core A*0201
14 peptide revealed that a concentration of 1.5 μ M of the wild-type NLVPMVATV peptide
15 reduced the binding of the fluoresceinated reference peptide by 15% (Figure 5a). We then
16 used this concentration for the three mutants peptides; M5C, V3L and A7G, which induced
17 higher level of HLA A*0201 surface expression in the T2 assay. The M5C mutant was a
18 more potent competitor than the wild-type NLVPMVATV peptide and inhibited binding of
19 the FL-fluorescent peptide to 32%. The V3L mutant also showed some enhanced
20 competition (22%) although the A&G mutant was comparable to the wild type in this assay
21 (Figure 5b). These results are consistent with the T2 cell stabilisation assay and indicate
22 that the M5C peptide has a higher affinity for HLA A*0201 resulting in increased surface
23 expression.

24 **Mutations in ppUL83 A*0201 epitopes in vivo**

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1 In order to investigate whether mutations in the specific ppUL83 epitopes were being
2 produced in vivo we focused on patients who were undergoing rapid and/or persistent
3 HCMV replication in the presence of a ppUL83 specific CD8 T-cell response (enumerated
4 by class I HLA tetramers). DNA sequences encoding three HLA-A*0201 and one HLA-
5 B*3501-restricted CD8 T-cell epitopes within ppUL83 were PCR-amplified from the
6 patient's blood, cloned and then multiple clones subjected to DNA sequence analysis. No
7 mutations in the sequences of any of the epitopes analysed were detected in either the
8 four long-term HCMV-PCR positive patients or five transplant patients with rapidly
9 increasing HCMV loads indicating that the major class I HLA A*0201 CD8 T-cell epitope
10 at amino acids 495-503 of ppUL83 was genetically stable despite being under high
11 immune pressure. These data are summarised in Table III.

1 Discussion

2 Analysis of the sequence constraints within immunodominant epitopes of viral proteins
3 provides insight into the role of key residues in class I HLA binding, in the engagement
4 with the TCR and can inform on the likely pattern of escape mutants in vivo (for example
5 see [Bertoletti et al., 1994]). In this study we performed a detailed investigation of the HLA
6 A*0201 restricted epitope within the HCMV ppUL83 protein that is one of the most
7 immunodominant viral epitopes so far characterised. Our results show that the majority of
8 mutations had a deleterious effect on IFN γ production with values ranging from <10% to
9 ~80% of the wild type peptide induced IFN γ levels. Thus, if such mutations occurred in vivo
10 it is likely that they would escape recognition by CD8 T-cells specific for the wild type
11 epitope (see later). To ascertain the mechanism(s) by which the mutant peptides resulted
12 in the reduction of IFN γ production (i.e. at the level of class I HLA stabilisation or TCR
13 engagement) we first investigated the ability of the peptides to bind to and stabilise cell
14 surface expression of class I HLA molecules. In virtually all cases, the mutant peptides
15 were compromised in their abilities to stabilise cell surface class I display. This frequently
16 correlated with the ability of the same peptide to elicit IFN γ production. However, there
17 were some notable exceptions. Mutations at the G6 and S8 positions which only weakly
18 stabilised class I HLA cell surface expression were relatively potent at eliciting IFN γ
19 production (~80% wild type levels) whereas the M5C mutant peptide showed high level
20 stabilisation of cell surface class I HLA and yet elicited very low levels of IFN γ production.
21 It remains unclear as to why the C5M mutant was so potent at stabilising the cell surface
22 expression of the HLA class I molecule since peptide binding affinity algorithms do not
23 indicate a particularly high binding affinity for this peptide. Further investigations showed
24 that this mutant peptide was about 125 times more potent at binding class I HLA than the
25 wild type peptide and was able to competitively inhibit IFN γ production by the wild type

