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Synthesis and *ex vivo* profiling of chemically modified cytomegalovirus CMVpp65 epitopes

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Abstract: The effect of substituting unnatural hydrophobic amino acids into the critical MHC binding residues of an HLA-A*0201-restricted cytomegalovirus CMVpp65 epitope, NLVPMVATV, has been investigated. A new set of peptides containing the amino acids *tert*-butyl glycine (Tgl), cyclohexyl glycine (Chg), *neo*-pentyl glycine (Npg), cyclohexyl alanine (Cha) and cyclo leucine (Cyl), at either position 2, to mimic Leu, or position 9, to mimic Val, have been synthesised. Immunological profiling using class I MHC stabilisation assays to assess MHC binding affinity, and enzyme-linked immunospot (ELISPOT) assays to assess the ability of the modified peptides to re-stimulate a specific cytotoxic T-lymphocyte (CTL) response, compared to the native epitope, have been performed. It was found that the majority of the unnatural substitutions resulted in a decrease in either HLA-A*0201 binding affinity or cytotoxic T-cell activity. However, the HLA-A*0201 binding affinity was unrelated to the ability to re-stimulate a T-cell response. Minimisation and molecular dynamics studies proved helpful in dissecting the ELISPOT responses. Two principal peptide binding modes were found by minimisation, designated kinked and straight. Peptides that bound in a kinked conformation were poor at re-stimulating a T-cell response. Of the peptides that bound in a straight conformation, molecular dynamics (MD) simulations revealed that those capable of re-stimulating the strongest responses had the greatest degree of flexibility (as determined by RMSD values across the MD simulation) around the P6 residue, one of the residues important for T-cell receptor recognition. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chemically modified peptide epitopes; cytomegalovirus CMVpp65 epitope; synthetic antigens; hydrophobic amino acids

INTRODUCTION

Human cytomegalovirus (CMV) is an important pathogen, geographically widespread and infecting the majority of the general population across all socio-economic groups [1,2]. Viral latency *in vivo* is maintained in healthy individuals by T-cell immune surveillance, resulting in very few, if any, symptoms and no long-term health consequences. However, if T-cell function is impaired, viral activation and clinical disease may develop. In immunocompromised patients, activation of latent CMV can lead to potentially life-threatening diseases [2,3]. Immunisation of CMV seropositive individuals could achieve a reduction in CMV load through boosting existing responses, thereby providing a significant therapeutic benefit to these high-risk individuals [4–6].

Classical immunisation strategies involving treatment with attenuated viral preparations have had limited success in combating CMV infection, and carry with them safety implications [7]. In the case of chronic infections such as CMV, evolution of the virus under the selective pressure of the host's immune system has led to the expression of sub-optimal epitopes and the development of other strategies to evade immune surveillance [8].

The cytotoxic T-lymphocyte (CTL) response generated to peptide epitopes plays a crucial role in suppressing viral activation, and an alternative vaccination modality employing T-cell epitopes rather than attenuated viral preparations has shown considerable promise in animal models [9]. To date, the majority of studies on CTL responses towards CMV have focused on epitopes generated from the CMV pp65 protein [10–12]. The viral tegument protein pp65 is recognised by most CMV^+ individuals, and an epitope from this protein (NLVPMVATV, residues 495–503) is immunodominant in the context of the human leukocyte antigen HLA-A*0201, the most common HLA allele.

Recent advances in our understanding of the cellular immune response at the molecular level have led to a new strategy for vaccine development, based on modification of epitopes such as $pp65_{495-503}$ to make them more immunogenic, a process termed

Abbreviations: BSA, bovine serum albumin; FCS, foetal calf serum; PBS, phosphate buffered saline; RPMI media, Roswell Park Memorial Institute media; AIM-V media, L-glutamine (50 μg/ml), streptomycin sulfate and gentamycin sulfate (10 μg/ml); IFN-γ, gamma interferon; p/s, penicillin/streptomycin; Tween 20, polyoxyethylene sorbitan monolaurate; MHC, major histocompatibility (molecule); TCR, T-cell receptor; PBMC, peripheral blood mononuclear cells.

