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Murdoch UNIVERSITY

Abacavir Impairs an HLA-B*57:01-restricted CD8+ T Cell Response to an Immunodominant Epstein-Barr Virus Epitope

Correspondence:
e.phillips@iiid.com.au

A. Lucas[1], V. Meyer-Pannwitt[1], E. McKinnon[1], S. Burrows[2], M. Rist[2], S. Leary[1], M. Lucas[1], S. Gaudieri[1,4], S. Mallal[1,2,5] *E. Phillips[1-5] [1]Institute for Immunology & Infectious Diseases, Murdoch University, [2]Royal Perth Hospital, [3]Sir Charles Gairdner Hospital, [4]University of Western Australia, [5]PathWest, Perth, Western Australia, Australia

ABSTRACT

Background: Structural and functional studies show that abacavir (ABC) binds non-covalently to HLA-B*57:01 and alters the repertoire of peptide presented, some of which may re-stimulate memory CD8+ T-cells to cause ABC hypersensitivity. We questioned whether the binding of ABC to HLA-B*57:01 might also impair an HLA-B*57:01 restricted CD8+ T-cell anti-viral response. **Methods**: 52 putative B95-8 EBV strain HLA-B*57:01 restricted epitopes were identified using NetMHC3.2 (binding score<400 nM). 7 healthy adult HLA-B*57:01+ donors were screened with EBV peptide pools followed by confirmatory ELISpot with individual EBV peptides and avidity testing to 0.001 mg/ml peptide. In addition, 4 HLA-B*57:01+ and 31 negative healthy donors were screened with 20mer peptides overlapping with 15 aa from EBNA3A, 3B, BZLF1 and BMLF1. Four short term lines were generated from 4 HLA-B*57:01+ ABC naive donors by pulsing PBMC with 20 mg/ml VSFIEFVGW for 1 hour, washing and then 9 days culture in RPMI-1640 media containing 10% FCS and 10% T-Stim media and 100 U/ml IL-2. The resulting cell lines contained 5% to 35% peptide specific CD8+ T-cells. Cell lines or PBMCs taken from HLA-B*57:01+ donors were tested for peptide specific responses ± ABC at a range of concentrations previously shown not to affect cell viability, by intra-cellular cytokine re-stimulation assays (ICS) or ELISpot. Peptide concentrations of 0.01, 0.1, 1 and 10 mg/ml were used in the ICS assay and 1 mg/ml in the ELISpot assay. Expression of interferon-γ in the presence of increasing concentrations of ABC relative to baseline (no ABC) and the respective ratios were analyzed using a linear mixed effects model with nesting for multiple peptide concentrations per individual. **Results:** Four HLA-B*57:01 restricted epitopes were confirmed of which 2 were independently detected using overlapping peptides: VSFIEFVGW from EBNA3A and VAAHPEIGAW from BMLF1. Responses to VSFIEFVGW were of high avidity and detectable in all 11 donors confirming it as an immunodominant peptide. IFN-γ responses to VSFIEFVGW were reduced relative to baseline with increasing ABC concentrations using both ICS and ELISpot assay. **Conclusions:** A new immunodominant HLA-B*57:01 restricted EBV epitope was identified. ABC was shown to impair the CD8+ T cell response to this epitope in a dose dependent manner, suggesting that drugs that bind non-covalently to HLA molecules may modulate immune responses.

Background

- ABC causes a drug hypersensitivity syndrome which is known to be CD8+T cell dependent and specifically restricted through the class I allele HLA-B*57:01.
- More recently the crystal structure of ABC-peptide-HLA-B*57:01 has been resolved and ABC has been shown to rapidly and non-covalently bind to HLA-B*57:01 (Figure 1 &2; [1,2]) and alter the repertoire of peptides presented from predominantly aromatic hydrophobic amino acids such as tryptophan (W) and phenylalanine (F) at the C-terminal position to aliphatic hydrophobic amino acids such as isoleucine(I) and valine(V)(Table 1; [1-3]). This interaction essentially creates a new HLA allele creating an allograft type reaction which phenotypically manifests as ABC hypersensitivity.
- We have previously demonstrated that ABC reactive memory CD8+ T cells can be detected in ABC unexposed healthy HLA-B*57:01 positive donors and that ABC reactive CD8+ T cells can be expanded from sorted naive and memory T cells from these donors without the need for CD4+ T cell help supporting a potential heterologous immunity model for ABC hypersensitivity whereby a newly presented self-peptide is cross-recognized by a pre-existing memory response to a prevalent antigen.
- We aimed to determine whether the binding of ABC to HLA-B*57:01 has the potential to impact on a specific HLA-B*57:01 restricted CD8+ T-cell antiviral response.

