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# PAD-2-mediated citrullination of nucleophosmin provides an effective target for tumor immunotherapy

Ruhul H Choudhury ,[1] Peter Symonds,[1] Samantha J Paston,[1] Ian Daniels,[1] Katherine W Cook ,[1] Mohamed Gijon,[1] Rachael L Metheringham,[1] Victoria A Brentville ,[1] Lindy G Durrant[1,2]

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[1]Scancell Ltd, Nottingham, UK
[2]University of Nottingham Biodiscovery Institute, Scancell Ltd, Nottingham, UK

**Correspondence to**
Dr Lindy G Durrant;
lindy.durrant@nottingham.ac.uk

## ABSTRACT

**Background** The enzymatic conversion of arginine to citrulline is involved in gene and protein regulation and in alerting the immune system to stressed cells, including tumor cells. Nucleophosmin (NPM) is a nuclear protein that plays key roles in cellular metabolism including ribosome biogenesis, mRNA processing and chromatin remodeling and is regulated by citrullination. In this study, we explored if the same citrullinated arginines within NPM are involved in gene regulation and immune activation.

**Methods** HLA-DP4 and HLA-DR4 transgenic mice were immunized with 22 citrullinated NPM overlapping peptides and immune responses to the peptides were assessed by ex vivo ELISpot assays. Antitumor immunity of NPM targeted vaccination was assessed by challenging transgenic mice with B16F1 HHDII/iDP4, B16F1 HHDII/PAD2KOcDP4, B16F1 HHDII and Lewis lung carcinoma cells/cDP4 cells subcutaneously. Peripheral blood mononuclear cells isolated from healthy donors were stimulated with NPM266-285cit peptides with/without CD45RO+memory cell depletion to assess if the responses in human were naïve or memory.

**Results** In contrast to NPM regulation, which is mediated by peptidylarginine deiminase (PAD4) citrullination of arginine at position 197, only citrullinated NPM266-285 peptide induced a citrulline-specific CD4 T cell response in transgenic mice models expressing human HLA-DP4 or HLA-DR4. Vaccinations with the NPM266-285cit peptide stimulated antitumor responses that resulted in dramatic tumor therapy, greatly improved survival, and protected against rechallenge without further vaccination. The antitumor response was lost if MHCII expression on the tumor cells was knocked out demonstrating direct presentation of the NPM266-285cit epitope in tumors. This antitumor response was lost in B16 tumors lacking PAD2 enzyme indicating NPM266cit is citrullinated by PAD2 in this model. Assessment of the T cell repertoire in healthy individuals and patients with lung cancer also showed CD4 T cells that respond to NPM266-285cit. The proliferative CD4 responses displayed a Th1 profile as they were accompanied with increased IFNγ and granzyme B expression. Depletion of CD45RO+ memory cells prior to stimulation suggested that responses originated from a naïve population in healthy donors.

**Conclusion** This study indicates PAD2 can citrullinate the nuclear antigen NPM at position 277 which can be targeted by CD4 T cells for antitumor therapy. This is distinct from PAD4 citrullination of arginine 197 within NPM which results in its transport from the nucleoli to the nucleoplasm.

## INTRODUCTION

Immune-based therapy serves a distinct advantage over traditional treatment regimens because of its specificity in killing tumor cells without harming healthy tissues. There is a growing interest in harnessing CD4 T cells for immunotherapy as they have been shown to play a critical role in antitumor immunity.[1] Many major histocompatibility complex class II (MHCII) restricted tumor epitopes have been identified[2] and the effectiveness of CD4 T cells targeting MHCII restricted epitopes in animal models has previously been demonstrated.[3] Recently, adoptive T cell therapy with tumor infiltrating lymphocytes containing mutated tumor antigen-specific Th1 cells showed success against metastatic epithelial cancer.[4] During their development T cells undergo positive and negative selection in the thymus to allow the maturation of low affinity T cells. High affinity T cells are deleted while thymocytes with moderate affinity T cell receptors are redirected towards a regulatory T cell developmental pathway to play an immunosuppressive function in the periphery.[5] CD4 T cells recognizing modified self-epitopes often escape thymic deletion and have been shown to play a role in the pathophysiology of several autoimmune diseases such as rheumatoid arthritis, collagen II-induced arthritis, sarcoidosis, celiac disease and psoriasis.[6–9] One of these common modifications is the citrullination of arginine, which involves the conversion of the positively charged aldimine group (=NH) of arginine to the neutrally charged ketone group (=O) of citrulline (cit). Citrullination is mediated by peptidylarginine deiminases (PADs), which are a family of calcium

dependent enzymes found in a variety of tissues. Ireland and Unanue[10] demonstrated that the presentation of citrullinated T cell epitopes on antigen presenting cells (APCs) is dependent on both autophagy and PAD activity. This pathway is thought to enable processing of endogenous antigens for presentation by MHCII molecules on professional APCs and epithelial cells.[11 12] However, normally cytoplasmic antigens are presented on MHCII through autophagy and the only known nuclear antigens to follow the same pathway are viral nuclear proteins.[13] We have previously demonstrated epitopes from the cytoplasmic antigens vimentin and α-enolase can be citrullinated and are presented on B16F1 melanoma tumors.[14 15] The antigens also induce T cell responses in healthy donors with various HLA backgrounds indicating they can be presented by different HLA alleles.[16] Autophagy is constitutive in APCs, but in other cells it is only induced by stress such as nutrient deprivation, hypoxia, DNA damage, reactive oxygen species, protein aggregation and organelle damage.[17] Importantly, in tumor cells, autophagy is upregulated to promote survival.[17]

Nucleophosmin (NPM) is an ubiquitously expressed protein which plays a key role in the regulation of cell growth, proliferation and transformation.[18] Recently overexpression of NPM has been reported in multiple human cancers including those of the pancreas,[19] prostate,[20] liver,[21] colon,[22] stomach,[23] thyroid,[24] and in glioblastoma.[25] Furthermore, in some cancers, such as bladder carcinoma, the progression of the disease to an advance stage correlates with NPM expression.[26] NPM has also been implicated in chromosomal translocations in acute myeloid leukemia (AML) and non-Hodgkin lymphomas. In 35% of AML patients, NPM is mutated and aberrantly located in the cytoplasm of leukemic cells.[27] These AML patients have a normal karyotype and the NPM shift to the cytoplasm is due to a mutation in exon 12.[27] NPM is citrullinated at position 197 by PAD4 within the nucleus resulting in its transport from the nucleoli to the nucleoplasm[28] and subsequently to the cytoplasm for degradation whereby it could be presented on MHC-II for CD4 T cell recognition.

In this study, we demonstrate that the citrullination of arginine 197 in NPM does not appear to be a target for CD4 T cells, at least in HLA-DP4 or HLA-DR4 mice. In contrast, citrullination of arginine 277 by PAD2 offers a good target for CD4 T cell recognition and may be exploited to impart strong antitumor immunity.

## METHODS

### Peptides

Peptide panel spanning the NPM sequence with 20aa peptides overlapping by 5aa was created and those containing an arginine (table 1) were synthesized by Genscript with arginine replaced by citrulline. Peptides were synthesized at >95% purity and stored in lyophilized form at −80°C. Peptides were dissolved in phosphate buffered saline (PBS) at 1 mg/mL prior usage.