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epitope enhancement [13]. Epitope enhancement can be achieved by increasing the affinity of the epitope for an MHC molecule (e.g. HLA-A*0201), by increasing the affinity of the peptide–MHC complex for the TCR, or by a combination of the two [14]. The approach that has been most widely adopted, and that has been shown to produce significantly improved vaccines, is to increase the affinity of the epitope for the MHC molecule. Raising the affinity, and so making an epitope more competitive for available MHC molecules, increases the concentration and duration of presentation of that specific peptide–MHC complex at the cell surface and reduces the amount of peptide required for immunisation. Epitope enhancement has been demonstrated in the case of HIV [15], hepatitis C virus [16] and cancer [17,18]. Peptides interact with MHC molecules primarily through the *C*- and *N*-termini and a number of anchor residues that bind in specific pockets in the protein [19]. An increase in the affinity of a peptide epitope for its MHC restriction element can be achieved by modification of the primary and/or secondary [20] anchor residues to produce a stronger interaction with the binding pocket.

The class I MHC molecule HLA-A*0201 is one of the most extensively studied human alleles and was among the first to be crystallised [21]. The length of peptides bound to A*0201, in common with most class I MHC molecules, has been shown to be between eight and ten residues. Examination of several peptide-A*0201 crystal structures has revealed two conserved features. The second residue (P2) of the peptide chain is bound in a pocket defined by the residues Tyr7, Phe9, Met45, Glu63, Lys66, Val67, His70 and Tyr99, specific for Leu or Ile at P2 of the epitope. At the *C*-terminus of the peptide, the pocket accommodating P9 is defined by Asp77, Thr80, Leu81, Tyr116, Tyr123, Thr143 and Trp147, and is specific for Val as the *C*-terminal residue [22].

Stabilisation of peptide–MHC complexes by filling a hydrophobic binding pocket with non-encoded amino acids has previously been demonstrated for an epitope restricted to HLA-B*2705 [23]. Replacement of a glycine residue by various aromatic amino acids exploited a hydrophobic interaction with a secondary anchor pocket. The modified peptides exhibited an enhanced affinity for HLA-B*2705, although the ability of the peptides to re-stimulate a T-cell response was not determined. We considered that a similar strategy could be used to increase the affinity of $pp65_{495-503}$ for HLA-A*0201. Guided by the crystallographic data for a number of peptide–A*0201 complexes [22], we synthesised a series of modified epitopes incorporating unnatural hydrophobic and conformationally restricted amino acids, **1**–**5** (Figure 1) at the P2 and P9 anchor positions, and studied the effect of these substitutions on MHC binding affinity and T-cell response.

MATERIALS AND METHODS

Amino acids were purchased from Aldrich Chemicals or AnaSpec. Peptide synthesis reagents and solvents were purchased from Applied Biosystems. All other reagents and solvents were purchased from Aldrich Chemicals.

General Fmoc Protection Procedure for Amino Acids 1–5

The amino acid (4 mmol) was dissolved in acetone : H_2O (1 : 1, 20 ml), and $NaHCO_3$ (1 M, 4.4 ml) was added, followed by Fmoc-OSu (1.41 g, 4.4 mmol). The mixture was stirred at room temperature for 16 h. Following concentration *in vacuo*, the crude residue was partitioned between CH_2Cl_2 and H_2O and the organic layer separated. After further extractions with CH_2Cl_2 (3×30 ml) the combined organic extracts were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to provide the crude product. Purification by recrystallisation from EtOAc : Hexane 1 : 1 gave the *N*-protected amino acid as a white crystalline solid. Data were in agreement with the previously reported values [24–27].

