The diversity of the immunogenic components of the melanoma immunopeptidome.

Katherine Woods[1,2,9], Pouya Faridi[3,9], Simone Ostrouska[1,2], Cyril Deceneux[1,2], Stephen Q. Wong[4,5], Weisan Chen[6], Ritchlynn Aranha[3], Nathan P. Croft[3], Divya Duscharla[3], Chen Li[3,7], Rochelle Ayala[3], Jonathan Cebon[1,2], Anthony W. Purcell[3*], Ralf B. Schittenhelm[3,8*], Andreas Behren[1,2*]

[1]Cancer Immunobiology, Olivia Newton-John Cancer Research Institute, Austin Hospital, Heidelberg, Victoria, Australia.

[2]School of Cancer Medicine, La Trobe University, Bundoora, Victoria, Australia.

[3]Department of Biochemistry and Molecular Biology, Monash Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia.

[4]Cancer Research Division, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia.

[5]Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia.

[6]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia.

[7]Current Address: Department of Biology, Institute of Molecular Systems Biology, ETH Zürich, Switzerland

[8]Monash Biomedical Proteomics Facility, Monash Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia.

[9]These authors contributed equally

*corresponding authors

Andreas.behren@onjcri.org.au; ralf.schittenhelm@monash.edu.au and

anthony.purcell@monash.edu

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Summary

Antigen-recognition by $CD8^+$ T cells is governed largely by the pool of peptide antigens presented on the cell surface in the context of MHC class I complexes. Recent studies have shown not only a high degree of plasticity in the immunopeptidome, but also that a considerable fraction of all presented peptides are generated through proteasome-mediated splicing of non-contiguous regions of proteins to form novel peptide antigens. Here we used high-resolution mass-spectrometry combined with new bioinformatic approaches to characterize the immunopeptidome of melanoma cells in the presence or absence of interferon-γ. In total, we identified 30,120 peptides and demonstrate that interferon-γ induces marked changes in the peptidome (with an overlap of only 49.3% between conditions as revealed by data independent acquisition mass spectrometry). Moreover, around 6% (1,821) of the peptides were identified as *cis*-spliced peptides with 712 derived from known melanoma-associated antigens. Several of these peptides were shown to be immunogenic in unrelated melanoma patients. These observations highlight the

breadth and complexity of the repertoire of immunogenic peptides that may be exploited therapeutically and suggest that spliced peptides may be a major class of tumour antigens.

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Introduction:

Antigen recognition by cytotoxic T cells and subsequent tumour cell destruction is the key component underlying cancer immunotherapy strategies. Its importance has been widely demonstrated, and loss-of-function of elements in the antigen processing and presentation pathways has been shown to confer therapeutic resistance[1]. Correlative findings point to neo-antigens arising from tumour mutations as an important source of immunogenic antigens in the context of melanoma and other cancers[2]. Nonetheless, tumours with low mutational burdens can respond to checkpoint inhibitor therapy, and the presence of a high tumour-mutational load does not necessarily correspond to the efficacy of treatment[3]. HLA-presented peptides (*p*-HLA-I) arising from the mutant proteins are mostly heterogeneously expressed and additionally determined by the patient-specific HLA-subtypes, making predictions about their presentation and immunogenicity unreliable. While a number of recent studies have reported the utility of mass spectrometry combined with exome sequencing in identifying HLA-presented peptides derived from mutated proteins[4-6], the analysis of the contribution of mutational neo-antigens to the overall tumour immunogenicity remains complicated and unresolved.

Against this background, the composition of the immunopeptidome, or the repertoire of HLA-bound peptides presented on the surface of the cell and their contribution to tumour immune recognition, becomes significant. The immunopeptidome is largely shaped by antigen processing through the proteasome complex for subsequent presentation of short peptide epitopes on MHC molecules[7]. Several forms of the proteasome complex exist, each with differing enzymatic activities[8]. In melanoma cells, the constitutive proteasome (cP) is expressed under steady state conditions. The expression of an immunoproteasome (IP), the subtype expressed by dendritic cells

and other cells of the immune system, may be induced in tumour cells in a cytokine-dependant manner [9], leading ultimately to changes in the peptides presented to the immune system[10, 11]. We have previously demonstrated induction of the IP in a range of human melanoma cell lines in the presence of the inflammatory cytokine IFN *in vitro*, and in melanoma patient inflamed tumours (characterised by presence of tumour infiltrating lymphocytes (TILs)) *ex vivo*[12]. Dependant on the proteasome subtype expressed by the cell, we have subsequently shown that a single melanoma antigen (NY-ESO-1) has the potential for cleavage into multiple differing epitopes. These differences in antigen processing led to concomitant change in the ability of antigen specific T cells to target the tumour cell. Thus, the potential for a tumour cell to 'look' substantially different to $CD8^+$ T cells, depending on inflammation at the tumour site, arises. Moreover, recent studies by ourselves and others[13, 14] have shown that a significant proportion of *p*-HLA-I are not genomically templated and result from post-translational proteasome splicing (ligation of non-contiguous small polypeptide segments from the same or different proteins). To date, these peptides have been missed in most neoantigen discovery studies due to the lack of appropriate bioinformatics tools[15, 16]. In this study we have used high resolution mass spectrometry approaches (both data-dependent and data-independent acquisition mass spectrometry) combined with a novel bioinformatics workflow to not only demonstrate significant changes in the melanoma immunopeptidome based on presence or absence of the cytokine IFNγ, but also to identify and quantify *cis*-spliced *p*-HLA-I presented by melanoma cells under both conditions. Those peptides derived from melanoma-associated or over-expressed proteins were tested for *ex vivo* immunogenicity in melanoma patients and healthy donors. Both linear and spliced peptides were found to be immunogenic in both donor groups. Notably, T lymphocyte

responses to pools of IFNγ upregulated peptides were not seen in healthy donors. We also demonstrate for the first time that *cis*-spliced peptides are widely presented by melanoma cells and immunogenic in multiple donors. These findings have significant implications for cancer immunotherapy as well as for fundamental questions such as induction of immune-tolerance, T cell repertoires and pathogen recognition.

Materials and Methods

Human Ethics Approval

Samples used in this study were derived from patients who consented to participate in a clinical research protocol approved by Austin Health Human Research Ethics Committee (HREC H2006/02633).

Melanoma cell line culture

Establishment and characterization of the melanoma cell lines used has been previously described[17, 18]. Cells were cultured in RF10 consisting of RPMI 1640, 2mM Glutamax, 100 IU/ml Penicillin, 100 µg/ml Streptomycin and 10% heat-inactivated fetal calf serum (all Invitrogen). For induction of immunoproteasome catalytic subunits cells were incubated with 100 ng/ml IFNγ (Peprotech) for 72 h prior to experiments.

Melanoma cell line sequencing

Whole exome sequencing of the LM-MEL-44 cell line was performed using the NimbleGen EZ Exome Library v2.0 kit and run on a Illumina Hiseq2000 instrument as previously described[19]. Sequence reads were aligned to the human genome (hg19 assembly) using the Burrows–Wheeler Aligner (BWA) program[20]. Single

nucleotide variants (SNVs) and indels were identified using the GATK Unified Genotyper[21], Somatic Indel Detector[22] and MuTect (Broad Institute)[23].

Isolation of peptides bound to HLA class I molecules

HLA class I peptides were eluted from LM-MEL-44 cells (prior to or after treatment with IFNγ) as described previously[24-26]. In brief, 3 x 10^9 cells were lysed in 0.5% IGEPAL, 50 mM Tris-HCl pH 8.0, 150 mM NaCl supplemented with protease inhibitors (CompleteProtease Inhibitor Cocktail Tablet; Roche Molecular Biochemicals) for 45 min at 4 °C. Lysates were cleared by ultracentrifugation at 40,000 *g* and HLA class I complexes were immunoaffinity purified using W6/32 (pan anti-HLA-A, B, C) and DT9 (anti HLA-C) monoclonal antibodies.

