Improving selection criteria for post translationally modified CD4 epitopes using computer algorithms.

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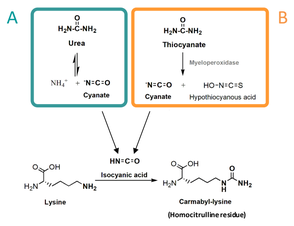
INTRODUCTION

- Targeting post translationally modified epitopes may provide new targets for cancer vaccines that avoid the attenuation often seen with responses to self antigens.[1, 2]
- Carbamylation of lysine residues leads to formation of homocitrulline (Hcit), converting the positively charged amino acid to a neutral amino acid which can generate a new epitope that is recognised by T-cells and B-cells.
- We have previously shown that peptides containing homocitrulline residues can induce CD4 responses restricted through specific HLA alleles that mediate tumour therapy.
- However, screening using overlapping peptides gave a low hit-rate for identifying HLA-specific immunogenic modified peptides.
- Here we show that combining online prediction software increases the likelihood of selecting modified peptides that can induce an immune response.



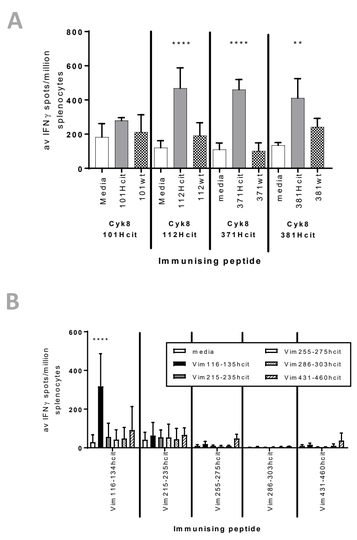
Carbamylation. A modification that is driven by increased isocyanic acid levels. This occurs physiologically due to the breakdown of Urea (A) or the actions of MPO enzyme on thiocyanate (B). Carbamylation converts positively charged lysine to neutrally charged homocitrulline.[4]

IEDB HLA-binding predictions

- Studies were carried out using HLA-DP4 transgenic mice.
- IEDB[3] was used to select cytokerating (Cyk8) or Vimentin peptides with a strong predicted binding score for MHC-II allele; HLA-DP4.

Protein	Sequence	DP4 prediction score	DP4 predicted cores
Cyk 8 101-120	KFASFID-Hcit-VRFLEQQN-Hcit-MLE	0.97 – 17.18 0.97 – 1.68 6.08	IDKVRFLEQ SFIDKVRFL FIDKVRFLE
Cyk8 112-131	LEQQN-hcit-MLET-hcit-WSLLQQQ-hcit-T	3.16 – 4.72 3.16 – 4.72	KMLETKWSL MLETKWSLL
Cyk8 182-202	EIN-hcit-RTEMENEFVLI-hcit-hcit-DVDE		
Cyk 8 371-388	LREYQELMNV-hcit-LALDIEI	24.36 – 26.69 24.36 – 26.69	LMNVKLALD ELMNVKLAL
Cyk 8 381-399	hcit-LALDIEIATYR-hcit-LLEGEE	18.10 – 24.62 24.62	IEIATYRKL IATYRKLLE
Vimentin116-135	NYID-hcit-VRFLEQQN-hcit-ILLAEL	2.12 – 17.18 2.12	IDKVRFLEQ YIDKVRFLE
Vimentin215-235	LARLDLER-hcit-VESLQEEIAFL-hcit	21.67 - 49.11 44.08 – 60.82	KVESLQEEI LERKVESLQ
Vimentin255-275	QIDVDVS-hcit-PDLTAALRDVRQQ	82.48 – 88.15	VSKPDLTAA
Vimentin 286-303	EAEEWY-hcit-S-hcit-FADLSEAAN	2.06 – 2.22 2.06 – 5.69	KSKFADLSE WYKSKFADL
Vimentin 431-454	LPLVDTHS-hcit-RTLLI-hcit-TVETRDGQV	35.09 – 40.42 41.46 – 60.30	THSKRTLLI TLLIKTVET

- Mice were immunised with Hcit peptides with adjuvant CpG/MPLA.
- IFNγ responses were then detected by ELISpot.
- HLA-specific responses were observed to 4/9 peptides.