General Synthesis and Purification of Modified CMVpp65 Epitopes

Solid phase peptide synthesis (SPPS) on a 0.1-mmol scale, using Fmoc-protected amino acids and HBTU/HOBt-mediated coupling in NMP as solvent, was carried out on an Applied Biosystems 433A peptide synthesiser. Piperidine (20%) in NMP was used for Fmoc deprotection. The Thr side chain was protected as a *tert*-butyl ether, and the Asn side chain was protected with Trt. Wang resin preloaded with Fmoc-protected *C*-terminal amino acid was used in the synthesis of the peptides **6**–**10**. Following resin swelling by washing with NMP, the *N*-terminal Fmoc-protecting group was removed. Coupling using 10 equivalents of the appropriate Fmoc-protected amino acid, preactivated with HBTU/HOBt, was performed with a coupling time of 15 min. A double coupling protocol was used for all residues following Pro. On completion of the synthesis, the resin was washed with CH_2Cl_2 and dried *in vacuo* for 2 h. The resin was placed in a cleavage vessel comprising a glass tube with glass sinter and three-way tap [28]. The

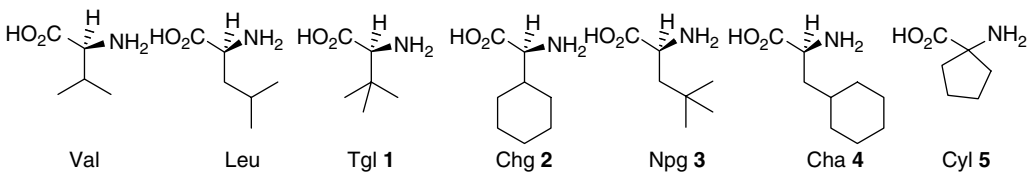

Figure 1 Valine, leucine and the unnatural amino acids **1**–**5**.

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peptide was cleaved with reagent B (TFA : phenol : water : triisopropylsilane [88 : 5 : 5 : 2, 10 ml]) at room temperature for 2 h. The cleavage cocktail was removed by filtration and the resin washed with TFA (2 × 5 ml). The combined filtrate was evaporated to dryness and the residue was triturated with Et_2O (5 ml). The resultant solid was dissolved in water (5 ml) and lyophilised to afford the fully deprotected crude peptide as a white solid.

Resin Loading of C-terminal Residues

For peptides **11**–**13**, loading of the appropriate *C*-terminal amino acid (0.1 mmol scale) was carried out on an Applied Biosystems 433A peptide synthesiser. Unloaded Wang resin was swollen by washing with NMP, and following activation with a solution of DCC (1 mmol) and DMAP (0.1 mmol) the appropriate Fmoc-protected *C*-terminal amino acid (1 mmol) was coupled to the resin. Unreacted resin sites were capped by the addition of benzoic anhydride (3 mmol). The loading level of the resin was measured by Fmoc number [29].

High-Pressure Liquid Chromatography (HPLC)

All HPLC was performed using a Dionex summit HPLC system with Chromeleon software. Analytical and semi-preparative HPLC was carried out using a Summit P580 quaternary low-pressure-gradient pump with a built-in vacuum degasser. A P580P high-pressure binary gradient pump with built-in vacuum degasser was employed for preparative HPLC. A UVD 170s UV/VIS multi-channel detector was used to monitor all HPLC. Luna 10 μ columns, supplied by Phenomenex, containing C18 as the sorbent were used for all HPLC (250 × 4.6 mm, 250 × 10 mm and 250 × 21.2 mm columns were used for analytical, semi-preparative, and preparative HPLC respectively). All HPLC was performed using the following eluents: eluent A (water : TFA (99.95 : 0.05)); and eluent B (MeCN : TFA (99.95 : 0.05)). Method 1 utilised a gradient of eluent A : B 80 : 20 to 25 : 75 over 30 min; method 2 utilised a gradient of eluent A : B 100 : 0 to 0 : 100 over 60 min.

Mass Spectrometry

Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry was carried out using a Bruker Biflex IV instrument using either sinapinic acid or α-cyano-4-hydroxy-*trans* cinnamic acid : nitrocellulose (3 : 1) as a thin layer matrix.