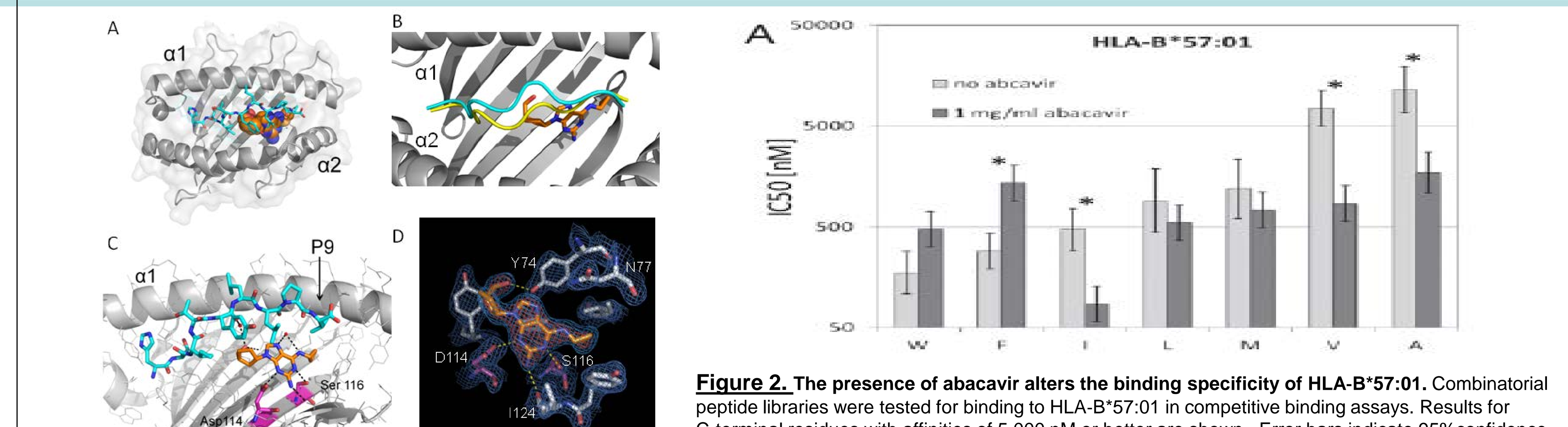

Figure 1. Identification of abacavir binding to HLA-B*57:01
The crystal structure of the abacavir:peptide:MHC complex. A. Cartoon diagram of HLA-B*57:01 in gray, peptide HSITYLLPV in cyan and abacavir shown as spheres. B. drug binding influences the peptide backbone conformation by shifting the main chain. Peptide bound to abacavir and HLA-B*57:01 shown in cyan. Peptide bound in the absence of abacavir is shown in yellow C. H bond interactions (black dashes) with peptide and the antigen-binding cleft of HLA-B*57:01. D. experimental electron density corresponding to abacavir in an Fo-Fc difference map contoured at 5σ (red mesh) following molecular replacement. Blue mesh depicts the final 2Fo-Fc electron density map of abacavir in the antigen-binding cleft of HLA-B*57:01 (contour level 1.5σ)