**Table 1** Sequences of the peptides used in the study

| Coordinates | Sequence |
|---|---|
| 1–20 | MEDSMDMDMSPL-cit-PQNYLFG |
| 6–25 | DMDMSPL-cit-PQNYLFGCELKA |
| 31–50 | FKVDNDENEHQLSL-cit-TVSLG |
| 36–55 | DENEHQLSL-cit-TVSLGAGAKD |
| 41–60 | QLSL-cit-TVSLGAGAKDELHIV |
| 86–105 | TVSLGGFEITPPVVL-cit-LKCG |
| 91–110 | GFETPPVVL-cit-LKCGSGPVH |
| 96–115 | PPVVL-cit-LKCGSGPVHISGQH |
| 126–145 | EDEEEEDVKLLSISGK-cit-SAP |
| 131–150 | EDVKLLSISGK-cit-SAPGGGSK |
| 136–155 | LSISGK-cit-SAPGGGSKVPQKK |
| 181–200 | FDDEEAEEKAPVKKSI-cit-DTP |
| 186–205 | AEEKAPVKKSI-cit-DTPAKNAQ |
| 191–210 | PVKKSI-cit-DTPAKNAQKSNQN |
| 206–225 | KSNQNGKDSKPSSTP-cit-SKGQ |
| 211–230 | GKDSKPSSTP-cit-SKGQESFKK |
| 216–235 | PSSTP-cit-SKGQESFKKQEKTP |
| 261–280 | LPKVEAKFINYVKNCF-cit-MTD |
| 266–280 | AKFINYVKNCF-cit-MTD |
| 266–285 | AKFINYVKNCF-cit-MTDQEAIQ |
| 271–290 | YVKNCF-cit-MTDQEAIQDLWQW |
| 276–294 | F-cit-MTDQEAIQDLWQWcitKSL |

### Generation of PAD2KO cell lines and transfection

The generation of the plasmid pVITRO2 Human HLA-DP4 and pVITRO2 Chimeric HLA-DR401 have been described previously.[14 16] The HLA-A0201 transgenic (HHDII) plasmid pCDNA3 HHDII is also described in previous literature,[29] as has B16F1 melanoma cells expressing HLA-DP4 under expression of IFNγ inducible promoter.[16]

PAD2 knock out of B16F1 cells was performed by Sigma-Aldrich Cell Design Studio. CompoZr zinc finger nuclease technology was used targeting NPM exon 1 with pair sequences NM008812-r649a1: CTGCAGCC GCACGGTCCGTTCCCGCAGC and NM008812-656a1: TGGGAGCCGCGTGGAGGCGGTGTACGTG. Following several rounds of retargeting, 89% cutting was achieved and single cell cloning was then performed to establish a stable clone. ddPCR, flow cytometry and western blot (ab50257 and ab150063, Abcam) (12110-AP, Proteintech) were used by Sigma-Aldrich Cell Design Studio to assess the PAD2 knock out of the clone. The primers and probes used for ddPCR were obtained from Thermo Fisher (Mm00447012_m1 & Mm00447020_m1). The PAD2 knock out clone were engineered to express HHDII and HLA-DP4 under a constitutive promoter as described previously.[16] Cloning was performed and expression was confirmed as described previously.[30] PAD4 expressions in the parental and knock out lines were confirmed using

Flow cytometry (PA5-29880, ThermoFisher) (ab181346, Abcam).

### Immunisation protocol

HLA-DP4 (EM:02221, European Mouse Mutant Archive) and HLA-DR4 mice (Model #4149, Taconic) transgenic mice aged between 8 and 12 weeks were used. All work was carried out at Nottingham Trent University under a Home Office approved project license. To screen responses to NPM peptides mice received 25 µg each of peptides in of non-overlapping pools and 5 µg each of CpG/MPLA subcutaneously at the base of the tail. The peptides were dissolved in PBS. Mice were immunized three times on day 1, 8 and 15, and spleens were removed for analysis on day 21. Groups of n=3 selected through statistical power calculations were used on independent occasions.

For tumor challenge experiments, mice were challenged with $1\times10^5$ B16F1 HHDII/iDP4, B16F1 HHDII/PAD2KOcDP4 and B16F1 HHDII, and $2.5\times10^6$ Lewis lung carcinoma cells (LLC2)/cDP4 cells subcutaneously on the right flank 3 days before primary immunization and then immunized as described above. Mice were also immunized on day 8, 15 and 22 post-tumor implants to assess responses to delayed immunization. To assess if the antitumor activity is driven by CD4 or CD8 cells mice were also treated with anti-CD4 or anti-CD8 depleting antibodies (BE0003-2 & BE0061, BioXCell). For tumor rechallenge studies, mice that had rejected the initial tumor challenge were implanted with B16F1 HHDII/iDP4 or B16F1 HHDII/PAD2KOcDP4 cells on day 49 without any further immunization. Tumor growth was monitored at 3–4 days intervals and mice humanely euthanised once tumor approached ≥15 mm in diameter. Groups of n=5–10 selected through statistical power calculations were used on independent occasions.

### Ex vivo ELispot

ELISpot assays were performed using murine IFNγ capture and detection reagents according to the manufacturer's instructions (Mabtech, Sweden). In brief, capture antibodies (10 µg/mL) were coated onto wells of 96-well Immobilin-P plate and quadruplicate wells were seeded with $5\times10^5$ per well splenocytes. Lipopolysaccharide (LPS) at 10 µg/mL was used as a positive control. Peptides were added at 10 µg/mL where relevant and plates incubated for 40 hours at 37°C. After incubation, captured IFNγ was detected by a biotinylated anti-IFNγ antibody and developed with a streptavidin alkaline phosphatase and chromogenic substrate. For blocking studies, anti-CD4 blocking antibody (RPA-T4) and anti-CD8 blocking antibody (2.43) from BioXCell were used at 20 µg/mL. Spots were analyzed and counted using an automated plate reader (Cellular Technologies).

### U-PLEX biomarker assay

U-PLEX biomarker assay (MSD) was carried out according to manufacturer guidance to assess granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)1-β, IL-2, IL-4, IL-10, tumor necrosis factor α (TNFα), interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP1α) and regulated on activation, normal T expressed and secreted (RANTES) in the culture supernatant taken from the ELISpot assay plates after 40 hours stimulation.

### Western blot

Cell lysates were prepared using RIPA buffer containing protease inhibitor cocktail (Sigma). The lysates were loaded and separated on a 4%–12% NuPAGE Bis-Tris gel (Invitrogen), followed by transfer onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 hour with 3% BSA then probed with human anti-NPM antibodies (clone FC82291, Abcam) 1:1000 and anti-β actin antibodies (clone AC-15, Sigma) 1:15 000. Proteins were visualized using the fluorescent secondary antibody IRDye 800RD and IRDye 680RD secondary anti-mouse (for β actin). Membranes were imaged using a Licor Odyssey scanner. NPM recombinant protein (ab114194, Abcam) was used as a positive control.

### In vitro citrullination

The citrullination of recombinant NPM (ab126664, Abcam) protein was performed in 0.1M Tris-HCl pH 7.5 (Fisher), 10 mM $CaCl_2$ (Sigma) and 5 mM DTT (Sigma). The final concentration of the solution was 376 mM Tris-HCl pH 7.5, 3.76 mM $CaCl_2$, 1.88 mM DTT. Samples were incubated with PAD enzymes for 2 hours at 37°C before storing at −80°C overnight or until use. PAD2 (MQ16.201–2.5, Modiquest) enzyme was used at a final concentration of 148mU and PAD4 (MQ16.103–2.5, Modiquest) at a final concentration of 152mU. PAD enzymes were purchased from Modiquest at 37mU/µl hPAD2 and 38mU/µL hPAD4.

### Mass spectrometry

Samples were prepared by trypsin digest at a ratio of 1:50 trypsin to protein overnight at 37°C. Samples were then dried under vacuum and resuspended in 0.1% formic acid/5% acetonitrile in liquid chromatography and mass spectrometry (LCMS) grade water before MS analysis. For MS Analysis, samples were injected via autosampler (Eksigent Ekspert nanoLC 425 LC system utilizing a 1–10 µL/min pump module running at 5 µL/min) with a 2 min wash trap/elute configuration onto a YMC Triart C18 column (300 um i.d., 3 µm particle size, 15 cm) in a column oven at 35°C. Samples were gradient eluted over an 87 min runtime into a SCIEX 6600 TripleT mass spectrometer via a Duospray (TurboV) source with a 50 µm electrode. The SCIEX 6600 TripleT was set up in IDA mode (Independent Data Acquisition/Data Dependent Acquisition) for 30 ions per cycle fragmentation. Total cycle time 1.8 s, TOFMS scan 250 ms accumulation; 50 ms for each product ion scan.

Data were analyzed using PEAKS Studio V.8.0 (Bioinformatic Solution, Waterloo, Canada) searching the SwissProt human (Uniprot manually annotated/curated) database, 25 ppm parent mass error tolerance, 0.1 Da fragment mass error tolerance searching for modifications for citrullination (R), deamidation (NQR), oxidation (M). Sites were identified as a confident modification site with a minimum ion intensity of 5%.