Fractionation of HLA-bound peptides by reversed-phase high-performance liquid chromatography (RP-HPLC)

The HLA-peptide eluates were loaded onto a 4.6 mm internal diameter x 50 mm monolithic C18 RP-HPLC column (Chromolith Speed Rod; Merck) at a flow rate of 1 ml/min using an EttanLC HPLC system (GE Healthcare) with buffer A (0.1% trifluoroacetic acid (TFA)) and buffer B (80% ACN / 0.1% TFA) as mobile phases. The bound peptides were separated from the class I heavy chains and ß2m molecules using increasing concentration of buffer B. Peptide-containing fractions (500 µl) were collected, vacuum concentrated to ~5 µl and reconstituted to 15 µl with 0.1 % formic acid (FA). Indexed retention time (iRT) peptides[27] were spiked in for retention time alignment.

Identification of HLA bound-peptides using data-dependent acquisition (DDA)

Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, the samples were loaded via an Acclaim PepMap 100 trap column (100 µm x 2 cm, nanoViper, C18, 5 µm, 100Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75 µm x 50 cm, nanoViper, C18, 2 µm, 100Å; Thermo Scientific). The peptides were separated by increasing concentrations of 80% ACN / 0.1% FA at a flow of 250 nl/min for 65 min and analyzed with a QExactive Plus mass spectrometer (Thermo Scientific). In each cycle, a full ms1 scan (resolution: 70.000; AGC target: 3e6; maximum IT: 120 ms; scan range: 375-1800 m/z) preceded up to 12 subsequent ms2 scans (resolution: 17.500; AGC target: 1e5; maximum IT: 120 ms; isolation window: 1.8 m/z; scan range: 200-2000 m/z; NCE: 27). To minimize repeated sequencing of the same peptides, dynamic exclusion was set to 15 sec and the 'exclude isotopes' option was activated.

Quantification of HLA bound-peptides using data-independent acquisition mass spectrometry (DIA-MS)

The identical instrument setup as described above (Dionex UltiMate 3000 LC system coupled to a QExactive Plus mass spectrometer) was used to quantify HLA-bound peptides using data-independent acquisition (DIA). In each cycle, 28 sequential DIA windows with an isolation width of 25 m/z between 300 - 1000 Da were acquired (resolution: 17.500; AGC target: 2e5; maximum IT: auto; NCE: 27) following a full ms1 scan (resolution: 70.000; AGC target: 3e6; maximum IT: 55 ms; scan range: 300-1000 m/z). A 90 min gradient of increasing concentrations of 80% ACN / 0.1% FA was used to separate the peptides for the DIA acquisition.

DDA data analysis

Linear and cis-spliced peptide sequences were identified as described previously[14]. In brief, the acquired .raw files were searched with PEAKS Studio 8.5 (Bioinformatics Solutions) against the human UniProtKB/TrEMBL UniProtKB/SwissProt database (v2017_10). The parent mass error tolerance was set to 10 ppm and the fragment mass error tolerance to 0.02 Da. Oxidation of M, deamidation of N & Q and phosphorylation of S, T & Y were set as variable modifications and a FDR cutoff of 1% was applied. High confidence *de-novo* peptide sequences without any linear peptide match in the provided database were further interrogated with the "Hybrid finder" algorithm[28] and the identified *cis*-spliced peptide sequences added back to the original UniProtKB/SwissProt database and all data researched using PEAKS DB . Linear and spliced peptides in this search were extracted at 1% FDR to create the final list of identified peptides.

DIA data analysis

Spectronaut 11 Pulsar (Biognosys) was used to generate a spectral library, which contained information of all linear and *cis*-spliced HLA class I peptides identified in this study, as well as to quantify peptides within the all DIA dataset.

T cell stimulation assay

To assess T cell responses selected peptides were synthesised (Mimotopes, VIC, Australia). PBMC from healthy donors (Australian Red Cross, VIC, Australia), or melanoma patients (Austin Health HREC approved protocol HREC H2006/02633) were purified by density centrifugation over Ficoll Hi-Paque. Cells were cultured in TCRPMI consisting of RPMI 1640, 2mM Glutamax, 100 IU/ml Penicillin, 100 µg/ml

Streptomycin, 20 mM HEPES, 1% nonessential amino acids, 1 mM sodium pyruvate, 55 µM β-mercaptoethanol, and 10% human AB serum (Australian Red Cross, VIC, Australia). Peptides were combined into pools of 5-9 peptides as outlined in Supplementary Table 6. 10^6 PBMC/ml were incubated with 10µM each peptide in pools for 10 - 12 days at 37 °C. IL-2 (100 IU/ml) was added and replaced every 3 days.

Intracellular cytokine staining (ICS) of antigen-activated T-lymphocytes

To assess antigen responses, T-lymphocytes were restimulated (following 10 - 12 days incubation as outlined above) with peptide pools for 4-8 h in TCRPMI in presence of 10 µg/ml brefeldin A (BFA, Golgi plug). Cells were washed with PBS (Invitrogen) labeled with live/dead fixable violet stain (Invitrogen), then incubated with antibodies against CD3 and CD8 (BD biosciences) for 15 min at 4°C. Samples were washed and fixed for 20 min at 4°C. Cells were permeabilised and stained with anti-TNFα (eBiosciences) for 25 min at 4°C. The gating strategy was: SSC/FSC; Singlets; SSC/LD^-; $CD3^+/CD8^+$; $CD8^+/TNF\alpha^+$. Data were acquired on a FACSCanto (BD biosciences, VIC Australia) and analyzed with FlowJo software (Version 10, FlowJo, Ashland OR, USA). To account for the large variation in DMSO background $CD8^+$ T cell activity across multiple donors, signals were normalized by subtracting the background from DMSO control treated samples in each case.

HLA-A2 stabilisation assays

The binding activity of the peptides was assayed by measuring peptide-induced stabilization of HLA-A2 on TAP-deficient T2 cells by flow cytometry. T2 cells were cultured in RF-10 (RPMI with 10% serum, 5% Glutamine, 5% Pen/Strep) in T25

flasks for 2 – 3 days before the assay. T2 cells (2x 10^5 cells/well) were cultured for 16 h at 37 ^oC in 5% CO_2 in 200 µL RF-10 in 96-well U-bottomed plates in presence or absence of 10 µg/ml of synthetic peptides. Peptides from Melan-A (modified aa26-35 ELA……and aa 60-72) served as a positive or negative controls respectively. All peptides were tested in triplicate.

After 16 h stimulation the cells were washed and stained with anti-HLA A2 monoclonal antibody BB7.2 (Biolegend) for 30 min. at 4 ^oC. Cells were subsequently stained with Fixable Viability Kit (Zombie NIR™, Biolegend) for 15 min. at 4 ^oC before flow cytometry on a FacsCanto (BD). Data was analysed using FlowJo software (Version 10, FlowJo, Ashland OR, USA).

Results:

The melanoma immunopeptidome contains a large proportion of cis-spliced peptides.

To establish a comprehensive repository of HLA class I peptide ligands presented by melanoma cells utilizing either the canonical proteasome (cP) or the immunoproteasome (IP), we cultured the patient-derived melanoma cell line LM-MEL-44[18] in the absence or presence of IFN for 3 days to allow for turnover of the cell-surface presented epitopes[29], and then subjected to the workflow outlined in Figure 1a. Briefly, HLA class I molecules were affinity purified with the HLA-C (DT-9) and pan-HLA-ABC antibodies (W6/32) and HLA-bound peptides were pre-fractionated by HPLC prior to analysis by data-dependent acquisition mass spectrometry (DDA-MS). Data was analysed by Peaks 8.5 software and spliced peptides further characterised using the HybridFinder algorithm[14].