IFNγ ELISpot responses to peptide. Immunisation with Cyk8 Hcit (A) or Vimentin Hcit (B) peptides induced responses in HLA-DP4 mice. Responses were Hcit peptide specific and did not cross react with the wild type (WT) peptide.

PEP-FOLD 3 binding predictions

- Analysis was performed using PEP-FOLD 3 software[4], a freely available online resource.
- Peptide folding analysis showed good responses often correlated with a spiral shape.

Protein	sequence	PEP-FOLD prediction	T cell response
Vimentin 116-135	NYID-hcit-VRFLEQQN-hcit-ILLAEL		yes
Vimentin 431-454	LPLVDTHS-hcit-RTLLI-hcit-TVETRDGQV		no

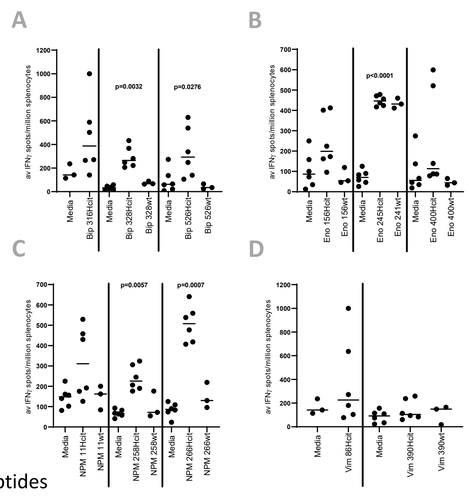
- Given this observation new Hcit peptide predictions were made based on IEDB prediction and spiral shape.

New selection criteria – Peptide selection.

Protein	sequence	DP4 prediction score	DP4 predicted cores	Spiral
BiP 316-336	EDFSETLTRA-Hcit-FEELNMDLFR	0.13 – 1.31	LTRAKFEEL	
BiP 328-346	EELNMDLFRSTM-Hcit-PVQ-Hcit-VL	19.99 – 23.39 21.61 – 23.39	LFRSTMKPV FRSTMKPVQ	
BiP 562-579	RNELESYAYSL-Hcit-NQIGD-Hcit	21.01 21.01	LESYAYSLK YAYSLKNQI	
Alpha Enolase 156-176	GSHAGN-Hcit-LAMQEFMILPVGAA	1.2 – 22.16 22.16	KLAMQEFMI HAGNKLAMQ	
Alpha Enolase 245-264	DVAASEFFRSG-Hcit-YDLDF-Hcit-SP	10.04 – 19.07 10.04 – 19.07	FRSGKYDLD FFRSGKYDL	
Alpha Enolase 400-419	RSERLA-Hcit-YNQLLRIEEELGS	11.8 – 12.36 11.8 – 18.57	RLAKYNQLL AKYNQLLRI	
Nucleophosmin 11-27	PLRPQNYLFGCEL-Hcit-AD-Hcit-	26.13 – 33.98 26.54	YLFGCELKA LFGCELKAD	
Nucleophosmin 258-277	GGSLP-Hcit-VEA-Hcit-FINYV-Hcit-NCFR	24.31 – 30.51 24.31 – 30.51 28.92 – 30.51	KVEAKFINY VEAKFINYV FINYVKNCF	
Nucleophosmin 266-287	A-Hcit-FINYV-Hcit-NCFRMTDQEAIQDL	2.93 – 3.06 2.93 – 14.95	KFINYVKNC YVKNCFRMT	
Vimentin 86-108	FSLADAINTEF-Hcit-NTRTNE-Hcit-VELQ	25.28 – 29.44 25.28 – 27.76 25.28 – 27.76 25.32 – 27.76	AINTEFKNT FKNTRTNEK EFKNTRTNE TRTNEKVEL	
Vimentin 390-408	Hcit-MALDIEIATYR-Hcit-LLEGEE	18.19 – 24.62 24.62	IEIATYRKL IATYRKLLE	