### Peripheral blood mononuclear cell Isolation, CD25 depletion, CD45RO+ depletion, CFSE proliferation assay and restimulation assay

Patients with lung cancer and healthy donors were bled (20–60 mL) at Nottingham City Hospital. Following the separation using Histopaque, Peripheral blood mononuclear cells (PBMCs) were depleted of CD25 +T cells and/ or CD45RO+T cells using CD25 depletion kit (Miltenyi) and CD45RO depletion kit (Miltenyi), respectively. CD14 + cells were positively isolated (Miltenyi) from PBMCs and then reintroduced to the PBMCs after CD45RO depletion as CD14 +cells express CD45RO. PBMCs were carboxyfluorescein succinimidyl ester (CFSE) loaded and plated out as described previously.[16] PBMCs were stimulated with NPM266-285cit peptide at 10 µg/mL. PHA and medium were used as positive and negative controls, respectively. Proliferation responses which were double background control were considered to be significant. Buffy coats were purchased from NBT NHS Sheffield and PBMCs were isolated as described earlier. Following depletion of CD25 +T cells, PBMCs were stimulated with NPM266-285cit peptide at 10 µg/mL for 11 days. PBMCs were then restimulated with 10 µg/mL of NPM266-285cit or NPM266-285wt peptides to assess citrulline-specific response. The responses were measured using ELISpot assays with human IFNγ capture and detection reagents according to the manufacturer's instructions (Mabtech, Sweden).

### Flow cytometry staining

CD25, and CD45RO depletion was confirmed by staining with anti-CD4 eFluor 450, anti-CD25-PE and anti-CD45RO-VioGreen antibodies. Immunostaining for activation and cytotoxic markers were carried out using anti-CD134-PEcy7, anti-IFNy-APCe780 and anti-GranB-PE antibodies. PBMCs were stained as described previously and antibodies details are in online supplemental table 1.[15] Stained samples were analyzed on a MACSQuant 10 flow cytometer equipped with MACSQuant software version 2.8.168.16380 using stained vehicle stimulated controls to determine suitable gates.

### Statistics

Statistical analyses were carried out using Graphpad prism software V.8. Kruskal-Wallis was used for comparison of T cell responses to peptides in HLA transgenic mice. Tumor survival in different groups were compared using log-rank Mantel-Cox test. Mann-Whitney U test was used to compare tumor volume. To assess the difference in cytokine expression between proliferating and non-proliferating cells Wilcoxon test was used.

## RESULTS

### Citrullinated NPM peptides stimulate T cell responses in transgenic HLA-DP4 and HLA-DR4 mice

Citrullinated peptides from cytoplasmic proteins have previously been shown to stimulate immune responses in HLA transgenic mice.[14–16 31] To determine if citrullinated peptides from the nuclear antigen NPM can induce similar responses we designed a panel of overlapping peptides from NPM and selected the 22 that spanned all 7 arginine's within the protein. Peptides were tested with citrulline replacing the arginine residue. We immunized HLA-DP4 and HLA-DR4 transgenic mice with pools of 4–6 non overlapping NPM peptides and screened for IFNγ release to the specific peptides ex vivo. NPM261-280cit ($p<0.05$) and NPM266-285cit ($p<0.0007$) peptides induced high frequency responses in HLA-DP4 mice while moderate but non-significant responses to NPM31-50cit, NPM96-115cit, NPM131-150cit, NPM136-155cit, NPM186-205cit, NPM211-230cit and NPM276-294cit were also observed (figure 1A). In HLA-DR4 mice only the NPM266-285cit ($p<0.003$) peptide induced a high frequency response while a moderate response ($p=0.064$) to NPM261-280cit was also observed (figure 1B). Interestingly, no detectable IFNγ release to any peptide containing the citrulline modification at aa197, that is reported to be citrullinated in vivo, was observed in either strain of mice[28] suggesting that either peptides citrullinated at this residue are not presented by these HLA alleles, or T cell responses to this epitope are deleted/tolerised.

### TH1-type responses to NPM266-285cit epitope are CD4 mediated and citrulline specific

Identification of two peptides, 261-280cit and 266-285cit, that share a common 15 amino acid sequence suggests the possibility of a novel citrullinated region of NPM. The two peptides both generated high frequency IFNγ responses in HHDII/DP4 mice. Therefore, the core sequence is likely to lie between aa266-280. The core epitope was confirmed by testing mice immunized with NPM261-280cit or 266-285cit peptides for responses to the peptide spanning 260-280aa. HHDII/DP4 mice showed responses to both the immunizing peptides as well as peptide spanning 266-280, indicating the core sequence lies within this region (figure 2A). To determine if the same sequence was the likely core sequence in HLA-DR4 mice they were immunized with a combination of NPM261-280cit and 266-285cit peptides and immune responses assessed to the 266-280cit sequence. IFNγ release was detected to NPM261-280cit and NPM266-280cit peptides, however higher responses were observed to NPM266-285cit (figure 2A) indicating that the majority of the core sequence for HLA-DR4 is within 266-280 but possibly also includes amino acids QEAIQ (position 281-285).

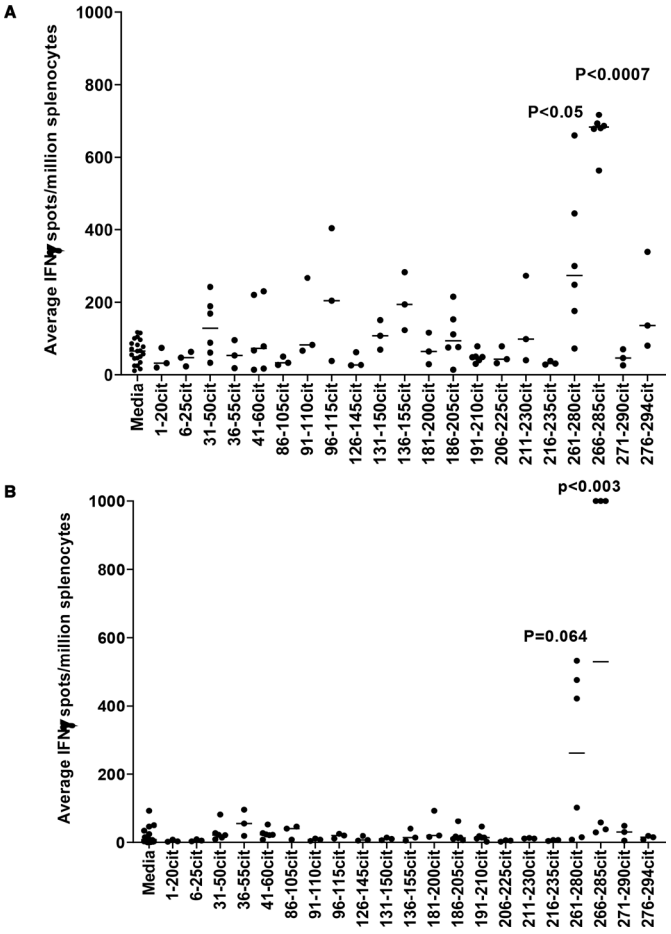


**Figure 1** Citrullinated NPM peptides stimulated T cells responses in transgenic HLA-DP4 and HLA-DR4 mice. HHDII/DP4 mice (A) or HLA-DR4 (B) transgenic mice were immunized with pools of 4–6 non-overlapping citrullinated peptides. Responses were measured at day 21 by IFNγ ELISpot assay. Responses were measured as spots/million splenocytes. Data are collated from independent studies where n=3–6. NPM, nucleophosmin.

The 266-285cit peptide stimulated the strongest immune response in both HLA-DR4 and HHDII/DP4 mice (figure 2A), therefore, this sequence was selected for further investigation. To determine if responses were CD4-specific they were assessed in the presence or absence of anti-CD4 or anti-CD8 blocking antibodies. The response to NPM266-285cit in HLA-DP4 mice was significantly blocked by an anti-CD4 (p=0.0056) antibody but not by an anti-CD8 antibody (figure 2B).