We identified a total of 30,120 peptides (derived from 12,025 source proteins) presented across all HLA class I allotypes expressed by the LM-MEL-44 cells at a false discovery rate (FDR) of 1% (Supplementary Table 1). Approximately 6% of these peptides (1,821 peptides) were assigned as *cis*-spliced in origin (Fig 1b, Supplementary Fig 1a, Supplementary Table 1), being derived from non-contiguous sequences of the same protein. While *trans*-splicing has been described to contribute to the cellular immunopeptidome as well[14], we have here focused on *cis*-spliced peptides only due to the availability of appropriate bioinformatics workflows. This proportion of peptides of *cis*-spliced origin is in agreement with our previous study[14] and others [30, 31]. As expected for HLA class I epitopes, the majority of peptides (27,321 peptides; ˜90%) were between 8-12 amino acids in length with no apparent difference between linear and *cis*-spliced sequences (Supplementary Figure 1b). Using the NetMHCpan binding algorithm[32], 91.2% of these 8-12mers were

predicted to bind to at least one of the HLA class I alleles expressed on the surface of the LM-MEL-44 cells (A*02:01,B*60/*44:02,C*03:04/*05:01)[33] suggesting that the majority of the identified peptide sequences can be considered genuine HLA class I ligands (Supplementary Table 2). The percentage of predicted binders was lower for *cis*-spliced peptides compared to linear peptides (68.5% vs. 92.9%), which can be attributed to the fact that NetMHCpan and other binding algorithms have been developed and based on consensus-binding motifs of linear epitopes, which can differ slightly from consensus-binding motifs of *cis*-spliced peptides[13, 14]. Indeed, similar marginal differences between the consensus-binding motifs of linear and *cis*-spliced epitopes are also evident in this dataset, which are more pronounced at the *N*-terminal anchor positions P2 / P3 compared to the *C*-terminal PΩ position (Figure 1c).

Taken together, we identified more than 30,000 predominantly novel HLA class I ligands presented on the surface of the LM-MEL-44 melanoma cells including nearly 2,000 *cis*-spliced peptides, which have not been described before. Of note, none of the mutational neoantigens predicted based on exome sequencing data of LM-MEL-44[34] were identified in our dataset (Supplementary table 3); however this cell line has a relatively low mutational load.

IFNγ-treatment leads to significant changes in the MHC class I presented peptide pool. Considering the well-described clinical relevance of so called “hot” versus “cold” tumour microenvironments and previous work demonstrating the influence of cytokine exposure on antigen-presentation pathways[10], we wanted to examine on a global scale the impact of IFNγ exposure on the immunopeptidome. An initial qualitative analysis revealed that only 49.3% of the 30,120 peptides were identified under both conditions demonstrating that the addition of IFN□ significantly changes

the composition of the immunopeptidome (Figure 2a). To measure these differences quantitatively, we combined all IFN□-treated or non-treated fractions and analysed these samples by data-independent acquisition mass spectrometry (DIA-MS; Figure 1a) using a spectral library, which has been created based on our DDA data in the presence of iRT peptides ([27]; Supplementary Figure 2). After applying stringent filter criteria, we were able to quantify a total of 9,799 peptides (Supplementary Table 4). 3,553 of these peptides (˜36%) were only quantified in one of the two conditions with a q-value of less than 1% and can be considered as condition-specific, which is in good agreement with our initial comparative analysis.

The remaining 6,246 peptides (˜64%) were quantified in both conditions with corresponding q-values of less than 1% suggesting that these peptides are presented by melanoma cells both in the presence and absence of IFN□. To determine their degree of variation, we calculated their log2 fold changes (+IFN□/- IFN□) and plotted these values as violin plots separately for linear and *cis*-spliced peptides (Figure 2b). A considerable proportion (2,183 peptides, ˜35%) were observed to change in abundance by at least by a factor of 2, confirming that the addition of IFN□ substantially changes HLA class I presentation, while not affecting the proportion of presented *cis*-spliced epitopes on LM-MEL-44 melanoma cells.

This DIA-MS dataset contains 439 peptides, which have been quantified based on their doubly as well as triply charged precursor ions in both conditions. The resulting fold changes are nearly identical with a Pearson correlation coefficient of 0.899 (p-value <0.001) and a near perfect linear regression curve (Supplementary Figure 3), which highlights the precision of the quantitation using DIA-MS data.

Identification of novel cancer specific peptides in the melanoma immunopeptidome.

Next we screened our results for peptides derived from proteins previously described as melanoma-associated antigens (MAA)[35, 36] as well as for tumour antigens with demonstrated immunogenicity[37-39]. We identified a total of 712 peptides in our dataset (674 linear, 38 spliced peptides) derived from 96 different MAAs (Figure 3a, Supplementary Table 5). Approximately 10% of the linear MAA-derived peptides identified in our study have previously been described as $CD8^+$ T cell epitopes, thus a high proportion of the linear peptides and all of those generated by splicing, constitute novel peptides (Figure 3b). Of the previously reported epitopes, the majority were present in both the presence and absence of IFN□, whereas >28% of novel peptides were exclusive to IFN□ treated samples, demonstrating the importance of carefully considering experimental conditions for epitope identification (Figure 3c).

$CD8^+$ T lymphocytes frequently recognised novel linear melanoma-specific epitopes.

HLA-A2 is one of the most prevalent HLA-types worldwide, and efforts to identify common HLA- peptides for a large number of patients often focus on this serotype. Therefore, we addressed functional immunogenicity of a selection of predicted HLA-A2 binding, novel linear melanoma-specific epitopes by using them to stimulate $CD8^+$ T lymphocytes in PBMC derived from healthy donors or melanoma patients (Supplementary table 6). To confirm their A2 specificity, we have included HLA-A2 negative melanoma patients and healthy donors as controls wherever feasible, here represented by melanoma patient 6. Alongside these assays, we also assessed differences in functional immunogenicity of IP or cP processed epitopes by pooling these peptides into groups of those which were up- or down- regulated following IFN□ treatment (Figure 4). Of note, the LM-MEL-44 cell line was derived from

melanoma patient 2. We found that $CD8^+$ T lymphocyte responses to the tested peptides were more frequently seen in melanoma patients (Figure 4a (individual) and 4b (pooled donors)). Novel peptides derived from 15 of the melanoma antigens identified in our screen stimulated specific $CD8^+$ T lymphocyte responses (over 2% $TNF\alpha^+$ cells) in three or more donors, demonstrating novel, functional, melanoma T cell epitopes (highlighted in Supplementary Table 6, Representative examples, Figure 4c). The strongest responses were induced by the peptides derived from SART1 (U4/U6.U5 tri-snRNP-associated protein 1) and PGK1 (Phosphoglycerate kinase 1), both of which stimulated responses in 2-7% of T lymphocytes from 4 melanoma patients. When the same selection of peptides were pooled in groups of those up/down regulated, or unchanged following IFN treatment no appreciable difference in functional immunogenicity in melanoma patients was observed between groups. Interestingly however, T lymphocyte responses to pools of IFNγ upregulated peptides were not seen in healthy donors. One pool in each group was made on the basis of higher *in silico*-predicted immunogenicity (www.iedb.org[40], Figure 4a,b, asterisks). However, these groups did not display enhanced ability to activate $CD8^+$ T lymphocytes in either melanoma patients or healthy donors.

Melanoma patients expressing immunoproteasome genes have a survival advantage.

Tumour recognition *in vivo* relies on the processing and generation of cognate peptides within the tumour cells. As antigen presenting cells (APCs) use the immunoproteasome for protein processing and subsequent HLA-loading, tumours that express the IP as opposed to the constitutive form should display a similar peptide repertoire as APCs, which should avoid T-cell-p-HLA mismatch and therefore lead to a greater likelihood of encountering matched T cells. Using OncoLnc[41], we mined

gene-expression data generated by the TCGA Research Network (http://cancergenome.nih.gov/) for correlation of both IP and cP-specific genes with survival in melanoma patients. We found that expression of all three IP-specific subunits was highly significantly associated with survival in melanoma patients. Conversely, cP-specific subunits were associated with decreased melanoma patient survival (Figure 5).