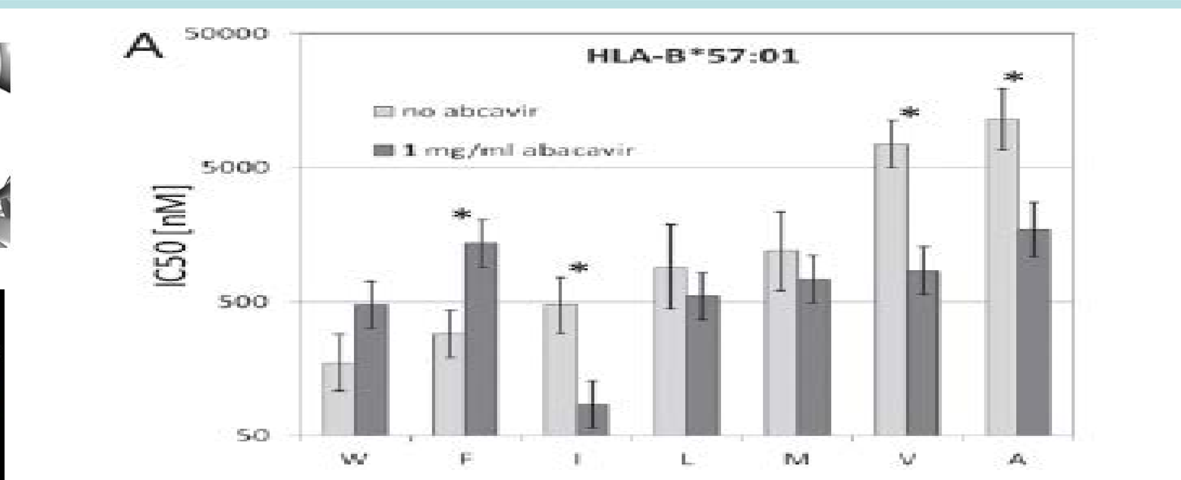

Figure 2. The presence of abacavir alters the binding specificity of HLA-B*57:01. Combinatorial peptide libraries were tested for binding to HLA-B*57:01 in competitive binding assays. Results for C-terminal residues with affinities of 5,000 nM or better are shown . Error bars indicate 95%confidence intervals for the mean, an asterisk * indicates a significantly different IC50 values in the presence vs. absence of abacavir (p < 0.001, two-tailed Student's t-test comparing log(IC50) values).

	Abacavir		
C-terminal residue	Untreated	Treated	P value*
W	218	↓95	2E-05
F	89	↓31	1E-03
Y	42	33	0.38
L	25	25	0.10
I	14	↑45	1E-09
M	6	7	0.39
V	0	↑15	5E-07

•**Table 1.** Distribution of C-terminal residues in peptides uniquely presented by abacavir treated and untreated cells. * Two-tailed Fishers exact test. All C-terminal residues for which two or more peptides were identified are listed.

Methods

Screening for HLA risk allele restricted CD8+ memory T-cell responses

To identify prospective HLA B*57:01 epitopes from EBV (strain B95-8), we utilised the NetMHC 3.2 Server (NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. Lundegaard C, et al. Nucleic Acids Res. 1;36(Web Server issue):W509-12. 2008) to compile a list of peptides with binding affinities <400 nM. 52 EBV derived peptides were synthesized (Mimotopes, Melbourne, Australia) and 18 B*57:01+ donors were screened to identify the immunodominant response, VSFIEFVGW.

Generation of peptide specific T-cell lines

Short term lines were generated from cryopreserved B*57:01+ donor PBMC. Two to five $\times 10^6$ cells were incubated with 20 μg/ml of virus specific peptide and incubated for 1 hour at 37°C in a CO_2 incubator, washed and then cultured for 3 days in stimulating media (SM) consisting of RPMI-1640 media containing 10% FCS, 10% T-Stim supernatant , 50 U/ml IL-2 (Chiron Corp., USA), 2 mM l-glutamine, 1 mM Na-pyruvate, 100U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Mulgrave, Australia). On day 4, SM was supplemented with IL-2 at final concentration of 100 IU/mL. Cultures were fed or split as required every 2 – 3 days up to day 9.