Class I MHC Surface Stabilisation Assays

T2-A*0201 cells (2×10^5) were incubated in serum-free AIM-V medium in the presence of 100 μg/ml of the peptide (or, for the control, an equivalent amount of DMSO, the solvent used to dissolve the peptides) for 14–16 h at 26 °C, after which the cells were incubated at 37 °C for 2 h prior to immunofluorescent staining. Cells were washed free of unbound peptide with growth medium prior to the addition of primary antibody. Anti-MHC allele-specific monoclonal antibody, W6/32, was added to the T2-A*0201 cells and incubated at 4 °C for 30 min. To detect binding of the W6/32 monoclonal antibody, these cells were washed and incubated with an anti-mouse fluorescein isothiocyanate-labelled antibody at 4 °C for 30 min. Finally, cells were washed and resuspended in 500 μl of cold PBS supplemented with 1% FCS. Mean fluorescence intensities of the stained T2-A*0201 cells were then measured using flow cytometry. To determine basal A*0201 stabilisation, a sample of T2-A*0201 cells was incubated with AIM-V medium alone at 26 °C for 14–16 h and the levels of cell-surface MHC determined as above. An additional negative control comprised a sample of T2-A*0201 cells that had been cultured in growth medium without the peptide at 37 °C. The MHC stabilisation efficiency (MSE) for each peptide was calculated as the percentage increase of the mean fluorescence above that of the basal levels.

IFN-γ ELISPOT Assays

The ELISPOT assay was used to determine whether the peptides could re-stimulate a memory T-cell response (as determined by the production of IFN-γ) in PBMC from a CMV seropositive donor. PBMC were purified from the blood of CMV-infected individuals using standard procedures [30]. Informed consent was taken from all blood donors prior to collection, and all experiments were approved by the South Birmingham Local Research Ethics Committee. A 96-well nitrocellulose plate (MAIP N45, Millipore) was coated overnight at 4 °C with anti-IFN-γ monoclonal antibody 1-D1K (Mabtech; 15 μg/ml, filtered PBS). The excess antibody was flicked off the plate and the plate was washed 6 times with filtered RPMI medium (200 μl). Blocking of each well of the plate was achieved with the use of RPMI medium supplemented with 10% FCS solution (200 μl) over a period of 1 h. The blocking solution was removed, and PBMC from a healthy CMV seropositive donor was added at a concentration of 2×10^5 cells per well in filtered RPMI medium supplemented with 10% FCS and 1% p/s. These cells were incubated for 18 h at 37 °C in a 5% CO_2 atmosphere in the presence of the synthetic peptides (10 μg/ml). After incubation, the plate was washed six times with filtered 0.05% Tween 20 diluted in PBS (150 μl). Biotinylated detection antibody, anti-IFN-γ, 7-B6-1 (50 μl) was added to each well at a final concentration of 1 μg/ml in filtered PBS. The plates were incubated at room temperature for 4 h and then washed, as described above. Streptavidin-alkaline phosphatase (50 μl) was added to each well at a final concentration of 1 μg/ml in filtered PBS and incubated at room temperature for 2 h. After a final wash with PBS, the alkaline phosphatase substrate (Biorad, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium) was added to each well, and the plates were incubated for 10 min at room temperature. Cells that produced IFN-γ in response to the presence of peptides were detected as purple spots on the nitrocellulose surface of each well. Results are expressed as number of spot-forming cells (SFC)/10^5 PBMC.

Energy Minimisation and Molecular Dynamics

Energy minimisations and molecular dynamics (MD) simulations were carried out using the AMBER v4.1 and the Amber94 forcefields [31,32]. The X-ray crystal structure of HLA-A*0201 containing the peptide TLTSCVTSV (pdb code: 1hhg) [22] was chosen as the starting point for calculations since the CMVpp65$_{495-503}$ epitope (NLVPMVATV) is not represented in the currently determined X-ray structures. The epitope-binding domain, complete with bound peptide, was

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extracted from the complete structure and the bound peptide converted to the NLVPMVATV epitope using the biopolymer and builder modules in InsightII [33]. The resulting complex was solvated in a water box with at least 12 Å water between the protein and the boundaries of the box before minimisation to default criteria with a 12-Å non-bonded cut-off, 4r distance-dependent dielectric and periodic boundary conditions. Complexes containing modified peptides were generated and minimised in the same way.

Following minimisation, all structures were subjected to MD simulations at 300 K, again with periodic boundary conditions. Simulations were run for 100 ps equilibration, followed by 500 ps for data gathering. Analysis of MD trajectories was carried out using the Mdanal module of Amber, determining the average coordinates and rms deviations of atom positions during the simulation (translation and rotation of the whole protein complex were eliminated from the analysis).