Since IP subunits are also expressed by immune cells, including TILs, we removed the top quartile of samples with the highest CD3 expression. In these tumours IP expression to a large extent is likely generated by immune infiltrates themselves. Following removal of these samples, we found that a significant survival benefit, associated with 2/3 IP subunits, was maintained (Supplementary Figure 4). Furthermore, presence of the 3 cP subunits was associated with a trend towards decreased survival. This indicates that patients whose tumours express an IP have survival benefit which is specifically associated with this proteasome type. As IP expression in tumours is driven by cytokine exposure it remains unclear if this is merely a footprint of a successful immune recognition or if it is part of the pre-conditions to allow for it.

Spliced peptides are immunogenic across patients and represent novel targets for immunotherapy

The potential implications of the presence of spliced peptides for all facets of immunity have sparked intense discussions in the last 2 years[14, 42-44]. In cancer, their presence would dramatically widen the repertoire of potentially targetable epitopes and may allow for many more tumour-specific antigens (including mutational derived neoantigens) being presented in various HLA-contexts. So far only

6 immunogenic *cis*-spliced peptides derived from 4 different proteins[42] have been described and most of them have been discovered by T cell assays rather than by mass spectrometry[45-50]. To test some of the identified spliced peptides for their ability to activate $CD8^+$ T cells *in-vitro* we synthesized 26 *cis*-spliced peptides based on (i) their *de-novo* sequencing confidence score, (ii) their NetMHC4 binding prediction score for 3 HLA alleles expressed on LM-MEL-44 cells (HLA-A*02:01, HLA-B*44:02 or HLA-C*05:01) and (iii) the quality of their peptide spectrum matches (PSMs). When employed as pools of 8-9 individual peptides, all 3 pools evoked immune-responses as measured by intracellular TNFα production in $CD8^+$ T cells (Figure 6a) in multiple HLA-A2 positive melanoma patients. These responses were more prominent in patients compared to healthy HLA-$A2^+$ donors (Figure 6b).

Given the differences in the potential to stimulate HLA-A2 positive vs. negative patient and healthy donor samples, most of the immunogenic peptides in our assays seem to be HLA-A2 associated. To identify specific immunogenic peptides, PBMCs were stimulated with the peptide pools (Figure 7a) for 10-12 days followed by single peptide re-stimulation. Six out of 26 peptides induced a TNF-α response above background (Figure 7b) in at least one melanoma patient. Of note, the peptide demonstrating the highest immunogenicity based on these assays is a spliced peptide derived from a cancer-testis antigen, MAGE-C2 and showed $CD8^+$ T cell activation across 3 patients. These data show that these spliced peptides can serve as *bona fide* anti-cancer targets and provide a large number of additional targets that have been previously unknown. Of note, all immunogenic peptides that tested positive for immunogenicity in our assays were subjected to T2 peptide binding assays to confirm their HLA A2 specificity. As shown in Figure 7c, these peptides all stabilize HLA-

A2, albeit weaker than the well described modified ELAGIGILTV HLA-A2 peptide (aa26-35) from the melanoma antigen Melan-A[51].

Discussion:

In this study we have established a detailed and in-depth immunopeptidome presented on the LM-MEL-44 cell line, which is a patient-derived melanoma cell line derived from a lymph node metastasis. Our qualitative assessment of the immunopeptidome yielded over 30,000 high confidence peptide identifications that encompassed two culture conditions (+/- IFNγ). Surprisingly only 50% of these peptides were identified in both conditions. To more accurately assess the overlap and plasticity of the LM-MEL-44 immunopeptidome we used data-independent acquisition mass spectrometry (DIA-MS) to quantify the IFNγ induced changes in the melanoma immunopeptidome. Although this state-of-the-art, mass spectrometric technique has been developed several years ago[52, 53], only a handful of studies have exploited DIA-MS to quantify HLA peptide ligands so far[38, 54-58]. The well-described effect of IFN□ in mediating changes to the composition of the antigen processing machinery, coupled with reports of differences in antigen processing between the cP and the IP over several years, led us to expect a degree of difference between the two immunopeptidomes. Nevertheless, our observation that ~35% of the HLA class I epitopes were exclusive to either IFNγ treated or untreated conditions, and that an additional 35% were observed to change more than 2-fold in presence of IFN□, reflect the profound impact of this cytokine on the composition of the immunopeptidome. Our observations are also consistent with recent studies in ovarian and lung cancer[10, 11]. For example, in ovarian cancer, a 9% difference in peptides

presented between IFNγ treated/untreated conditions was observed following 24 h of IFNγ treatment (as opposed to 72 h in our study).

In our study, MAA-derived peptides were found in roughly equal proportions in IFNγ treated (28.8%), or untreated (20%) settings. Interestingly however, of the MAA epitopes that have been previously described in other studies, only 2.8% were present in IFNγ treated (IP) conditions (Figure 5c). This observation suggests that many IP processed epitopes may be as yet undescribed, since traditional approaches to identify tumour associated antigens have largely been undertaken using cells lines under steady state conditions (*i.e.* which express cP). Furthermore, of the previously described cancer *cis*-spliced peptides, 3 have been shown to be processed exclusively by the cP, and 2 by both the cP and the IP (and 1 undetermined)[42].

It is evident from our study that the steady state immunopeptidome may vary dramatically from the *in vivo* tumour scenario depending on the tumour microenvironment at any given time. Though our functional studies did not reveal a difference in the immunogenicity of peptides derived from either IFNγ treated or untreated conditions, in the *in vivo* setting a T cell response to IFNγ related epitopes is likely to be aided by correlative IFNγ influences, such as upregulation of surface HLA[59]. Taken together, it is tempting to speculate that antigen processed *via* the IP may represent an untapped resource of "IFNγ-associated neo-epitopes".

The potential for tumour escape from $CD8^+$ T lymphocyte killing due to whole scale change to the immunopeptidome upon initiation of an anti-tumour responses, and corresponding induction of IFNγ is clear. These data become particularly significant in the context of recent studies demonstrating that tumours with an IFN□-inflamed, or 'hot' microenvironment are associated with better prognosis, and are more likely to be amenable to treatment with immune checkpoint inhibitors[60]. It seems conceivable

that *in vivo* the difference between immunopeptidomes is indeed of immunological relevance to disease progression and overall patient prognosis.

The identification of spliced peptides as tumour antigens in cancer was first described in 2004 in both the FGF5 protein in renal cancer[45] and the gp100 protein in melanoma[46], and since then only a further 4 *cis*-spliced peptides have been described in cancer[42]. To date their identification has been hampered by lack of feasible identification methods. In this study, we have used high-resolution mass spectrometry to identify more than 30,000 endogenous HLA class I peptide ligands. Approximately 2,000 (6%) of these peptides represent *cis*-spliced peptides demonstrating that *cis*-spliced peptides are commonly generated in cancer cells, which broadens the repertoire of potential targets in cancer immunotherapy. Consistent with our observations a recent study by Liepe *et al*[13] identified 750 and 486 *cis*-spliced peptides in colon and breast cancer cell lines respectively – however their immunogenicity was not described. Of note, 38 of our 2,000 *cis*-spliced peptides (and 712 of all identified peptides) were derived from melanoma-associated antigens, and with the exception of a few linear epitopes, none of these epitopes have been described before. Interestingly, of those that had been described, most were identified by mass spectrometry based immunopeptidome screening studies[38, 39]. Although others have identified HLA-binding *cis*-spliced epitopes by bioinformatic approaches [11, 61], few have addressed the functional immunogenicity of the identified epitopes – *i.e.* can a T lymphocyte recognise and respond to it? Importantly, we demonstrated functional immunogenicity of 6 (23%) of the *cis*-spliced epitopes, which represents a significant advance in the field since it doubles the number of known immunogenic cancer *cis*-spliced peptides and validates a platform to identify these going forward.