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3 1 peptide but was unable to fully out-compete the wild type peptide under the assay
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6 2 conditions used. To our knowledge this effect has not been described before for HCMV
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8 3 peptides and this peptide may have utility as an antagonist in CD8 T-cell investigations.
9
10 4 Complementary to the current study, an alanine walking study has previously shown that
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12 5 certain variants within the ppUL83₄₉₅₋₅₀₃ could act as potent enhancers of the T-cell
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15 6 response [La et al., 2001]. Since the wild type ppUL83 peptide was not as effective at
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17 7 stabilising class I HLA surface expression as the HBV core₁₈₋₂₇ peptide, it is tempting to
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20 8 speculate that this is another way in which HCMV subverts the host immune response.
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24 10 Subsequently we investigated whether CD8 T-cell escape was evident in vivo. We
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27 11 concentrated our analyses on patients who had experienced persistent HCMV replication
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29 12 in the presence of antiviral CD8 T-cells specific for the
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32 13 ppUL83₄₉₅₋₅₀₃ epitope or patients who were experiencing rapid exponential increases in
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34 14 HCMV load. We, and others, have previously shown that drug resistant mutants can
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36 15 readily be generated following long periods of persistent replication prior to and following
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39 16 drug therapy [Emery and Griffiths, 2000; Bowen et al., 1998; Boivin et al., 2001; Baldanti et
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41 17 al., 1998; Chou et al., 2007; Chou et al., 2000; Erice et al., 1997]. In addition, the rapid
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44 18 replication dynamics of HCMV in vivo with doubling times of ~1 day will randomly generate
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46 19 most single site mutations at quasi-steady state loads [Whalley et al., 2004; Emery and
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48 20 Griffiths, 2000]. However, DNA sequence analysis of multiple clones from these patients
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51 21 did not reveal a substantial population of CD8 T-cell epitope escape mutants. Although we
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53 22 could have increased sensitivity by analysing more clones by the use of SSCP as
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55 23 previously used in our HBV analysis [Whalley et al., 2004] at present it seems unlikely that
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58 24 CTL escape is a major factor in HCMV pathogenesis. Few other studies have investigated
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60 25 the CD8 CTL escape in HCMV with one study in bone marrow transplant recipients
26 reporting the identification of a single mutation in the ppUL83₄₉₅₋₅₀₃ epitope (methionine to

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3 1 leucine at position 5; [Zaia et al., 2001]) although this study did not use a proof reading
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6 2 Taq polymerase and so error introduction at the PCR stage cannot be excluded. Indeed,
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8 3 when we amplified this region using a non-proofreading Taq polymerase the same
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10 4 mutation was observed (data not shown). In the case of both HBV and HIV where error
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13 5 prone replication is more evident than HCMV, CD8 T-cell escape has been demonstrated
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15 6 [Borrow et al., 1997; Karlsson et al., 2007; Pillay et al., 2005; Bertoletti et al., 1994;
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17 7 Whalley et al., 2004]. In contrast to the lack of evidence for T-cell escape in HCMV, there
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20 8 are reports of mutations segregating with antibody escape within the AD-1 epitope of
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22 9 glycoprotein B [Darlington et al., 1991; Roy et al., 1993] . These differences may reflect the
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24
25 10 complex mechanisms HCMV has evolved to manipulate the cell mediated and innate
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27 11 immune responses that result in a reduced selective pressure favouring the survival of
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29 12 CD8 T-cell escape mutants. In addition, it may reflect the functional competence of the
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32 13 CD8 T-cell population in vivo. Both of these factors need to be investigated further
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34 14 together with complimentary analysis of other CD8 and CD4 epitopes in vivo to gauge the
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36 15 full extent of T-cell escape in HCMV infection.

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39 16 Several mutant peptides could stabilise MHC class 1 A2 molecules better than native
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41 17 sequence, further investigations are needed to address whether these peptides would
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43 18 elicit CMV-specific T- cell recognition and cytotoxic lymphocytes more effectively than the
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46 19 native sequence. Such peptide sequences could be exploited in future HCMV vaccine
47
48 20 candidates.

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26 and discussions on the T2 cell assay.

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53 45 presented by the HLA A*0201 allele among human cytomegalovirus isolates. *Journal of*
54 46 *Virology* 75:2472-4.

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3 1 Table I. Peptide variants of the ppUL83₄₉₅₋₅₀₃ used in the study. The amino acid which has
4 2 been mutated is shown in a shadow font.
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Peptide Sequence	Identification
N L V P M V A T V	Wildtype (wt)
G L V P M V A T V	N1G
Q L V P M V A T V	N1Q
N L G P M V A T V	V3G
N L L P M V A T V	V3L
N L V G M V A T V	P4G
N L V P G V A T V	M5G
N L V P I V A T V	M5I
N L V P C V A T V	M5C
N L V P M G A T V	V6G
N L V P M L A T V	V6L
N L V P M V G T V	A7G
N L V P M V A G V	T8G
N L V P M V A S V	T8S

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3 1 Table II. Nested primer pairs used to amplify HLA A*0201 restricted epitopes in ppUL83
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6 2 from clinical samples. The amino acid position of the epitopes is shown is shown in
7
8 3 brackets for each epitope.
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ppUL83 Epitope	Outer primers	Inner primers	Amplicon size (base pairs)
A*0201 (14-22)	Forward 5'-AATCGGTGGTGG TGTGTGAT Reverse 5'-GATGTTCAGCATC TTGAGCGG	Forward 5'-CTGAATAATCC GCGGTTGTCT Reverse 5'-TTGACCGACAC GTTCTCCA	409
A*0201 (120-128)	Forward 5'-CCGTTGTCCCGA AATGAT Reverse 5'-GGCTCTTTCCAC TGGTTCTG	Forward 5'-AGCGAGGTGG AGAACGTGT Reverse 5'-GCGTGAATCA CAGCGTCA	194
A*0201 (495-503)	Forward 5'-CAGGTAACCTGT TGATGAACGG Reverse 5'-GCCAAAAGTTGC GTGTCTGA	Forward 5'-AGAGGACACC GACGAGGATT Reverse 5'-GAATTGGAAG GCGTATGGC	200