Mice immunized with NPM266-285cit peptide were also assessed to see if IFNγ release was specific to the citrullinated peptide. figure 2C shows strong IFNγ responses detected in HHDII/DP4 mice immunized with NPM266-285cit peptide that were significant when compared with the unmodified NPM266-285wt peptide (p=0.0092) and control (p=<0.0001). Similar observations were made in HLA-DR4 mice (figure 2C). No significant response to the NPM 266-285 wt peptide was seen suggesting the response in these mice is specific to the citrullinated peptide. In contrast, IFNγ responses were detected in mice immunized with NPM 266-285 wt peptide which exhibited cross reactivity with the citrullinated peptide (figure 2C) implying a different cross reactive T cell repertoire is stimulated by the NPM266-285wt peptide. This confirmed that the immune response generated in HLA-DP4 and HLA-DR4 mice to the citrullinated version of the NPM266-285 peptide is specific to the citrulline modification.

NPM266-285cit peptide also stimulated significant GM-CSF (p<0.0047), IL-10 (p<0.0267), TNFα (p<0.006), IP-10 (p<0.0008), MIP-1α (p<0.0029) and RANTES (p<0.0267) responses in NPM266-285cit immunized mice (figure 2D), thus confirming a Th1 phenotype. MCP-1 (p<0.0678) response to NPM266-285cit were strong but was not statistically significant. The median of GM-CSF, IL-10, TNFα, IP-10, MCP-1, MIP-1α, RANTES and MCP-1 in response to NPM266-285cit were 70.1, 204.6, 509.7, 392.6, 703.7, 178.5 and 532.5 pg/mL, respectively (figure 2D). Cytokine responses to NPM266-285wt peptide were not significantly different to unstimulated (medium) control. IL-1β, IL-2 and IL-4 responses were generally low to NPM266-285cit peptides (online supplemental figure 1).

### NPM is expressed in the B16F1 tumor cell line and NPM 266-285 can be citrullinated in vitro by both PAD2 and PAD4

Having confirmed the presence of a Th1 CD4 T cell repertoire in mice to the citrullinated NPM266-285 epitope and shown that in HLA transgenic mouse models this can be efficiently stimulated by vaccination, the expression of NPM on tumors was investigated. The expression of NPM on the B16F1 melanoma cell line was determined by Western blot. B16F1 cell lysates showed a band of approximately 35 kDa suggesting that this line does express NPM (figure 3A). Recombinant NPM used in this study contained His-Tag on the N terminal of the protein which may contributed to the slightly larger size visualized by Western blot. Previous reports have demonstrated the NPM protein is citrullinated in vivo on arginine aa197. In vitro citrullination of NPM recombinant protein using PAD2 and PAD4 enzymes confirmed the citrullination of arginine at position aa277 within the identified T cell epitope. The presence of citrullinated residues was determined by mass spectrometry (figure 3B,C). Six out of seven arginine residues within the NPM protein were citrullinated by PAD2, three of which (aa142, 197 and 277) with 100% confidence (figure 3B). In the presence of PAD4, citrullination was detected in four out of seven arginine residues (figure 3C). Residues 197 and 277 showed a high degree of confidence for citrullination. This data suggests that citrullination at aa277 may occur in vivo.

### Citrullinated NPM 266-285 vaccination mediates antitumor therapy in transgenic HLA-DP4 mice

To determine if the arginine at aa277 is citrullinated in vivo, NPM266-285cit-specific responses were stimulated by vaccination in a HLA-DP4 transgenic mouse

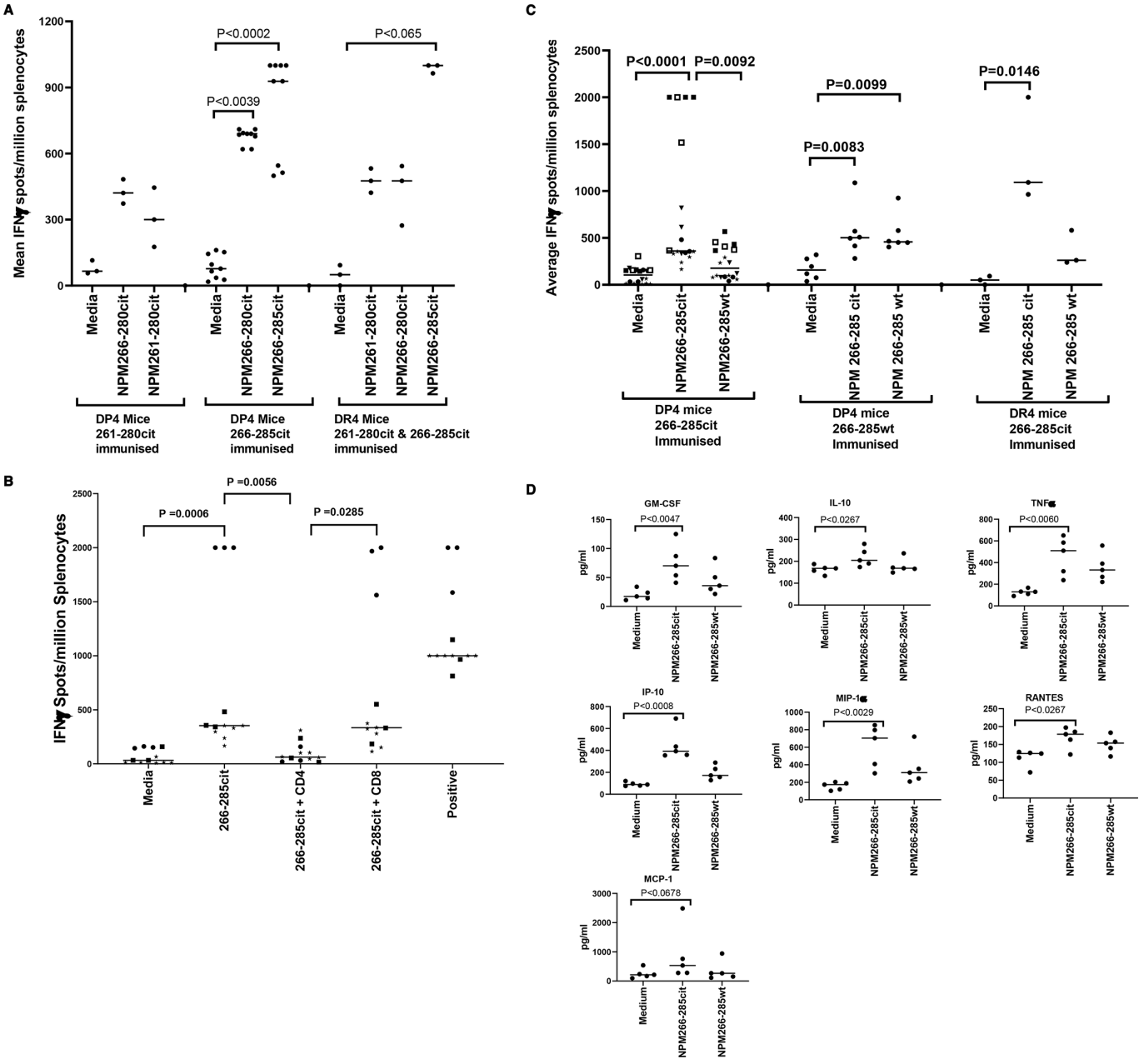


**Figure 2** NPM266-285cit induces CD4-specific response with no wild type cross reactivity. HHDII/DP4 mice (A–D) or HLA-DR4 (A, C) transgenic mice were immunized with NPM261-280cit (A), NPM266-285cit (A–D), NPM261-280cit and NPM266-285cit (A) or NPM266-285wt (C) peptides. Responses were measured at day 21 by IFNγ ELISpot assay to NPM261-280cit, NPM266-285cit, NPM266-280cit and NPM266-285wt peptides or to NPM266-285cit peptide in the presence of CD4 or CD8 blocking antibodies. Responses were measured as spots/million splenocytes. (B, C) Data points are formatted with symbols (● ■ □ ★ ▽) to group individual experiments. (D) GM-CSF, IL-10, TNFα, IP-10, MIP1α, RANTES and MCP-1 were also measured from the cell supernatant in response to NPM266-285cit and NPM266-285wt peptides. Data is collated from independent studies where n=3–18. NPM, nucleophosmin. HHDII, HLA-A0201 transgenic; GM-CSF, granulocyte macrophage colony stimulating factor; Interleukin-10, IL-10; tumor necrosis factor α, TNFα; interferon gamma-induced protein 10, IP-10; macrophage inflammatory protein 1α, MIP1α; monocyte chemoattractant protein-1, MCP-1.