Assessment of CD8+ T-cell responses following a 6h peptide re-stimulation assay

Aliquots of peptide specific short term lines were made into round bottom 96 well plates (Becton Dickinson,) and re-stimulated for 6 hours with final concentration of 0.1 – 10 μg/ml peptide in the presence or absence of increasing concentrations of abacavir (GSK, Brentford, UK ; 0.1 – 100 μg/ml final concentration) in F10 media consisting of RPMI-1640 media with 10% FCS, 2 mM l-glutamine, 1 mM Na-pyruvate, 100U/ml penicillin and 100 μg/ml streptomycin (Life Technologies), supplemented with 20 IU/ml IL-2. Brefeldin A (Sigma-Aldrich, St Louis, USA) was added for the final 4 hours of the incubation to a final concentration of 20 μg/ml. The plates were washed by the addition of 200 μl ice cold PBS containing 10% FCS and cells pelleted by centrifugation at 600g for 5 minutes. The supernatant was discarded by flicking, the plate was placed on ice and anti-CD4-PE and anti-CD8-APC-H7 added and incubated for 15 minutes. The cells were fixed and permeabilized using Intraprep following manufacturers directions and then anti-IFN-γ-Alexa 488 was added for 15 minutes at room temperature. Following washing in re-suspension in PBS containing 10% FCS the samples were analysed using a Gallios cytometer (Beckman Coulter, Atlanta, USA).

IFN-γ ELISpot assay

Interferon-γ ELISpot assays were performed with the following modifications. ELISpot plates (MAIPS4510; Millipore) were coated with 15 μg/ml anti-IFN-γ antibody (Mabtech) and used to assay 1 – 2 x 10^5 viable PBMC in duplicate were exposed to B*57:01 restricted peptide (VSFIEFVGW) or variant peptide (VSFIEFVGV) (1 – 10 μg/ml final concentration) in the presence or absence of ABC (0.1 – 100 μg/ml final concentration). PBMC were exposed to peptides ± ABC using 2 different protocols. Firstly for a 30 minute pulse followed by a wash with 30 volumes of ice cold RPMI-1640, centrifugation at 300g for 10 minutes, and re-suspension in F10 media supplemented with 20 IU/ml IL-2 and transfer to the ELISpot plate, or secondly by incubation with peptides ± abacavir for the entire overnight the ELISpot assay. ELISpot plates were incubated overnight at 37°C in a humidified 5% CO2 incubator before processing to detect IFN-γ secretion. Positive spots were enumerated using an automated plate reader (AID, Strassberg , Germany).