RESULTS AND DISCUSSION

Peptide Design and Synthesis

Inspection of a series of peptide–HLA-A*0201 crystal structures [22] suggested that the hydrophobic pockets in the protein accommodating residues P2 and P9 of the bound epitope could accommodate hydrophobic residues slightly larger than Leu or Ile (the most common P2 anchors) and Val (the P9 anchor). To this end, the synthesis of **6**–**13** provides the opportunity to probe the above premise. Amino-3,3-dimethylbutyric acid (*tert*-glycine, Tgl, **1**) and amino cyclohexylacetic acid (cyclohexyl glycine, Chg, **2**) were chosen as P9 Val replacements. The former is a more hydrophobic replacement, while the latter is more bulky and could give more information about the size of residues accommodated by the P9 pocket [34,35]. Similar reasoning was applied to the choice of P2 replacements, with 2-amino-4,4-dimethylpentanoic acid (neopentylglycine, Npg, **3**) designed to function as a hydrophobic Leu replacement and the more bulky cyclohexyl alanine (Cha, **4**) as a probe for the size of the P2 pocket. The effect of substituting 1-aminocyclopentanecarboxylic acid (cycloleucine, Cyl, **5**) at both P2 and P9 was also examined. Cyl is a secondary amino acid containing a quaternary centre, which could replace Val and Leu and may alter peptide conformation in a similar manner to proline [36].

The native and modified epitopes **6**–**13** were synthesised as described in 'Materials and Methods' and purified by reverse-phase HPLC. Following lyophilisation, the peptides were isolated as white solids and characterised by MALDI mass spectrometry, Table 1.

Class I MHC Surface Stabilisation Assays

The ability of the modified peptides and the native epitope to bind to HLA-A*0201 was assessed by a class I MHC surface stabilisation assay, as described in 'Materials and Methods'.

Briefly, cells that are deficient in components of the peptide-loading machinery, T2-A*0201, mostly present empty HLA-A*0201 molecules at the cell surface, which are unstable and are rapidly degraded. Upon binding of an appropriate peptide, the resultant complexes are stabilised, and these can be bound using the class I MHC conformation-specific monoclonal antibody, W6/32, the binding of which can be detected with a fluorescently labelled anti-mouse monoclonal antibody. The fluorescence intensities of these labelled cells can then be determined by flow cytometric analysis, giving a measure of the ability of the peptide to stabilise the complex. This is expressed as MSE, the percentage increase of the mean fluorescence above that of the negative control. All peptides that resulted in a fluorescence intensity greater than the mean +3 standard errors of the mean (SEM) of the fluorescence intensity resulting from the T2-A*0201 cells in the

Table 1 Characterisation data for peptides

Peptide sequence	Yield (%)	t_R (min)[a]	m/z[b]	
			Calculated	Found
Asn-Leu-Val-Pro-Met-Val-Ala-Thr-Val **6**	42	31.33[c]	945.2	945.0
Asn-Cha-Val-Pro-Met-Val-Ala-Thr-Val **7**	46	30.41	983.6	983.9
Asn-Tgl-Val-Pro-Met-Val-Ala-Thr-Val **8**	39	17.97	943.5	943.9
Asn-Cyl-Val-Pro-Met-Val-Ala-Thr-Val **9**	31	18.37	963.5[d]	963.8
Asn-Npg-Val-Pro-Met-Val-Ala-Thr-Val **10**	35	32.63[c]	957.2	958.1
Asn-Leu-Val-Pro-Met-Val-Ala-Thr-Chg **11**	16	19.03	1005.5[d]	1005.9
Asn-Leu-Val-Pro-Met-Val-Ala-Thr-Tgl **12**	52	20.43	957.5	957.6
Asn-Leu-Val-Pro-Met-Val-Ala-Thr-Cyl **13**	39	19.87	955.5	955.7

[a] HPLC retention time, method 1 (see 'Materials and Methods').
[b] $[M + H]^+$ ion from MALDI-TOF mass spectrum of the purified peptide.
[c] HPLC retention time, method 2 (see 'Materials and Methods').
[d] $[M + Na]^+$ ion from MALDI-TOF mass spectrum of the purified peptide.