model and assessed for epitope recognition presented within tumors and for delivery of tumor therapy. Mice were implanted with B16 melanoma cell line expressing IFNγ-inducible HLA-DP4 (iDP4) and the effect of immunization with NPM266-285cit peptide on tumor growth was assessed. Mice immunized with NPM266-285cit peptide showed a significant survival advantage over control mice immunized with CpG/MPLA only (figure 4A(i)). No control mice (0/10) were alive at 50 days compared with 65% (13/20) of the NPM266-285cit immunized mice (p≤0.0001) suggesting that this epitope is presented in the tumor and can be efficiently targeted by the vaccine induced CD4 response. Furthermore, 11 out 20 mice (55%) of the NPM266-285cit immunized group remained completely tumor free throughout the study. Analysis of tumor volumes

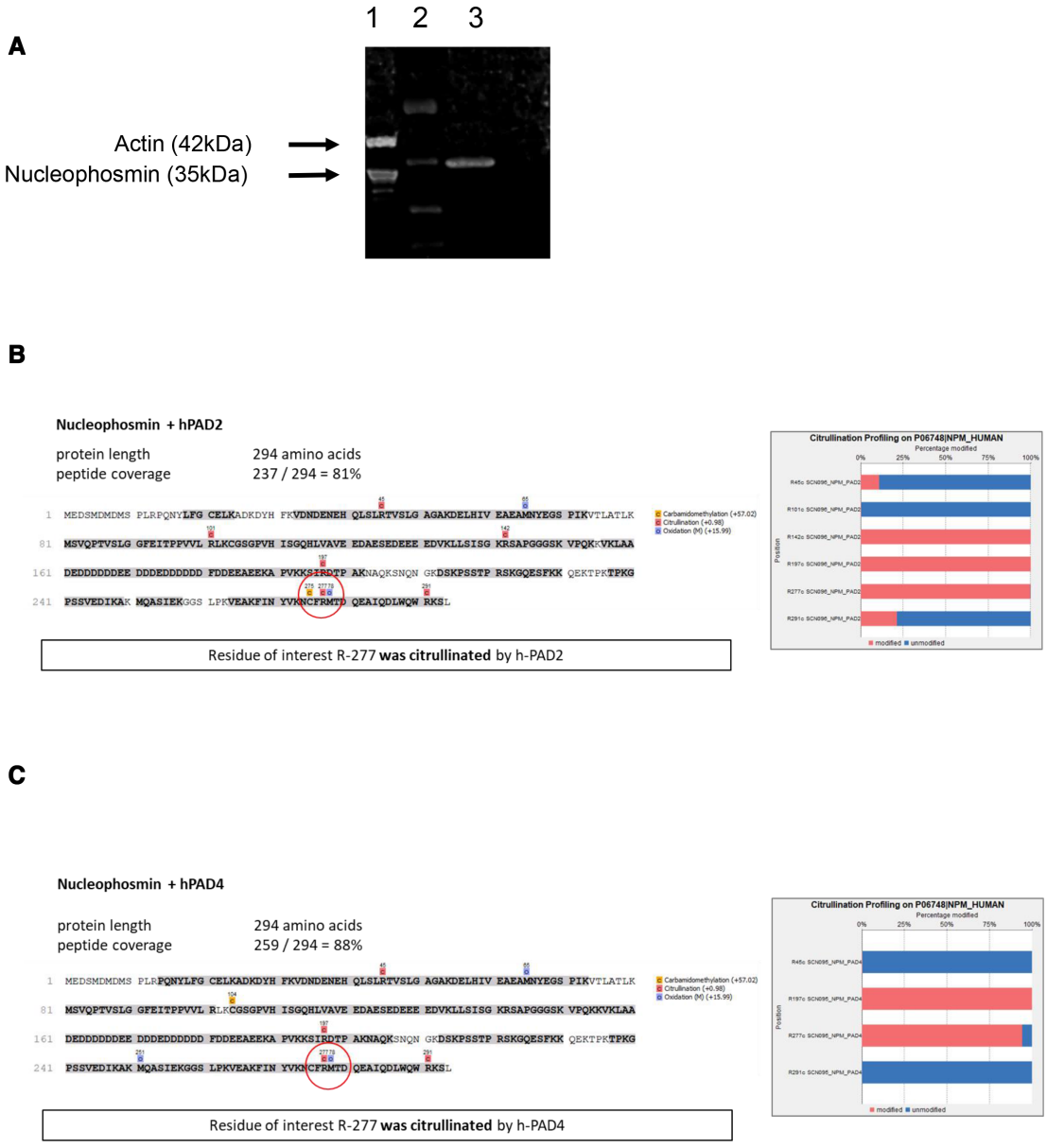


**Figure 3** NPM is expressed in B16F1 cells and can be citrullinated in vitro. Immunoblot (A) of lysates from B16F1 (lane 1), ladder (lane 2), recombinant NPM protein (lane 3) probed for NPM and β actin. The expected size for NPM (35 kDa) and β-actin (42 kDa) is shown. In vitro citrullination of recombinant NPM protein was performed in the presence of either (B) PAD2 or (C) PAD4, followed by mass spectrometry analysis to identify the sites of citrullination. NPM, nucleophosmin.

at day 24 post-tumor implant was also significantly lower (p=<0.0001) in the NPM266-285cit immunized mice ((figure 4A(ii)) compared with the CpG/MPLA control group. Mice were also implanted with LLC2 expressing constitutive HLA-DP4 and the effect of immunization with NPM266-285cit peptide on tumor growth was assessed. NPM266-285cit immunized mice showed slower tumor growth and a significant survival advantage over unimmunised, CpG/MPLA only, and irrelevant peptide immunized mice (online supplemental figure 2Ai,ii). This data confirms that the arginine at position 277 is citrullinated in vivo and can be efficiently targeted for immune therapy. The peptide was significantly (p<0.0374) protective against a more established B16F1 tumor as survival was observed with immunization delayed to day 8 post-tumor challenge (figure 4B(i)). Mice immunized with both regimens that survived the initial tumor challenge also developed 'immunological memory' as they were protected against rechallenge in the absence of any further vaccination (figure 4B(ii)). To determine if CD4 or CD8 cells play a role in tumor therapy mice were depleted of CD4 or CD8 T cells in combination with vaccination. CD4 (p<0.0001) and CD8 (p<0.0083) T cells depletion resulted in the loss of the protective effect of NPM266-285cit vaccination (figure 4C) indicating that although the immune response appears to be mainly CD4 T cell driven, CD8 T cells are also vital for anti-tumor activity in this model. Groups of mice were also implanted with B16F1 HHDII tumor that express the