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Figure legends:

Figure 1: Experimental Workflow & consensus-binding motifs

(A) Peptides presented by HLA class I molecules were isolated from melanoma cells, prior to or after treatment with IFNγ, by affinity chromatography using the antibodies DT9 and W632. The peptides were fractionated by RP-HPLC and analyzed on a QExactive Plus mass spectrometer (Thermo Scientific) operated in data-dependent acquisition (DDA) mode. Peptide sequences were obtained using PEAKS 8.5 (Bioinformatics Solutions) in combination with "Hybrid finder" to determine possible *cis*-spliced peptides[14] and a spectral library was created in Spectronaut 11 Pulsar (Biognosys). To identify quantitative differences in the immunopeptidome prior to or after treatment with IFNγ, the remaining DT9 and W632 eluates were pooled and analyzed by data-independent acquisition (DIA) mass spectrometry on a QExactive Plus mass spectrometer (Thermo Scientific). Data were imported and analyzed with Spectronaut 11 Pulsar (Biognosys).

(B) During proteolysis, the proteasome generates short linear peptides through hydrolysis of the peptide bonds. These peptides directly match the proteome. Alternatively, transpeptidation reactions can generate peptides which do not have a template in the genome. Such spliced peptides could originate from one (*cis*-spliced) or two (trans-spliced) distinct proteins.

(C) Consensus-binding motifs were generated for the identified linear and cis-spliced nonameric peptides. Note, de-novo sequencing cannot distinguish between leucine and isoleucine and therefore, L represents either a leucine or an isoleucine residue in case of the *cis*-spliced peptide motif.

Figure 2: Exposure to IFNγ largely alters the presented immunopeptidome

(A) The Venn diagram shows the overlap in the peptide identifications between the 2 analyzed conditions (prior to or after treatment with IFNγ).

(B) The log2 fold changes of all linear and cis-spliced peptides are shown as violin plots.

Figure 3: Identified cancer-specific peptides

(A) The number of identified linear or *cis*-spliced peptides derived from cancer-related proteins are shown. (B) The number of novel or previously described peptides identified, and the cancer-related proteins they are derived from is shown. (C) The number of previously reported, novel, or *cis*-spliced, cancer specific peptides were graphed based on their presence in IFNγ treated or untreated samples exclusively, or in both conditions.

Figure 4: Immunogenicity of identified melanoma-associated epitopes

Selected melanoma-associated peptides were pooled and incubated at 10 µM final concentration with PBMC from healthy donors (n=4) or melanoma patients (n=6) for 10 days in presence of IL-2. All patients were HLA-A2^{+}, with the exception of Melanoma patient 6, which was a HLA-A2^{-} control. On day 10, CD8^{+} T lymphocytes were re-stimulated with the individual peptides, or pools of those peptides which were up/down regulated or unchanged with IFN as indicated, in presence of brefeldin A. Cells were labeled with fluorescent antibodies for surface (CD3, CD8) and intracellular (TNFα) proteins, and analysed by flow cytometry. Data show the percentage of TNFα^{+} CD8^{+} T lymphocytes in response to each peptide combined (A) or as individual values (B). Representative plots from melanoma patient 2 are shown for negative (DMSO) and positive (FEC) controls, and 2 positive and 1 negative

peptide responses (C). * denotes peptides with the highest *in silico* predicted immunogenicity.

Figure 5: Immunoproteasome expression is associated with survival benefit in melanoma patients

Using the TCGA-SKCM and FM-AD datasets looking at nevi and melanomas, the top and bottom quartiles of samples expressing IP or cP subunits were plotted on a Kaplan Meier survival curve (www.onclnk.org).

Figure 6: Immunogenicity of cis-spliced peptide pools

PBMCs from 5 melanoma patients and 4 healthy donors (HD) were stimulated with pooled peptides (n=8-9) for 10 days in the presence of Il-2. Cells were re-stimulated for 8 hours in the presence of BFA and TNFα expression measured by ICS. DMSO and FEC served as negative or positive control respectively (A). Example of gating strategy and results for patient 2 (B).

Figure 7: Immunogenic cis-spliced peptides are HLA-A2 binders

Sequence of peptides in each pool (A).PBMCs from the same patients and donors pre-stimulated with the pooled peptides as in Figure 6 were re-stimulated with single peptides from the same pool after 12 days for 8 hours in the presence of BFA and TNFα expression measured by ICS. DMSO and FEC served as negative or positive control respectively (B). All peptides that stimulated $CD8^+$ T cells as measured by TNFα to a higher degree than DMSO in at least 1 patient plus some randomly picked cis-spliced peptides were subjected to HLA-A2 stabilization assays on T2 cells as described in M&M. None HLA-A2 binding peptides (B7) or the Melan-A modified

HLA-A2 epitope served as negative and positive control respectively.* denotes immunogenic peptides.

Supplementary Figures:

Supplementary Figure 1: Peptide length

(A) A pie chart showing the proportion of linear vs. *cis*-spliced peptides. Approx. 6% of the identified 30,120 peptides represent *cis*-spliced peptides. (B) Length distribution of the identified linear and *cis*-spliced peptides.

Supplementary Figure 2: iRT retention time predictor

(A) The table lists the 11 iRT peptides including their published iRT values [27]. For each iRT peptide, the observed retention time (in minutes) was averaged across the 4 DDA runs and the corresponding standard deviated was calculated.

(B) The graph shows the correlation between the published iRT values of the 11 iRT peptides[27] and their averaged retention times (in minutes) across the 4 DDA runs. As expected, a very strong, linear correlation (R^2 = 0.9824) was observed. Error bars represent standard deviations.

(C) The graph shows the correlation between the published iRT values of the 11 iRT peptides[27] and their observed retention times (in minutes) in the 2 DIA runs.

Supplementary Figure 3: Multiple charge states

Independent quantitative information was obtained for 439 peptides, which had both doubly and triply charged precursor ions. To assess the quantitative accuracy of our dataset, we plotted for each peptide the obtained log2 fold change of each charge state, which resulted in near identical quantitative results.

Supplementary Figure 4

Using the TCGA-SKCM and FM-AD datasets looking at nevi and melanomas, the top and bottom quartiles of samples expressing IP or cP subunits, and CD3g were identified, and the top quartile of the CD3g expressing samples were removed. The remainder of the top and bottom quartiles of samples expressing IP or cP subunits were plotted on a Kaplan Meier survival curve (astatsa.com/LogRankTest/).

Supplementary Tables:

