Citrullinated glucose-regulated protein 78 is a candidate target for cancer immunotherapy

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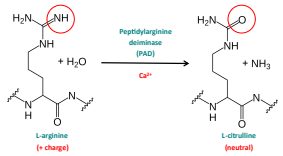
INTRODUCTION

- CD4 T cells are potent effector cells. But CD4 responses to self antigens are often attenuated.
- Cellular stress induces autophagy which leads to modification of proteins that can be recognised by the immune system [(1)]. One such modification is citrullination (cit).
- In the absence of inflammation immunity is regulated. But in the presence of inflammation CD4 responses to modified self-antigens are stimulated [(2)].
- Autophagy is upregulated in rapidly proliferating cancer cells. Cancer cells citrullinate proteins via PAD enzymes[(3)].
- Stressful conditions in the tumour microenvironment lead to presentation of modified peptides on MHC class II. These MHC II presented modified peptides are targets for CD4 T cells. We have shown that these T cells can be harnessed for tumour therapy [(4,5,6)].
- The ER chaperone protein glucose-regulated protein 78 (GRP78) is a master regulator of ER stress. ER stress triggered induction of GRP78 leads to enhanced survival of cancer cells and an association of GRP78 expression is linked to tumour progression [(7)].
- GRP78 has been shown to also be involved in Ca^{2+} homeostasis and is required for stress induced autophagy [(8)].
- We provide evidence for citrullinated GRP78 in tumours and identify T cell responses to a citrullinated GRP78 peptide in HLA transgenic mice that are restricted through multiple HLA alleles. We show a repertoire of CD4 T cells to citrullinated GRP78 in healthy donors and demonstrate in mice and humans that CD4 responses are citrulline specific. We provide evidence of citrullinated GRP78 in *in vivo* grown murine tumour and show in a mouse model that citrullinated GRP78 peptide vaccination mediates efficient tumour therapy.



Citrullination. A modification that occurs within stressed cells. Peptidylarginine deiminase (PADs) enzymes are activated and convert arginine to citrulline by altering the positively charge aldimine group (=NH) group of arginine to the neutrally charged ketone group (=O) of citrulline.

Detection of citrullinated GRP78 in tumours *in vitro*

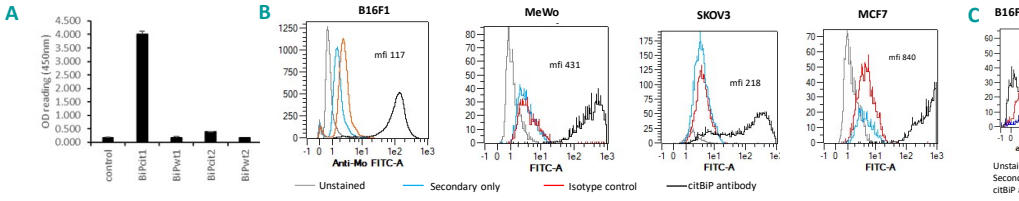


Figure 1. A, an antibody raised against citrullinated GRP78 peptide shows citrullinated GRP78 peptide specificity by ELISA. B, in vitro cultured murine B16 melanoma, human MeWo melanoma SKOV3 ovarian tumour or MCF7 breast tumour cells stained with citrullinated GRP78 antibody (black), secondary antibody only (blue), isotype control (red) or unstained (grey) and analysed by flow cytometry. C, Flow cytometry staining of B16 melanoma tumours ex vivo with citrullinated GRP78 antibody.