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absence of peptide at 26°C (negative control) were considered to be positive binders. The results of one representative assay of three are presented as MSE in Figure 2.

The results show that most of the modifications resulted in peptides that were weaker binders than the native epitope **6**, as determined by their ability to stabilise class I MHC protein on the cell surface. Peptides **7**, **8** and **9** (P2-Cha, P2-Tgl and P2-Cyl, respectively) showed comparable binding to that of the native epitope. All the modifications at P9 (Val in the native epitope) resulted in diminished binding, suggesting that the P9 pocket is highly specific for Val.

As peptides **7**, **8** and **9** stabilised surface class I MHC, we next sought to determine whether the modifications

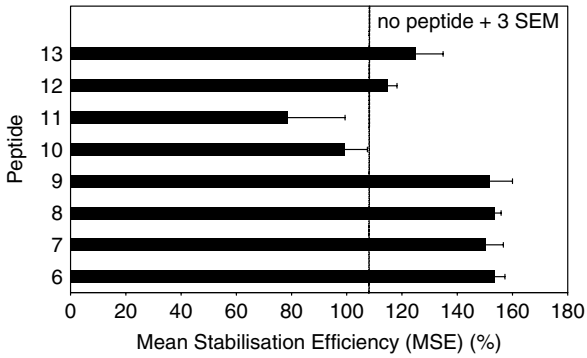

Figure 2 Class I MHC surface stabilisation on T2-A*0201 cells using the native epitope **6** or a series of unnatural analogues **7**–**13**. The dotted line (mean +3 standard errors of the mean (SEM)) indicates the background fluorescence intensity for HLA-A*0201 on T2 cells incubated at 26°C without peptide, which was the cut-off for a positive result.

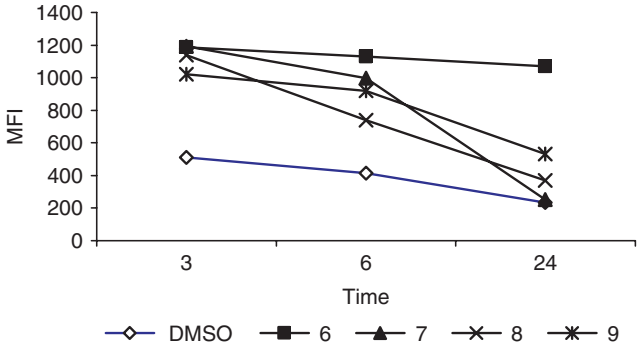

Figure 3 Mean fluorescence intensity (MFI) over a period of 24 h from class I MHC surface stabilisation on T2-A*0201 cells using the native epitope **6** or the unnatural analogues **7**–**9**. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

increased the stability of the class I MHC complex over time compared to the native peptide **6**. The T2-A*0201 cells were loaded with peptide, and the decay in mean fluorescence intensity was measured as before over a 24 h period (Figure 3). While all four peptides were able to stabilise class I MHC protein on the T2 cells to a similar degree after 3 h, the decay in mean fluorescence intensity for the three analogues **7**–**9** was much more rapid than for the native epitope **6**.

IFN-γ Elispot Assays

In a second set of experiments, the consequence of substituting the anchor residues of the epitope on T-cell recognition of the peptide-MHC complex was assessed. ELISPOT assays [37] were performed to assess the ability of the modified epitopes to re-stimulate a memory