Table 1: All linear and cis-spliced peptides identified

Table 2: NetMHCpan predicted class I binders from LM-MEL-44 HLAs

Table 3: mutated peptides based on LM-MEL-44 sequencing data

Table 4: DIA-MS quantified 9799 peptides

Table 5: 96 MAAs

Table 6: linear peptides tested in immunogenicity assay

FIGURE 1

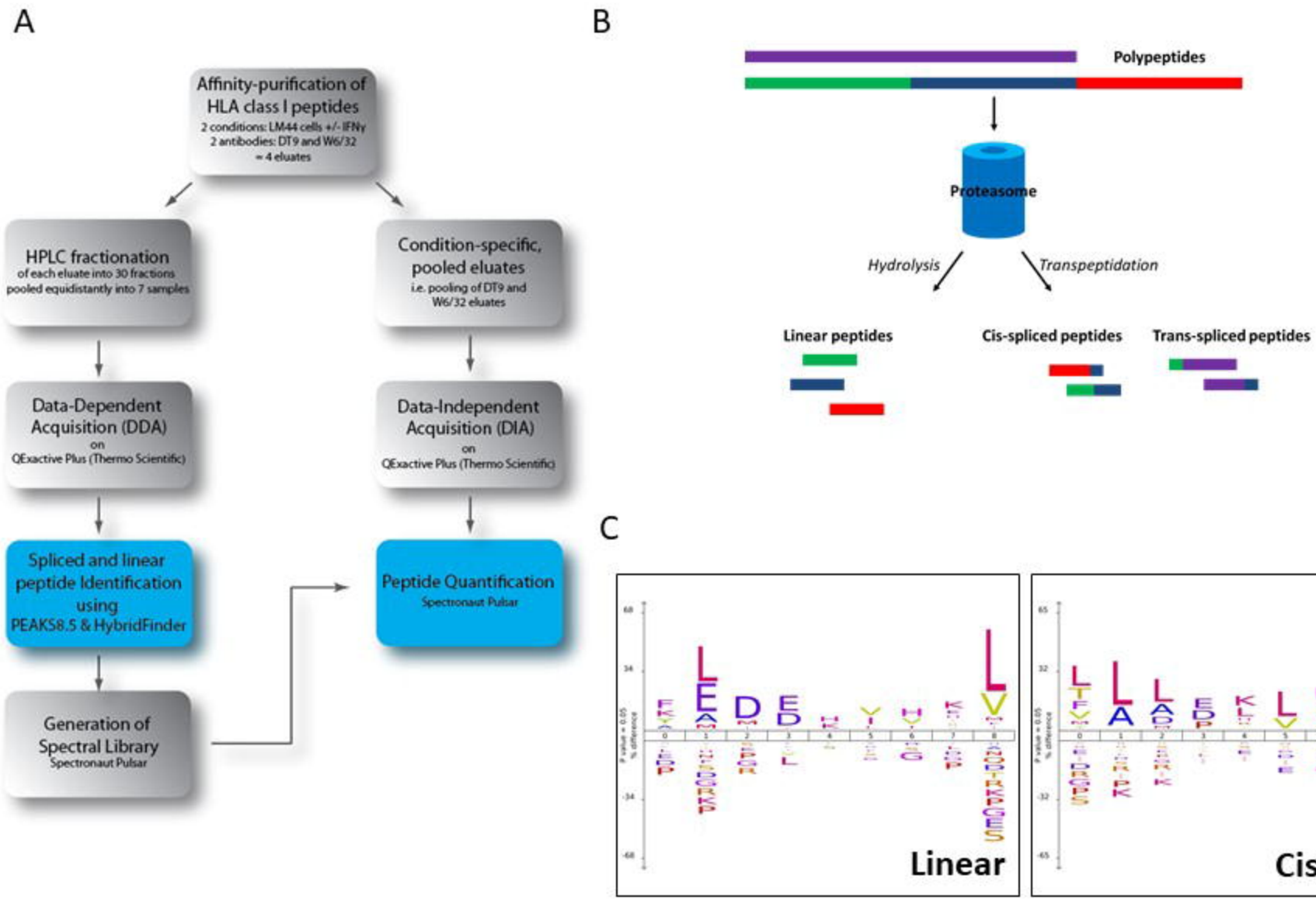
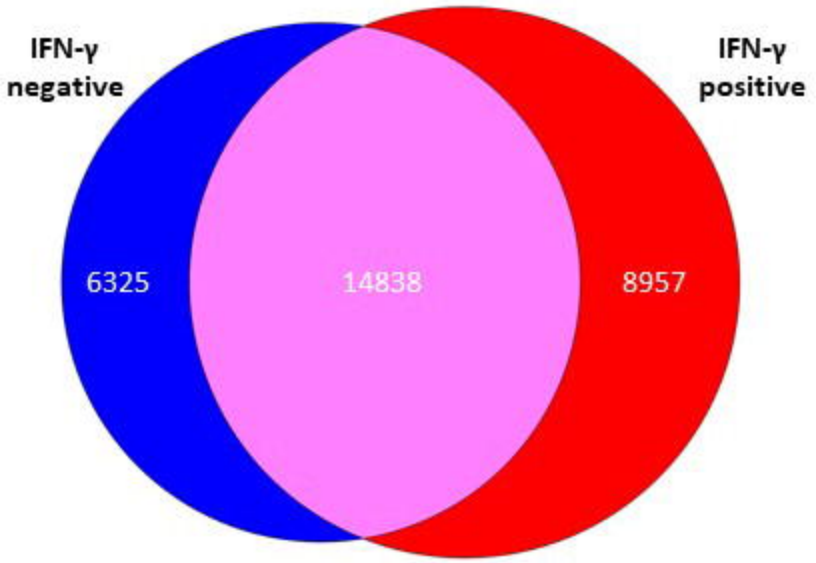

A
Affinity-purification of HLA class I peptides
2 conditions: LM44 cells +/- IFNγ
2 antibodies: DT9 and W6/32
= 4 eluates
HPLC fractionation
of each eluate into 30 fractions
pooled equidistantly into 7 samples
Condition-specific, pooled eluates
i.e. pooling of DT9 and W6/32 eluates
Data-Dependent Acquisition (DDA)
on
QExactive Plus (Thermo Scientific)
Data-Independent Acquisition (DIA)
on
QExactive Plus (Thermo Scientific)
Spliced and linear peptide Identification using
PEAKS8.5 & HybridFinder
Peptide Quantification
Spectronaut Pulsar
Generation of Spectral Library
Spectronaut Pulsar
B
Polypeptides
Proteasome
Hydrolysis
Transpeptidation
Linear peptides
Cis-spliced peptides
Trans-spliced peptides
C
Linear
Cis-spliced

FIGURE 2

A


IFN-γ negative
IFN-γ positive
6325
14838
8957

B

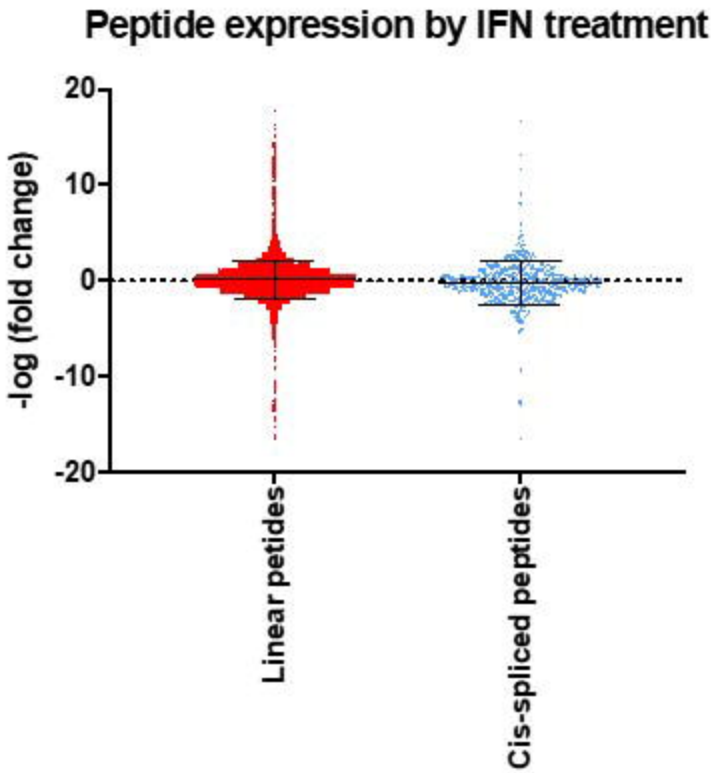

Peptide expression by IFN treatment
-log (fold change)
20
10
0
-10
-20
Linear petides
Cis-spliced peptides