RESULTS

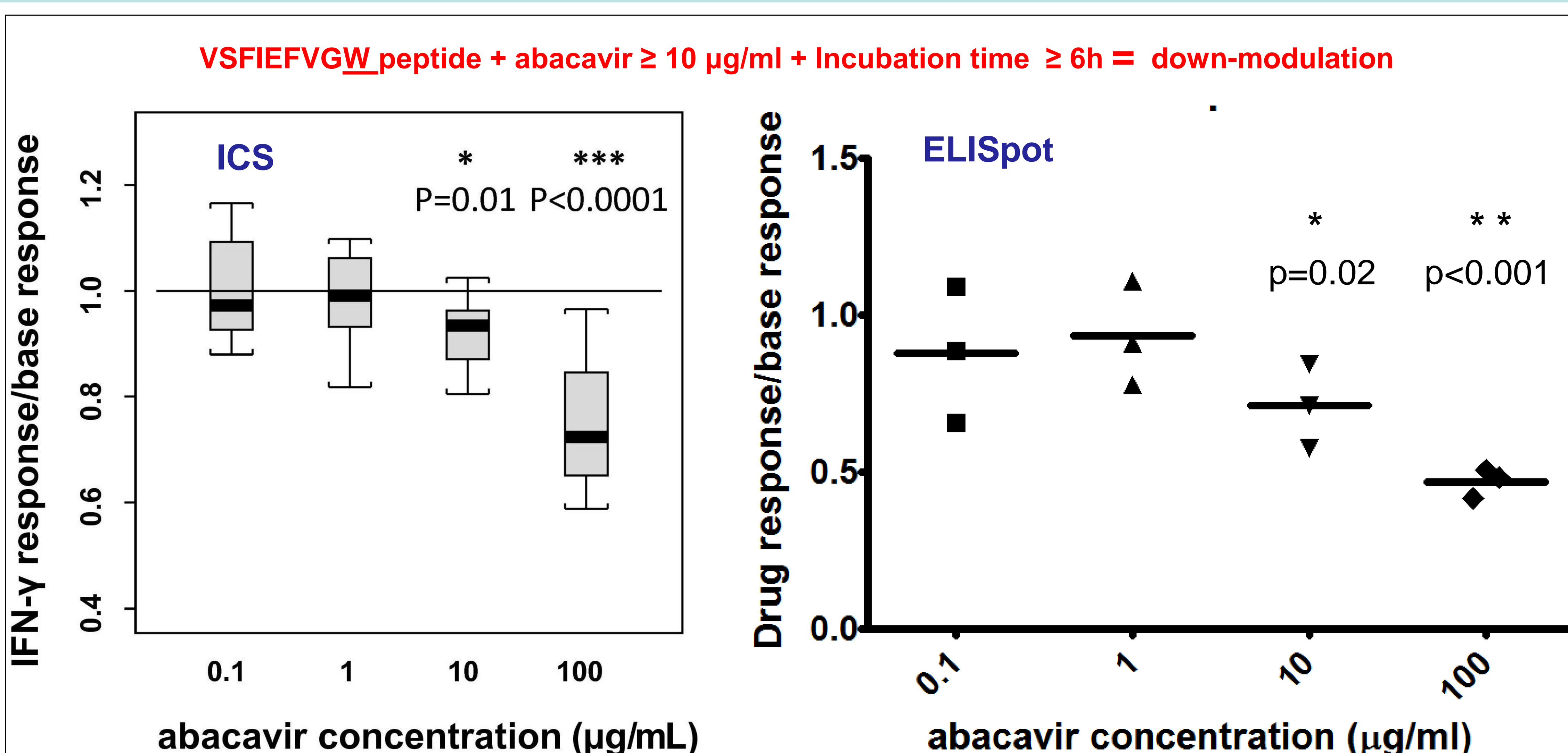

Figure .3 VSFIEFVG**W** expanded cell lines from B*57:01+ donors (n=4) were re-stimulated with combinations of VSFIEFVGW (0 – 10 μg/ml) and abacavir (0 – 100 μg/ml) and CD8+/IFN-γ+ T-cells determined by flow cytometry. Responses to treatment have been plotted as ratio of the no-drug control treatments and analysed in using a mixed effects model.

Figure .4 B*57:01+ donor PBMC (n=3) were re-stimulated with 1 μg/ml VSFIEFVG**W** and abacavir (0 – 100 μg/ml) and IFN-γ+ T-cells determined by IFN-γ ELISpot assay. Responses to treatment have been plotted as ratio of the no-drug control treatments and analysed using a paired Students t-test.