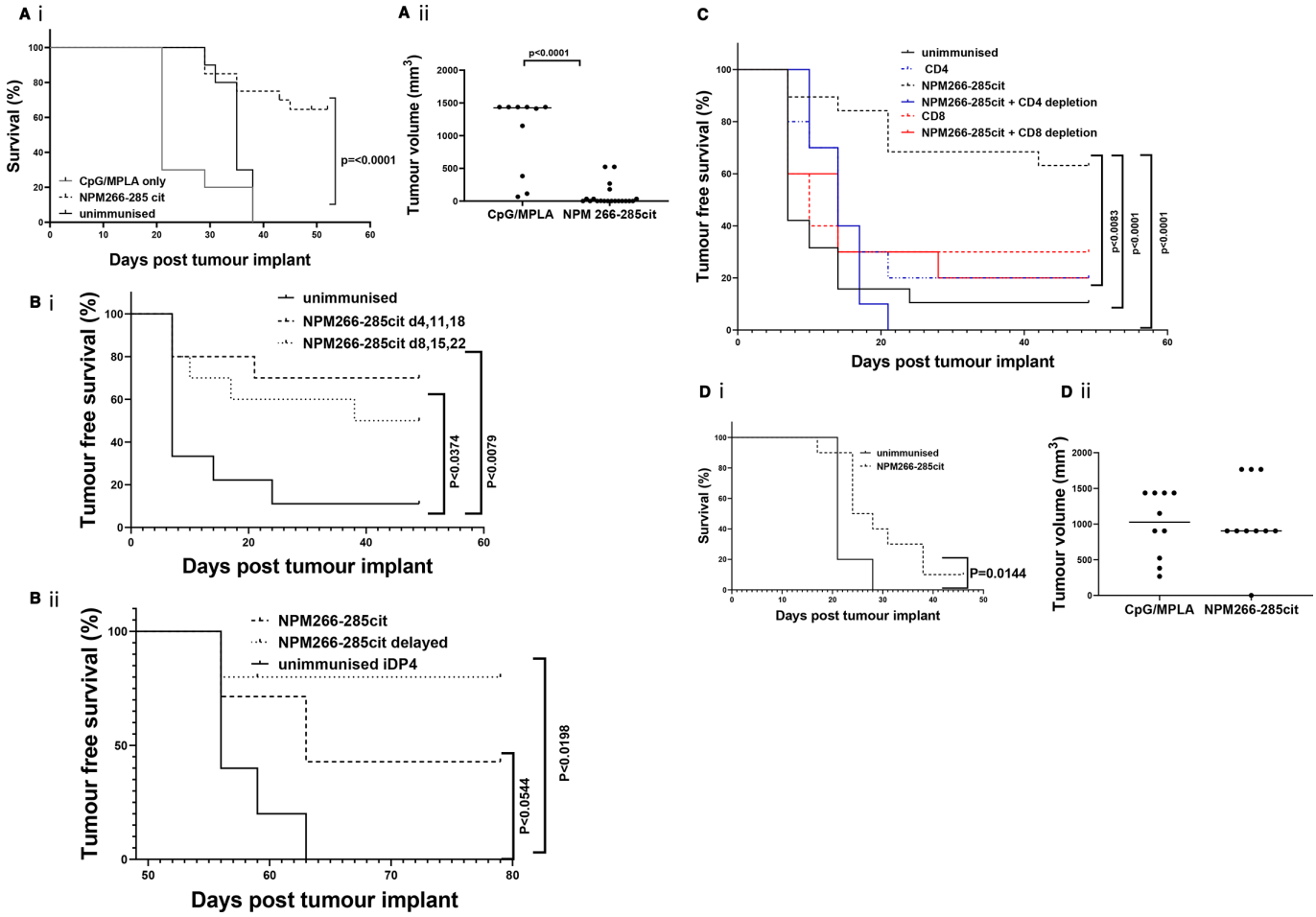


**Figure 4** Citrullinated NPM mediate antitumor therapy in transgenic HLA-DP4 mice. To assess if NPM266-285cit vaccination provides antitumor activity, HLA-DP4 mice were challenged with B16F1 tumor cells expressing inducible DP4 (B16F1 HHDII/iDP4) (A(i&ii), B(i&ii) and C) or B16F1 tumor cells lacking MHCII (D(i&ii)) followed by immunization on day 4, 11 and 18 (A(i&ii), B(i), C, D(i&ii)) or delayed immunization on day 8, 15 and 22 (B(i)). B16F1 HHDII/iDP4 (B(ii)) implanted on day 49 for rechallenge in surviving mice of the NPM266-285cit immunized group without further immunization. To assess if the antitumor activity is driven by CD4 or CD8 cells mice were also treated with CD4 or CD8 (C) antibodies. Tumor growth and survival was monitored and n=5–20 mice/group. Tumor volumes at day 24 (A(ii)) or day 38 (D(ii)) are shown. NPM, nucleophosmin.

relevant MHCI molecule but lack MHCII expression to assess if the peptide is presented and tumor therapy elicited in the absence of relevant MHCII molecules on the tumor cells. NPM266-285cit vaccination provided 10% (p=0.0144) (figure 4D(i)) survival advantage over CpG/MPLA immunized group, although there were no differences in tumor volume between the groups (figure 4D(ii)) suggesting antitumor activity is predominantly reliant on CD4 T cell recognition of tumor cells. Tumor growth curves are shown in online supplemental figure 3A–E.

## PAD2 is responsible for citrullination of arginine 277 in tumors in vivo

Arginine 277 can be citrullinated in vitro by both PAD2 and PAD4. In an attempt to determine which enzyme is responsible for the citrullination in vivo, we attempted to generate PAD2, PAD4 and double knock outs of B16F1 cells. The PAD2 knockout cell line was generated successfully (online supplemental figure 4) but PAD4 and double knock out B16F1 cells were unsuccessful as the double knock out was lethal. Despite this we examined tumor therapy in the model lacking expression of PAD2. Transgenic HLA-DP4 mice were implanted with B16F1 HHDII/PAD2KOcDP4 tumor lacking PAD2 enzyme and the tumor growth was assessed in presence or absence of NPM266-285cit vaccination. There was no significant survival advantage following NPM266-285cit immunization in B16F1 HHDII/PAD2KOcDP4 tumor bearing mice (p=0.6826) (figure 5A(i,ii)). Interestingly, the growth of tumor lacking PAD2 was slower than B16F1 HHDII/iDP4 (p<0.0001) and B16F1 HHDII (p=0.0060) tumors (figure 5B). Furthermore, in contrast to earlier results showing protection against rechallenge by mice that survived the initial tumor challenge, there was no protection in survivors against a rechallenge with tumors lacking PAD2 suggesting PAD2 is essential for NPM266-285cit citrullination in tumor (figure 5C). Tumor growth curves are shown in online supplemental figure 5A,B.

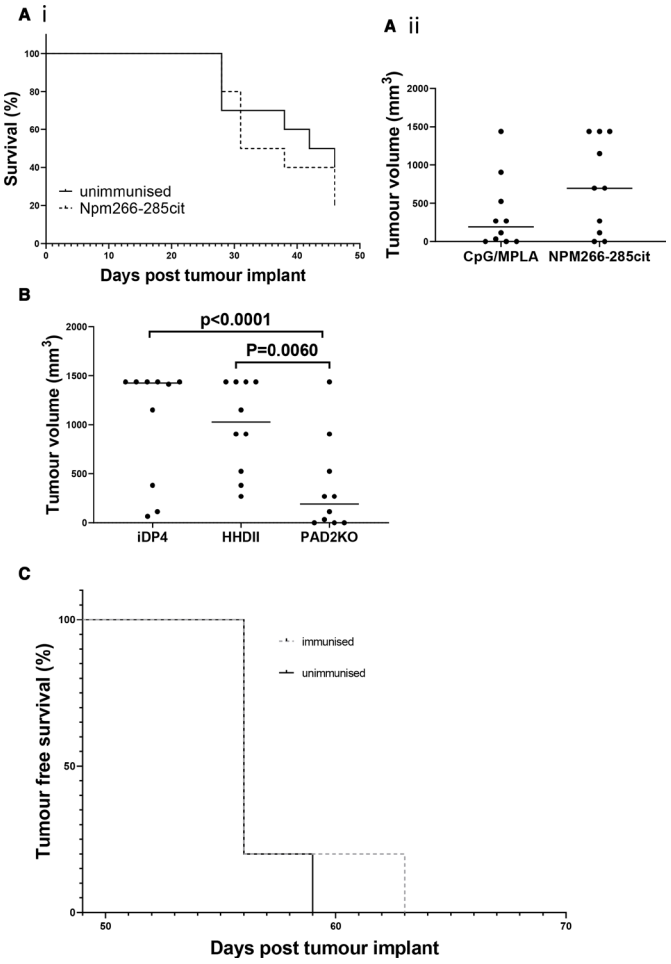


**Figure 5** PAD2 is responsible for citrullination of arginine 277 in tumors in vivo. HLA-DP4 mice were challenged with B16F1 tumor cells expressing constitutive DP4 but lacking PAD2 enzymes (A(i&ii) and C) followed by immunization on day 4, 11 and 18 (A(i&ii)). NPM26-285cit immunized HLA-DP4 mice that survived B16F1 HHDII/iDP4 implant were further rechallenged by implanting B16F1 tumor cells lacking PAD2 enzymes (C) on day 49 without further immunization. Tumor growth and survival was monitored and n=5–10 mice/group. Tumor volumes at day 38 (A(ii), B) are shown. HHDII, HLA-A0201 transgenic.