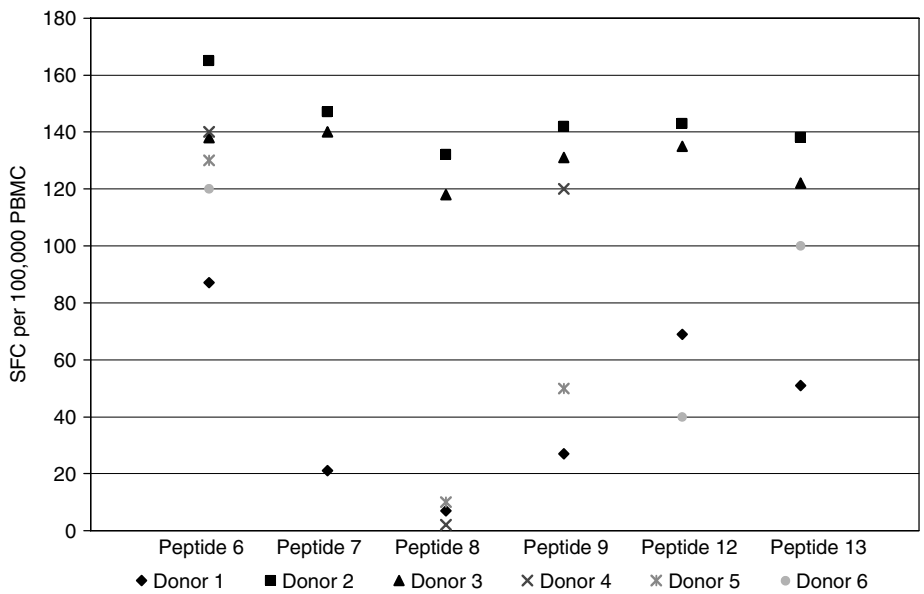

Figure 4 ELISPOT results for the peptides shown to be positive class I MHC binders by surface stabilisation assay. PBMC were obtained from six CMV seropositive donors; the results are expressed as spot-forming cells (SFC) per 100 000 PBMC.

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T-cell response, as measured by the production of IFN-γ. PMBC from CMV seropositive donors were incubated with peptides shown to be positive class I MHC binders and subjected to ELISPOT analysis; the results are represented graphically in Figure 4.

In total, six donors provided the PBMC for the ELISPOT assays, and while all responded strongly to the native epitope, there was a wide variation in response to the modified epitopes. Donors 2 and 3 were the least discriminating, while the others showed a broader panel of responses. This may be a function of different TCR usage by the T-cells of the donors, with the NLV-specific T-cells of Donors 2 and 3 using receptors that are more tolerant to the modifications. The ELISPOT results do not correlate with the ability of the peptides to bind HLA-A*0201, as determined by the class I MHC surface stabilisation assay, or with the stability of the peptide–MHC complex over an extended time period. Peptide **8**, despite being able to stabilise class I MHC molecules at the cell surface as well as the native epitope **6**, was among the poorest at being able to re-stimulate a T-cell response, and it was effectively unrecognised by three of the donors (Donors 1, 4 and 5). In an effort to account for the differences between the peptides, we turned to the results of our MD simulations.

Minimisation and MD

Comparison of the minimised structures of the binding domain of HLA-A*0201 containing peptides **6**–**13** with that of the minimised X-ray crystal structure containing the peptide TLTSCVTSV (pdb code: 1hhg) shows little difference in the binding of the various peptides. Inclusion of a proline at P4 (in place of serine) induces a change in the local conformation of the bound peptide (compared to TLTSCNTSV) to one of two forms – peptides **6**, **7**, **9** and **13** take up a largely linear form, whereas peptides **8**, **10**, **11** and **12** adopt a kinked conformation. With the exception of peptide **8**, these kinked peptides show quite poor binding to HLA-A*0201. In all cases, the bound peptide fits in the binding cavity, and where a larger group is presented to the binding pocket, there is sufficient alteration in the structure of the MHC molecule to accommodate it (such as peptide **7** and peptide **11**), though in these cases the increase in bulk of the residue is not particularly large.