Citrullinated GRP78 189-208 is detected in tumours *in vivo*

Table 2. Targeted detection of citrullinated GRP78 189-208 compared to other known citrullinated peptides[(4,5)] by mass spectrometry in *ex vivo* B16 tumour lysates

Antigen	Peptide coordinates	Sequence	Linearity (R^2)	Average pmol/mg
GRP78	189-208	TIAGLNVM-cit-IINEPTAAAIA	0.93	3.086
Vimentin	415-433	LPTFSSLNL-cit-ETNLESLPL	0.90	1.704
Vimentin	28-49	cit-SYVTTST-cit-TYSLGSAL-cit-PSTS	1.00	0.856
Alpha-enolase	241-260	VIGMDVAASEFY-cit-SGKYDLD	0.97	0.704

cit = citrulline

Immunisation with citrullinated GRP78 189-208 peptide elicits tumour therapy

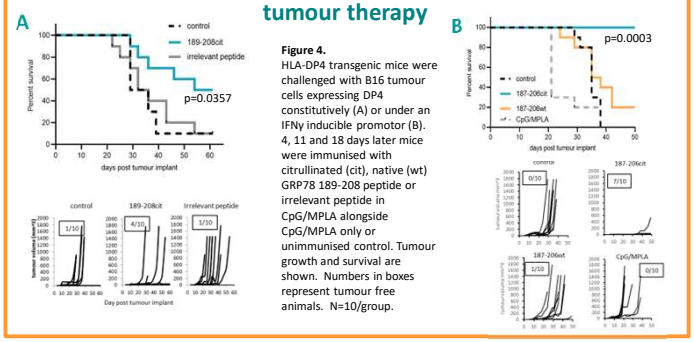


Figure 4.
HLA-DP4 transgenic mice were challenged with B16 tumour cells expressing DP4 constitutively (A) or under an IFNγ inducible promotor (B). 4, 11 and 18 days later mice were immunised with citrullinated (cit), native (wt) GRP78 189-208 peptide or irrelevant peptide in CpG/MPLA alongside CpG/MPLA only or unimmunised control. Tumour growth and survival are shown. Numbers in boxes represent tumour free animals. N=10/group.

Citrullinated GRP78 specific CD4 Th1 responses restricted through multiple HLA alleles can be stimulated in HLA transgenic mice.

Table 1. Predicted binding scores of 5 selected GRP78 peptides to HLA-DR4, DR1 and DP4 using IEDB prediction software. **R** = arginine changed to citrulline.

coordinates	sequence	DR*0101 binding score	DR*0101 predicted cores	DP*0401 binding score	DP*0401 predicted cores
189-208	TIAGLNVMRIIN EPTAAAIA	7.28	VMRIINEPT IAGLNVMRI	59.16	AGLNVMRII IAGLNVMRI LNVMRIINE
315-334	EDFSETLTRAKFE ELNMDLF	54.2	SETLTRAKF FSETLTRAK LTRAKFEEL	0.13	LTRAKFEEL
327-346	EELNMDLFRST MKPVQKVLE	28.99	FRSTMKPVQ LFRSTMKPV MDLFRSTMK	19.99	LFRSTMKPV LNMDLFRST
460-478	VTIKVYEGERPLT KDNHLL	44.67	KVYEGERPL YEGERPLTK	17.85	KVYEGERPL YEGERPLTK
501-520	EIDVNGILRVTAE DKGTGNK	35.86	IDVNGILRV ILRVTAEDK	59.87	DVNGILRVT IDVNGILRV

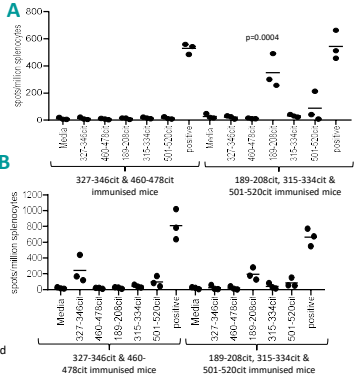


Figure 2.
HLA-DP4 (A) or HLA-DR1 (B) transgenic mice were immunised at days 1, 8 & 15 with pools of non overlapping citrullinated GPR78 peptides (10nmol each) in CpG/MPLA and immune responses to individual citrullinated peptides monitored at day 21 by IFNγ Elispot assay