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3 1 Table III. DNA sequence analysis of ppUL83 epitopes in patients experiencing HCMV
4 replication (acute or persistent). The days posttransplant (PT) for each sample is shown
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6 2 together with the HCMV load (\log_{10} genomes/ml of whole blood) and where available the
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8 3 CD8 T-cell frequency against the epitope being investigated using the relevant class I HLA
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10 4 tetramer.
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Table III.

Patients persistent HCMV PCR+ve	Haplotype	Viral Load (log ₁₀ ge/ml)	Days PT	ppUL83 ₍₁₄₋₂₂₎ HLA-A*0201	ppUL83 ₍₁₂₀₋₈₎ HLA-A*0201	ppUL83 ₍₄₉₅₋₅₀₃₎ HLA-A*0201	% NLV-specific CD8 cells	ppUL83 ₍₁₂₃₋₁₃₁₎ HLA-B*3501	% IPS-specific CD8 cells
				V L G P I S G H V	M L N I P S I N V	N L V P M V A T V		I P S I N V H H Y	
1	HLA-B*3501	3.4	54	N/A	N/A	N/A	N/A	-----	ND
			140	N/A	N/A	N/A	N/A	-----	2.3
		4.3	372	N/A	N/A	N/A	N/A	-----	4.1
		3.8	507	N/A	N/A	N/A	N/A	-----	3.0
2	HLA-A*0201	5.0	26	-----	-----	-----	0.4	ND	N/A
		3.3	48	-----	-----	-----	0.32	ND	N/A
		3.6	53	-----	-----	-----	0.28	ND	N/A
		3.2	93	-----	-----	-----	0.65	ND	N/A
3	HLA-A*0201	3.1	17	-----	-----	-----	0.33	ND	N/A
		3.4	38	-----	-----	-----	0.32	ND	N/A
		3.7	57	-----	-----	-----	0.43	ND	N/A
		2.8	67	-----	-----	-----	0.40	ND	N/A
4	HLA-A*0201	3.8	26	-----	-----	-----	0.34	ND	N/A
		4.0	30	-----	-----	-----	0.65	ND	N/A
		2.9	90	-----	-----	-----	N/A	ND	N/A
Log Phase									
5	HLA-A*0201	3.0	180	-----	-----	-----	N/A	ND	N/A
6	HLA-A*0201	5.2	41	-----	-----	-----	N/A	ND	N/A
7	HLA-A*0201	3.3	102	-----	-----	-----	N/A	N/A	N/A
8	HLA-A*0201	3.6	56	-----	-----	-----	N/A	N/A	N/A
9	HLA-A*0201	3.9	150	-----	-----	-----	N/A	N/A	N/A

Figure Legends

Figure 1

Induction of Interferon gamma production by the mutant ppUL83 peptides. The data are expressed relative to the wild type NLVPMATV peptide (100%) and are presented as the mean of triplicates. The results for two HLA A*0201 individuals are shown, together with a HCMV seronegative HLA A*0201 control and an individual restricted via HLA B*0701.

Figure 2

Stabilisation of class I HLA cell surface expression on T2 cells by wild type and mutant ppUL83₄₉₅₋₅₀₃ peptides. The stabilisation is expressed as the fold increase in mean fluorescence intensity (MFI for mutant peptide/MFI for wildtype peptide).

Figure 3

Relationship between the cell surface expression of HLA A*0201 on T2 cells and the concentration of the M5C mutant peptide.

Figure 4

Titration of the NLVPCATV mutant peptide against a fixed concentration (75uM) of wild type ppUL83₄₉₅₋₅₀₃ peptide in an ELISpot assay. The mean number of cells producing IFN γ is shown (spots/well). All assays were performed in triplicate.

Figure 5

Competition of binding of the HLA A2 FL-labelled reference peptide HBcAg FLPSDC(F1)FPSV by; (a) various concentrations of wild type ppUL₈₃₄₉₅₋₅₀₃ peptide, and M5C, V3L and A7G mutant peptides (b). EBV-transformed HAL-A2 cells were incubated with FL-labelled reference peptide and the test

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3 peptides for 24 hours at 4°C. Fluorescence was measured with flow
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6 cytometry using FACScan and the inhibition of binding was calculated.
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