Relating the computational results to the ELISPOT assays is rather more problematical since the TCRs of some donors are much more sensitive than others to peptide modifications and there are a number of factors that may contribute to the experimental result. While the affinity of the peptide for the MHC molecule does not correlate with the ELISPOT result, the conformation of the bound peptide may be important in recognition. Alternatively, the bound conformation may not correspond directly with the recognised conformation, so flexibility of the bound peptide may be important. In two cases, Donors 2 and 3, the ELISPOT assays show little variation for each of the peptides tested, but in the case of Donor 1, peptides **7**–**9** elicit a greatly reduced response compared with the unmodified peptide **6**. Though peptides **7**, **9** and **13** adopt a conformation similar to **6**, flexibility at P6 (as represented by RMSD values across the MD simulation) is reduced (Figure 5). Comparing the RMSD values for peptides **8** and **12** shows little difference in both

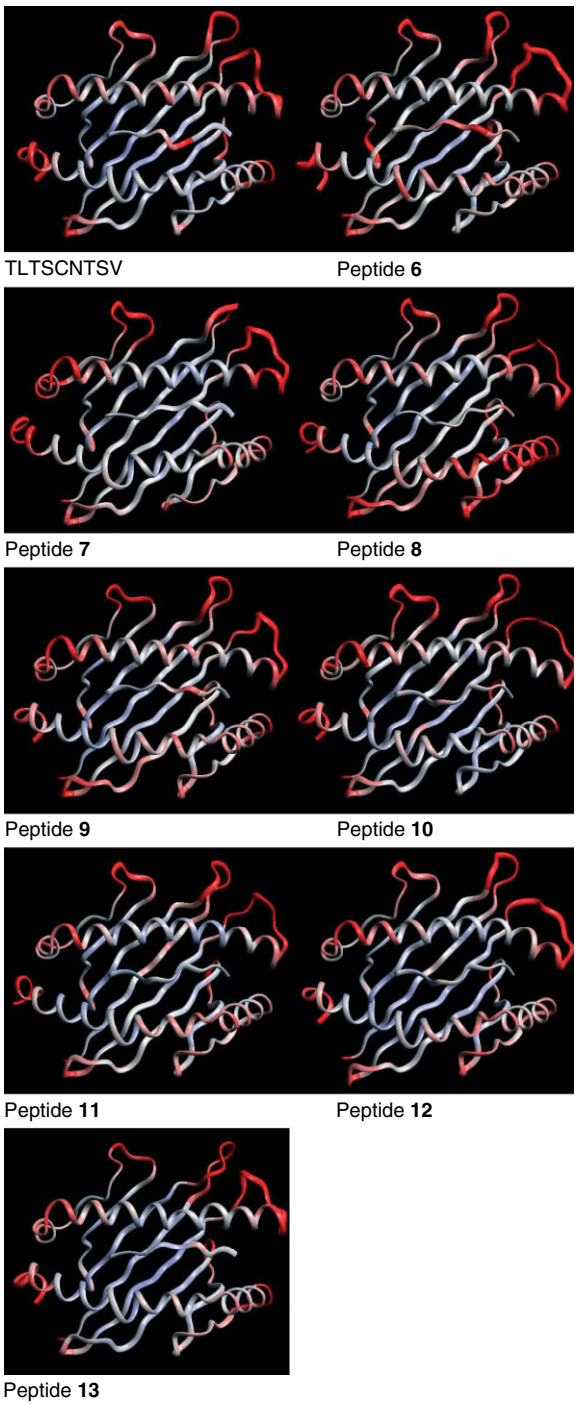

Figure 5 High resolution plots of Mdanal flexibilities; blue indicates low flexibility, red high flexibility.

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conformation and flexibility of the bound peptides but peptide **8** leads to a significant increase in the flexibility of the *N*-terminal end of helix α_2. The *N*-terminal end of helix α_2 lies close to CDR-1β, and so forms part of the TCR-MHC recognition sequence, for some, but not all, T-cell–HLA complexes [38]. Thus increased flexibility in this region may account for the greatly reduced Elispot response with peptide **8** for three of the donors.

CONCLUSIONS

Chemically modified peptide epitopes incorporating unnatural hydrophobic amino acids at the P2 or P9 anchor positions show an altered ability to re-stimulate a T-cell response compared to the native epitope, which is more consistent with changes in the short-timescale dynamics of the peptide–MHC complex than with changes in the bound conformation or affinity. In addition, variation in immune response to these peptides is highly dependent on TCR repertoire even though the sites of modification are not exposed to the TCR. MD simulations suggest that the flexibility of the bound peptide is important in determining the T-cell response to the peptide–MHC complex, and that this flexibility may be influenced by substitutions at peptide anchor residues.

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