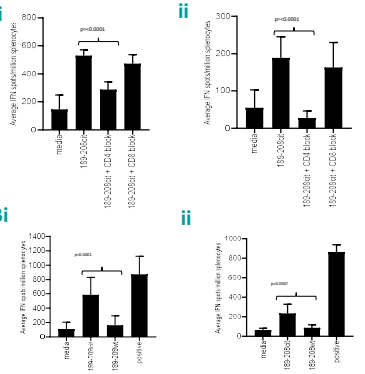


Figure 3.
HLA-DP4 (i) or HLA-DR1 (ii) transgenic mice were immunised with citrullinated (cit) 189-208 peptide in CpG/MPLA on days 1, 8 & 15. On day 21, immune responses to cit peptide were assessed by IFNγ Elispot assay (A) in the presence of CD4 (clone OKT4 or GK1.5) or CD8 (clone 2.43) blocking antibody (20ug/ml) and (B) compared to native (wt) peptide.

Healthy humans show a repertoire of CD4 T cells able to respond to citrullinated GRP78 189-208 peptide

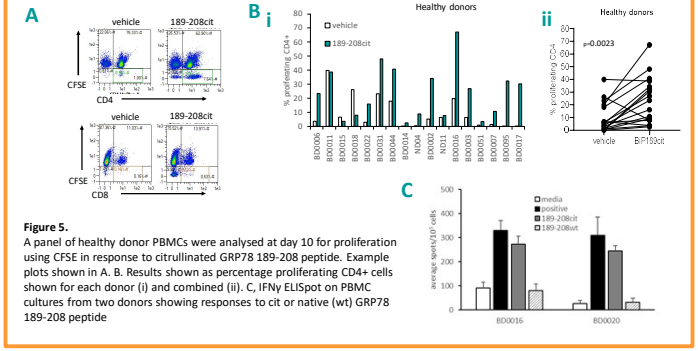


Figure 5.
A panel of healthy donor PBMCs were analysed at day 10 for proliferation using CFSE in response to citrullinated GRP78 189-208 peptide. Example plots shown in A. B. Results shown as percentage proliferating CD4+ cells shown for each donor (i) and combined (ii). C, IFNγ ELISpot on PBMC cultures from two donors showing responses to cit or native (wt) GRP78 189-208 peptide

CONCLUSIONS

- **Murine and human tumour cell lines stain with anti-citrullinated GRP78 antibody.**
- **Citrullinated GRP78 189-208 peptide stimulate CD4 T cell responses restricted through HLA-DP4 and HLA-DR1 that are citrulline specific.**
- **Citrullinated GRP78 189-208 peptide is detected in B16 tumours *ex vivo***
- **Vaccination with citrullinated GRP78 peptide mediates efficient tumour therapy.**
- **Healthy donors show a repertoire of CD4 T cells capable of responding to citrullinated GRP78 189-208 peptide.**
- **Citrullinated GRP78 is a promising immunotherapy target.**

References:

1. Ireland JM, Unanue ER. Autophagy in antigen presenting cells results in presentation of citrullinated peptides to CD4 T cells. J Exp Med. 2011 Dec 19;208(13):2625-32.
2. Feitsma AL et al. Identification of citrullinated vimentin peptides as T cell epitopes in HLA-DR4-positive patients with rheumatoid arthritis. Arthritis Rheum. 2010 Jan;62(1):117-25
3. Jiang Z et al. Investigating citrullinated proteins in tumour cell lines. World J Surg Oncol. 2013 Oct 7;11:260.
4. Brentville VA, et al. Citrullinated vimentin presented on MHC-II in tumor cells is a target for CD4+ T cell-mediated antitumor immunity. Cancer Research 2016 Feb 1;76(3):548-60
5. Cook K et al. Citrullinated alpha-enolase is an effective target for anti-cancer immunity. Oncoimmunology, 2018. 7(2): p. e1390642.
6. Brentville V et al. T cell repertoire to citrullinated self-peptides in healthy humans is not confined to the HLA-DR SE alleles; Targeting of citrullinated self-peptides presented by HLA-DP4 for tumour therapy. Oncoimmunology, 2019. 8(5): p. e1576490
7. Lee, A.S., GRP78 induction in cancer: therapeutic and prognostic implications. Cancer Res, 2007. 67(8): p. 3496-9.
8. Li, J., et al., The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ, 2008. 15(9): p. 1460-71