These data indicate that NPM266-285cit is citrullinated by PAD2 in tumor and vaccination with this epitope can provide antitumor immunity in animal model.

### Healthy donors and patients with lung cancer possess a naïve repertoire of CD4 T cells that recognise citrullinated NPM 266-285 peptide

We, and others, have previously demonstrated the presence of citrulline-specific CD4 T cells and CD4-specific responses in healthy donors.[16 30 32] The NPM 266-285 sequence is homologous in both mouse and humans. In order to assess the available T cell repertoire in humans, we stimulated PBMCs from 10 healthy donors with NPM266-285cit peptide in vitro after depletion of CD25 +cells (online supplemental figure 6) and showed seven donors generated a response to the peptide. An example of a FACS plot showing proliferation is given in figure 6A. The responses were observed by day 7 in four donors and by day 10 in the remaining three (figure 6B). Interestingly, all responding donors had the HLA-DP4 or HLA-DR4 haplotype, however, two of the non-responding donors were also HLA-DP4. This suggests that not all donors with these haplotypes are capable of responding and that the responses to NPM266-285cit may not be restricted to these HLA haplotypes. In a restimulation assay significant responses to NPM266-285cit peptide was observed but there was no response to the corresponding wild type sequence indicating a citrulline-specific response in healthy donors (online supplemental figure 7). T cells responses in 11 patients with lung cancer were also tested and 5 (45.5%) patients showed a CD4 proliferative response to NPM266-285cit which was lower than in healthy donors (figure 6C). However, the HLA status of the patients with cancer was not known and therefore HLA restriction of the peptide could not be determined in these donors. We further examined these responses after depletion of CD45RO+memory cells in healthy donors (online supplemental figure 6B). Data from donor 9 showed that the response persisted in the absence of memory cells suggesting it originated from a naïve T cell population (figure 6D). We assessed the phenotype of the proliferating cells for expression of IFNγ, granzyme B and CD134 markers at day 9–10. Despite some variation between individuals significantly higher proportions of proliferating cells expressed IFNγ, granzyme B and CD134 compared with non-proliferating cells (figure 6E(i,ii,iii)). These data demonstrate that healthy individuals possess a CD4 T cell repertoire that can respond to the citrullinated NPM266-285 peptide and display a Th1 cytotoxic phenotype. The memory/naïve status and Th1 profile in patients with cancer is yet to be determined but the lower response rate in patients compare to healthy donors may relate to the patient's treatment regimen.

## DISCUSSION

Accumulating evidence highlights an important role for CD4 T cell mediated immunity in the fight against cancer, however, only few tumor antigen-specific CD4 responses have been characterized. Post-translational modifications such as citrullination have been shown to stimulate strong self-antigen-specific CD4 responses that are particularly evident in autoimmune diseases.[32] We have previously shown that citrullination of proteins within tumor cells provide a good target for CD4 mediated immune therapy.[14–16 30] This previous work has focused on the citrullination of cytoplasmic proteins probably modified by PAD2. This study demonstrates that citrullination of the nuclear antigen NPM is present in the tumor microenvironment and is a target for immune therapy. NPM is known to be a target for PAD4-mediated citrullination modifying the residue at aa197.[28 33] PAD4 is well documented in cancer and its expression is increased in some cancers[34–36] especially during metastasis.[37] However, this study failed to show immune responses

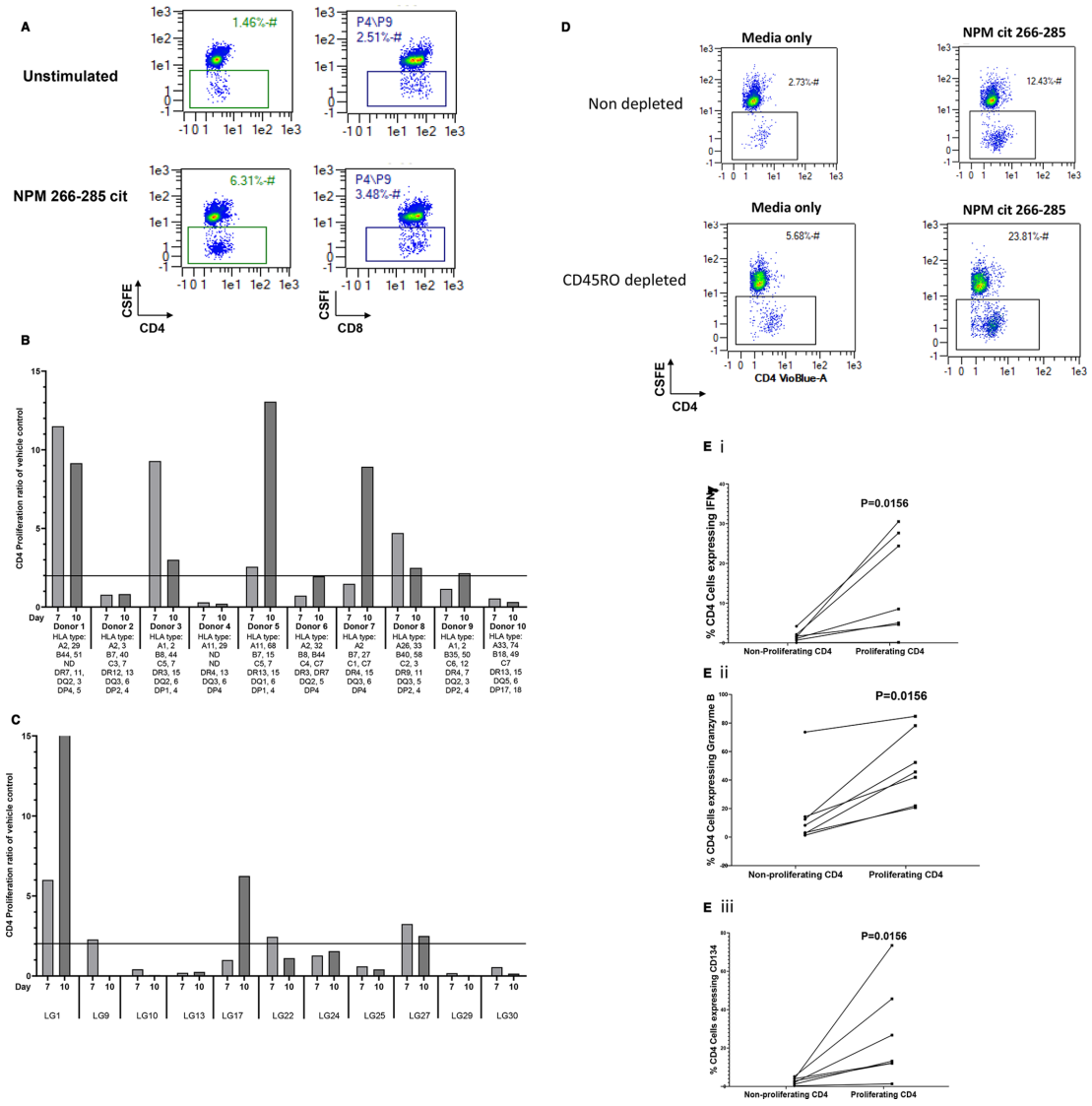


**Figure 6** Healthy donors and lung cancer patients have a repertoire of CD4 T cells that recognize citrullinated NPM. CFSE loaded PBMCs were stimulated with NPM266-285cit and proliferation was assessed on day 7–10. Responses double of medium control were considered to be significant. (A) FACs plot depicting representative CD4 and CD8 proliferation response to NPM266-285cit. Bar chart shows proliferation ratio of control in day 7 and 10 in (B) healthy donors and in (C) Lung cancer patients. (D) PBMCs were stimulated with NPM266-285cit with or without CD45RO depletion and responses were measured on days 7–11. Representative plots are shown. Proliferating CD4 T cells were phenotyped using (E(i)) IFNγ, (F(ii)) Granzyme B and (G(iii)) CD134 marker on day 10. NPM, nucleophosmin; PBMCs, peripheral blood mononuclear cells; CFSE,carboxyfluorescein succinimidyl ester.