Figure 3

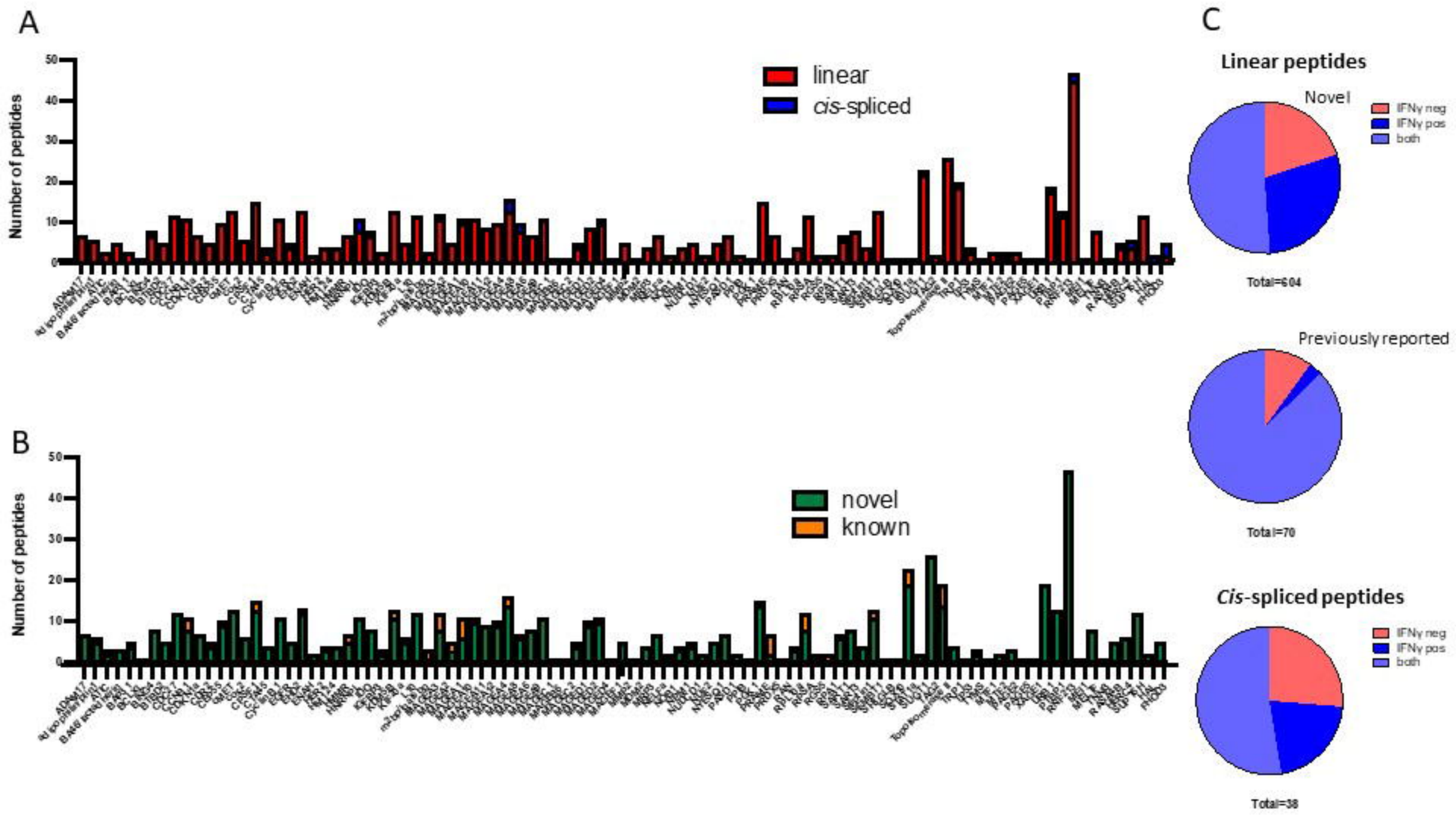

A
Number of peptides
linear
cis-spliced
B
Number of peptides
novel
known
C
Linear peptides
Novel
IFNγ neg
IFNγ pos
both
Total=604
Previously reported
Total=70
Cis-spliced peptides
IFNγ neg
IFNγ pos
both
Total=38

Figure 4

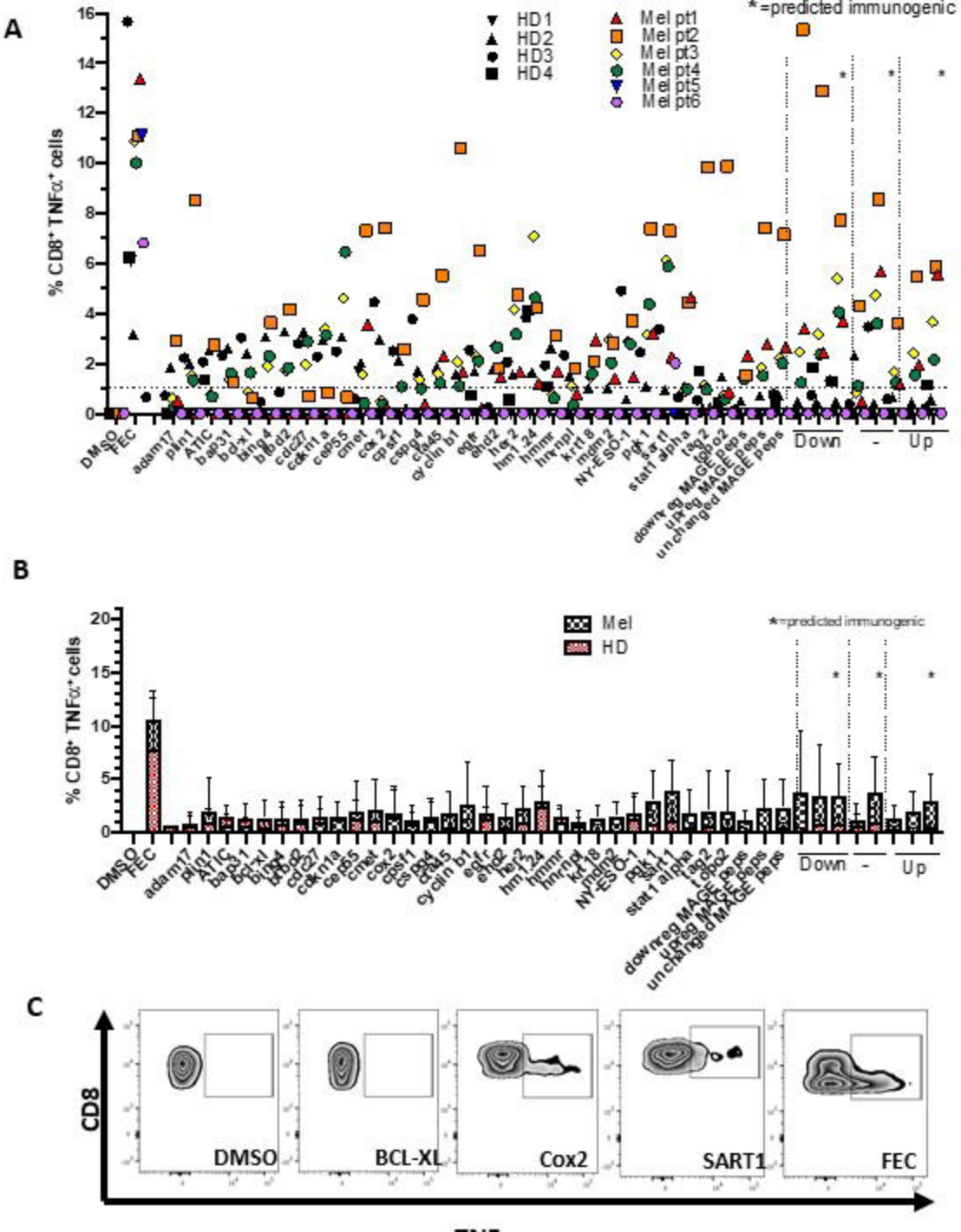

A
HD1
HD2
HD3
HD4
Mel pt1
Mel pt2
Mel pt3
Mel pt4
Mel pt5
Mel pt6
*=predicted immunogenic
% CD8+ TNFα+ cells
DMSO
FEC
Down
-
Up
B
Mel
HD
*=predicted immunogenic
% CD8+ TNFα+ cells
C
CD8
TNFα
DMSO
BCL-XL
Cox2
SART1
FEC