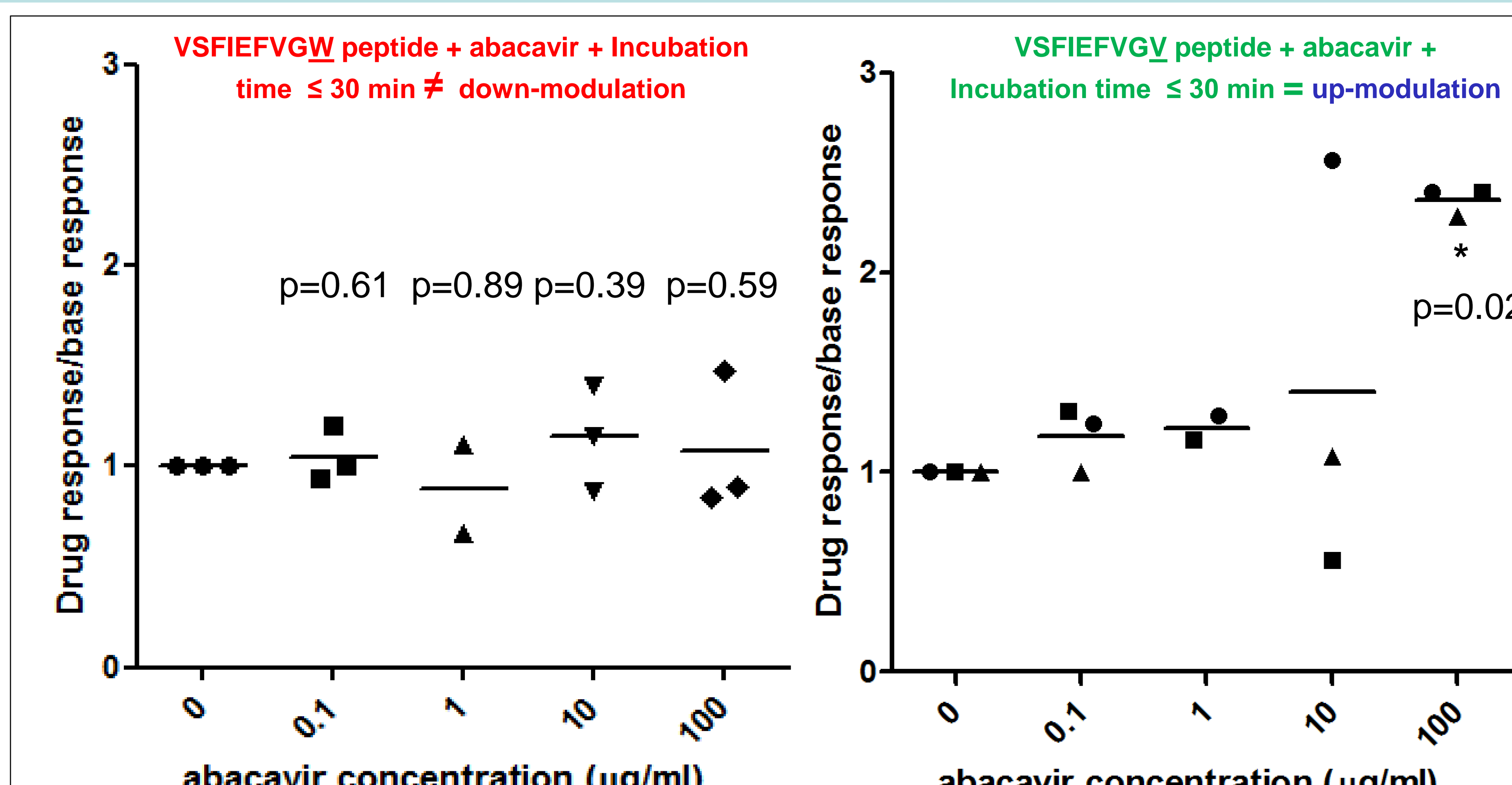

Figure 5 B*57:01+ donor PBMC (n=3) were re-stimulated with 1 μg/ml VSFIEFVG**W** and abacavir (0 – 100 μg/ml) for 30 minutes and then washed and IFN-γ+ T-cells determined by IFN-γ ELISpot assay. Responses to treatment have been plotted as ratio of the no-drug treatments and analysed using a paired Students t-test.

Figure 6 B*57:01+ donor PBMC (n=3) were re-stimulated with 10 μg/ml VSFIEFVG**V** and abacavir (0 – 100 μg/ml) for 30 minutes and then washed and IFN-γ+ T-cells determined by IFN-γ ELISpot assay. Responses to treatment have been plotted as ratio of the no-drug treatments and analysed using a paired Students t-test.

CONCLUSIONS

- Four HLA-B*57:01 restricted EBV epitopes were characterized using a combination of bioinformatic and functional approaches with two of the epitopes confirmed by an overlapping peptide screening approach.
- A CD8+ T-cell response to an immunodominant epitope VSFIEFVG**W** from EBNA3A was impaired by at least 6 hours of incubation with pharmacological concentrations of ABC.
- This ABC-induced down-modulation occurred in a dose dependent manner, suggesting that drugs that bind non-covalently to HLA molecules may modulate immune responses.
- In keeping with the altered peptide repertoire model, ABC incubation of 30 minutes or less increased CD8+ T cell responses to a variant of VSFIEFVG**W**, with a valine (V) substituted for a tryptophan (W) at the C-terminal position (VSFIEFVG**V**). This is consistent with recent biochemical and structural studies showing non-covalent binding of ABC to HLA-B*57:01 restricted epitopes with a W to V substitution at the C-terminal position in the presence of ABC.
- These findings suggest that small molecules such as ABC may non-covalently bind HLA molecules and result in up or down modulation of established functional HLA-restricted immune responses.

References

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