in transgenic mice to peptides containing a citrulline at aa197 that (known to be citrullinated by PAD4 in the nucleus) suggesting there is either no T cell repertoire to this epitope or it is not presented efficiently on HLA-DR4 or HLA-DP4 molecules. Here, a new citrullinated NPM epitope (aa266-285) is characterized which stimulates strong Th1 mediated immunity resulting in the secretion of IFNγ, GM-CSF, IL-10, TNFα, IP-10, MCP-1, MIP1α and RANTES. The physiological concentration of these cytokines varies greatly depending on the type of cancer and disease progression.[38–40] Cytokine and chemokine concentrations in response to NPM266-285cit were generally much higher than the reported physiological levels in healthy individuals which further suggests the peptide induces a strong Th1 response.[38–43] The antitumor role of IFNγ, GM-CSF and TNFα have been discussed elsewhere.[44–46] IP-10, MCP-1, MIP1α and RANTES are chemokines that can promote chemotaxis of T cells, natural killer cells, macrophages and dendritic cells, therefore, recruiting immune cells to tumor microenvironment.[47–50] NPM 266-285cit-specific CD4 responses mediate strong tumor therapy in both the aggressive and established B16 melanoma model providing evidence that this peptide is citrullinated and presented in the tumor environment. Immune responses generated were capable of protecting against a rechallenge without subsequent vaccination indicating the establishment of immunological memory.

This study suggests a role for PAD2 in citrullination of NPM 266-285 epitope within tumors since tumor therapy is dependent on PAD2 expression in the tumor both in the initial challenge and for a rechallenge. PAD2 is likely to be responsible for citrullination of arginine 277 in the cytoplasm while arginine 197 is citrullinated by PAD4 in the nucleus. In the study by Tanikawa *et al* transfection of HEK293T cells with wild type and mutant PAD4 demonstrated NPM was only citrullinated by PAD4.[28] Citrullination was assessed by Western blotting which can fail to detect lower level of citrullination by other PAD enzymes such as PAD2.[28] PAD2 is the most abundantly expressed PAD enzyme in human tissue and in several cancers, including breast and skin cancers, it is overexpressed.[51] Given the ubiquitous expression of NPM and overexpression of PAD2 in cancers, citrullinated NPM makes an attractive candidate for cancer vaccine.

To examine if tumor therapy was dependent on tumor expression of MHCII we examined therapy in a model where tumor cells were unable to express the relevant MHCII molecules. Tumor growth in this model was similar to that seen in MHCII-expressing tumor, showing similar growth kinetics and survival in the HLA-DP4 transgenic mouse model. In contrast to the efficient tumor therapy in the B16 tumor model capable of MHCII expression, NPM266-285cit immunization induced only 10% survival against B16F1 tumors unable to express MHCII suggesting an important role for CD4 recognition of tumor cells via MHCII. However, in spite of a reduction in tumor therapy in the model unable to express MHCII on the tumor, significant therapy was seen on vaccination compared with control indicating an additional role for CD8, NK cells or macrophage driven killing with or without CD4 support. Immune cells can naturally eliminate tumors as evidenced with carcinogen induced tumors in immune competent mice.[52–54] CD4 cells are important for CD8 cell survival, expansion and effector function, hence activated CD4 cells following NPM immunization could potentiate CD8 mediated antitumor immunity.[2] This was evident in this study as protection against tumor was lost with CD4 or CD8 depletion. NK cells also target tumors through a process known as missing-self recognition[55] therefore immune editing in tumors promotes evasion via downregulation, but not total elimination of, MHC molecules. Unlike T cells, NK cells do not require antigen specificity and kill tumors through secretion of cytolytic granules. Macrophages in the tumor environment can take up tumor-specific antigens that are then presented to CD4 cells which secrete IFNγ further activating macrophages allowing tumor killing through an inducible nitric oxide synthetase-dependent mechanism.[56] Indeed macrophage mediated tumor killing has been demonstrated in murine models of multiple myeloma.[57]

In this study, a slower growth of B16F1 HHDII/PAD2KOcDP4 tumor was observed in the transgenic animal model indicating PAD2 may have a role in melanoma tumorigenesis. Histone H3 citrullination by PAD2 can inhibit methylation[58] and can thus influence downstream gene expression. Indeed, a previous study described PAD2 mediated regulation of gene expression in tumorigenesis and promoting epithelial to mesenchymal transition in mammary tumor cells, suggesting its role in tumorigenesis.[59] Furthermore, PAD2 is known to play role in progression of prostate cancer through androgen receptor signaling[60] and in inhibition of the enzyme supresses cytoskeletal protein RhoA, Rac1 and cdc42 resulting in poor migration of mammary carcinoma cells.[61] However, although the tumor growth was slower, the majority of B16F1 HHDII/PAD2KOcDP4 tumor bearing mice were culled by day 46 indicating the effect of PAD2 knock out in B16F1 melanoma tumorigenesis does not prevent tumor growth.

NPM266-285wt peptide can also stimulate a cross reactive response as observed in DP4 mice but the response to immunization with the citrullinated peptide was very specific. This was consistent with restimulation assay showing NPM266-285cit-specific responses in healthy donors with no wild-type cross-reactivity. Thus, the repertoire to citrullinated and wild type peptide are likely to be very different. For future studies, isolation cloning and analysis of a NPM266-28cit-specific TCR would help determine the reactivity and repertoires responding to the citrullinated and wild type peptide.

NPM is overexpressed in several solid tumors[20 22–24] and is mutated or involved in chromosomal translocation in hematological malignancies.[27 62] One of the most frequently mutated genes in AML is NPM, having been found to be mutated and aberrantly localized in the cytoplasm of leukemic blasts in around 35% of patients.[27] The mutation is identified on exon 12, involving the tryptophan reside at 288 and/or 290 positions of the 294 amino acids protein. The NPM266-285cit epitope identified in this study is likely to be unaffected by the mutation and could therefore be used for vaccine-mediated therapy in these hematological malignancies. Previous studies showed targeting citrullinated cytoplasmic proteins vimentin and enolase induce strong antitumor immunity in mouse models.[16 30] Likewise, targeting the citrullinated nuclear antigen NPM also provides antitumor immunity. This study demonstrates a T cell repertoire to NPM 266-285cit in healthy donors with proliferating cells showing expression of the activation marker CD134, the Th1 cytokine IFNγ and the cytotoxic protease Granzyme B. Almost half of the patients with lung cancer also responded to NPM266-285cit despite being on treatment which is known to adversely affect immune cell function. This implies that the T cell repertoire to NPM266-285cit epitope in humans has not been deleted or tolerised and has the potential to be targeted by vaccination. Since NPM is a nuclear protein, combining NPM266-285cit with another antigen for vaccination may be advantageous. Furthermore, donors with various HLA-types responded to the citrullinated NPM peptide indicating that responses in humans may

not be solely restricted to the HLA-DP4 or HLA-DR4 haplotypes. We propose that targeting of citrullinated proteins for tumor therapy should not be restricted to cytoplasmic antigens and that targeting citrullinated nuclear protein NPM should be further investigated for immunotherapeutic approaches.

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**Contributors** RHC: methodology, investigation, writing-original draft, review and editing; PS: methodology, investigation; SJP: formal analysis, writing-original draft; ID: methodology, investigation; KWC: methodology, investigation; MG: methodology, investigation; RLM: conceptualization, methodology, resources; VAB: conceptualization, visualisation, supervision, writing-review editing; LGD: conceptualization, visualisation, supervision, writing-review editing. RHC accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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**Competing interests** RHC and LGD have ownership interest in the UK Patent Application No. 2005779.0. LGD is the joint CEO of Scancell has ownership interest (including patents) in Scancell, is a consultant/advisory board member of Scancell, and has provided expert testimony for Scancell.

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**ORCID iDs**
Ruhul H Choudhury http://orcid.org/0000-0003-1472-9743
Katherine W Cook http://orcid.org/0000-0002-7277-6700
Victoria A Brentville http://orcid.org/0000-0003-2000-5629

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