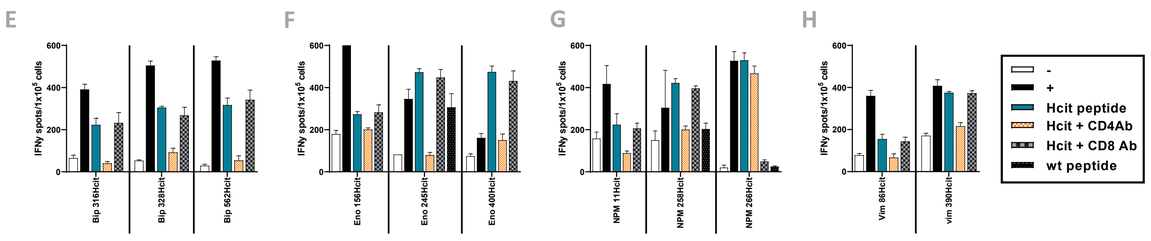
Immune responses to Homocitrullinated peptides

- Studies were carried out using transgenic mice expressing human HLA-DP4.
- Mice were immunised with Hcit peptides selected based on IEDB and PEP-FOLD predictions.
- IFNγ was determined ex vivo and after culture using ELISpot assays.
- 9/11 peptides showed responses *ex vivo*.



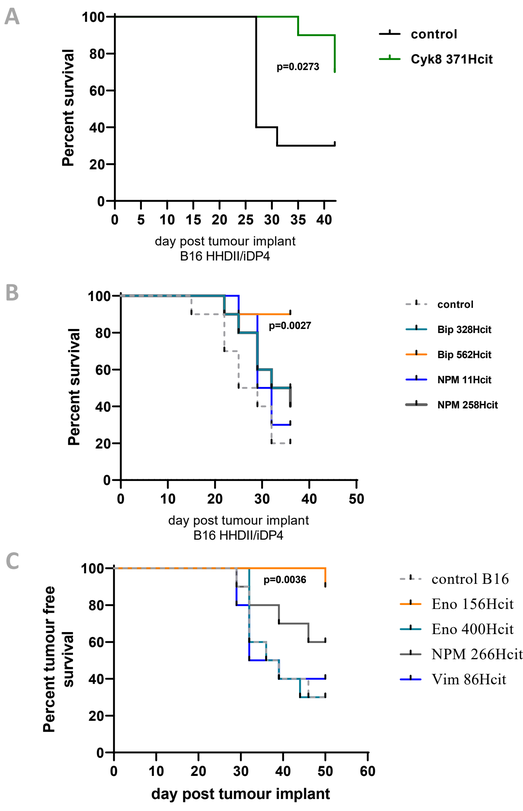
***Ex vivo* ELISpot responses**. IFNγ responses showing responses to BIP (A), enolase (B), NPM (C) and vimentin (D) homocitrullinated peptides.

Cultured ELISpot responses. Cultures were restimulated with Hcit or wt peptides with anti-CD4 or anti-CD8 blocking antibodies. IFNγ responses were detectable after culture and show that the majority of responses are CD4 mediated and Hcit-specific for BIP (E), enolase (F), NPM (G) and vimentin (H) peptides.



In vivo anti-tumour effect of Homocitrullinated peptides

- Mice were implanted with B16F1 tumour cells transfected with human HLA under an IFNγ inducible promoter on day 1 (B16F1 HHDII/iDP4).
- Treated mice were then immunised with Hcit peptides on day 4, 11 and 18. Tumour growth and survival was monitored.
- Significant anti-tumour responses were seen for multiple peptides.



Anti-tumour survival. HHDII/DP4 mice were implanted with B16F1 HHDII/iDP4 tumour. Increased survival when compared to control was seen for Cyk8 371Hcit (A), Bip 562Hcit (B) and Enolase 156Hcit (C). NPM 266Hcit showed no significant survival advantage over control.

CONCLUSIONS

Using online prediction software can improve selection of peptides that are capable of inducing immune responses

- **Homocitrullinated peptides to a number of proteins including vimentin, enolase, cytokeratin 8 (CyK8), immunoglobulin binding protein 35 (BiP) and nucleophosmin (NPM) are able to induce immune responses in HLA-DP4 mice.**
- **Selecting peptides to screen responses can be improved by including binding scores and peptide structure predictions.**

Homocitrullinated peptides selected in this way can induce immune responses which can give an anti-tumour response

- **One BIP peptide, one Cyk8 peptide and one enolase peptide selected using this prediction software showed a strong anti-tumour effect against the B16F1 model.**
- **As expected, not all Homocitrullinated peptides that induce an immune response give an anti-tumour response against B16F1.**

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