Figure 5

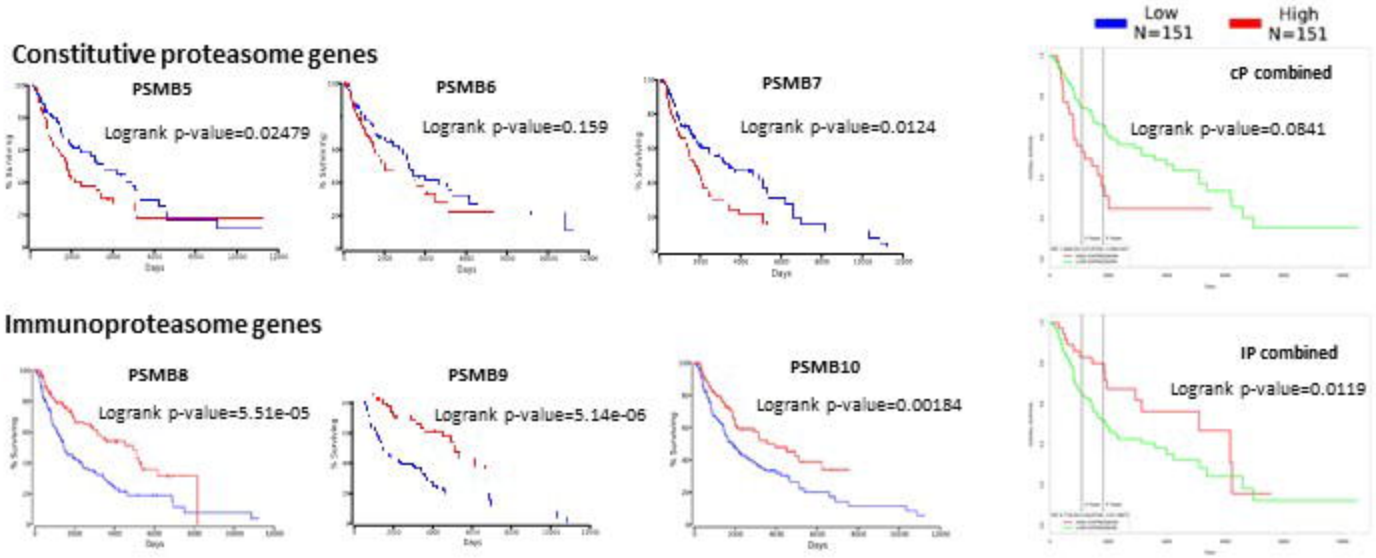

Constitutive proteasome genes
PSMB5
Logrank p-value=0.02479
PSMB6
Logrank p-value=0.159
PSMB7
Logrank p-value=0.0124
Low N=151
High N=151
cP combined
Logrank p-value=0.0841
Immunoproteasome genes
PSMB8
Logrank p-value=5.51e-05
PSMB9
Logrank p-value=5.14e-06
PSMB10
Logrank p-value=0.00184
IP combined
Logrank p-value=0.0119

Figure 6

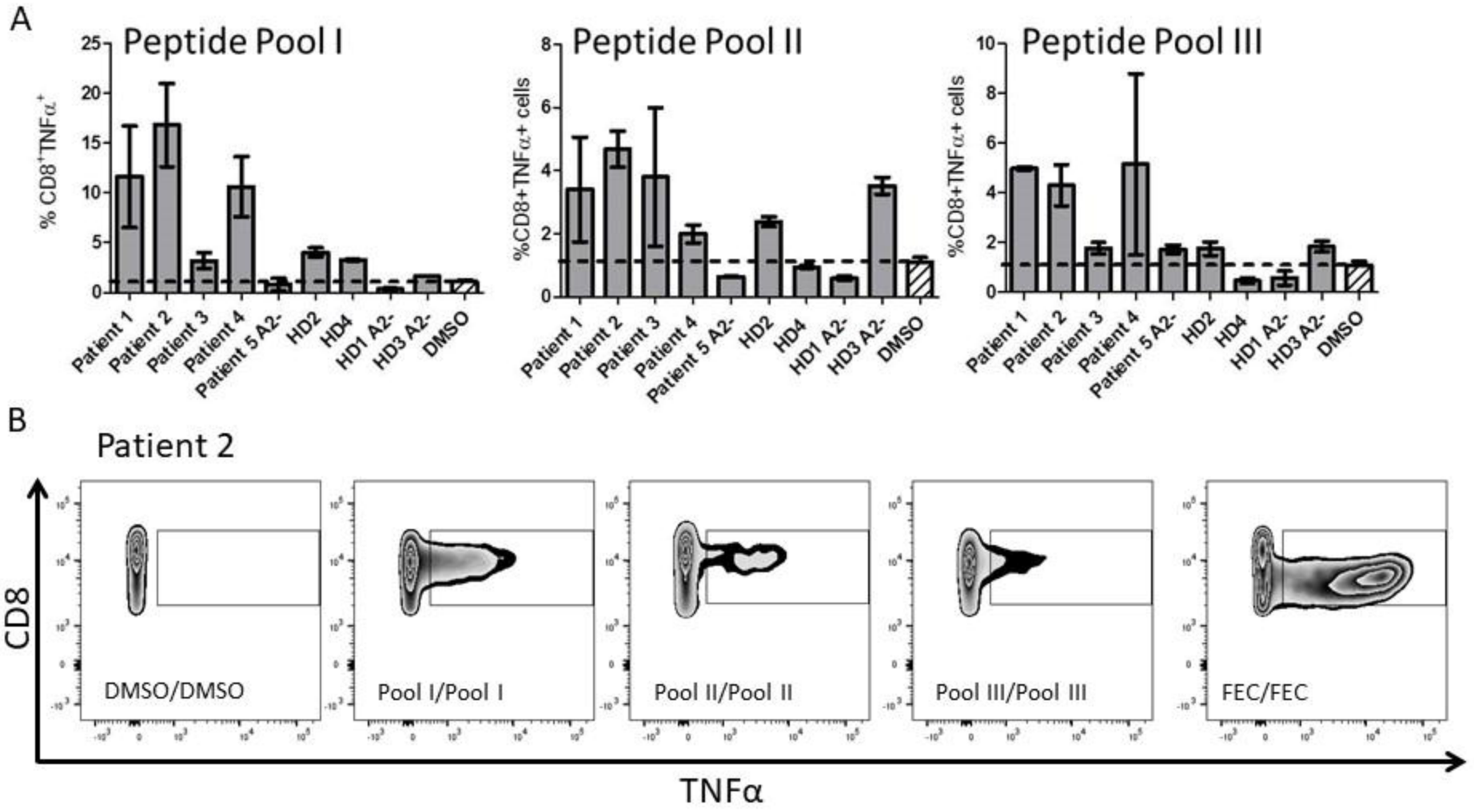

A
Peptide Pool I
% CD8+TNFα+
Peptide Pool II
%CD8+TNFα+ cells
Peptide Pool III
%CD8+TNFα+ cells
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5 A2-
HD2
HD4
HD1 A2-
HD3 A2-
DMSO
B
Patient 2
CD8
TNFα
DMSO/DMSO
Pool I/Pool I
Pool II/Pool II
Pool III/Pool III
FEC/FEC

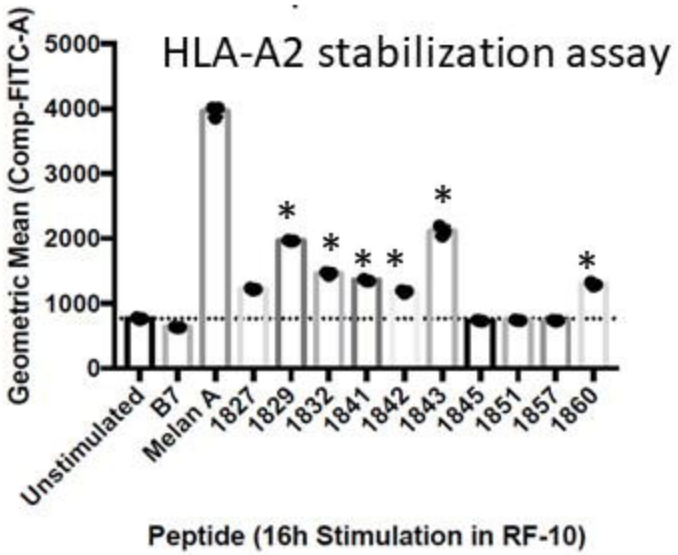
Figure 7

A

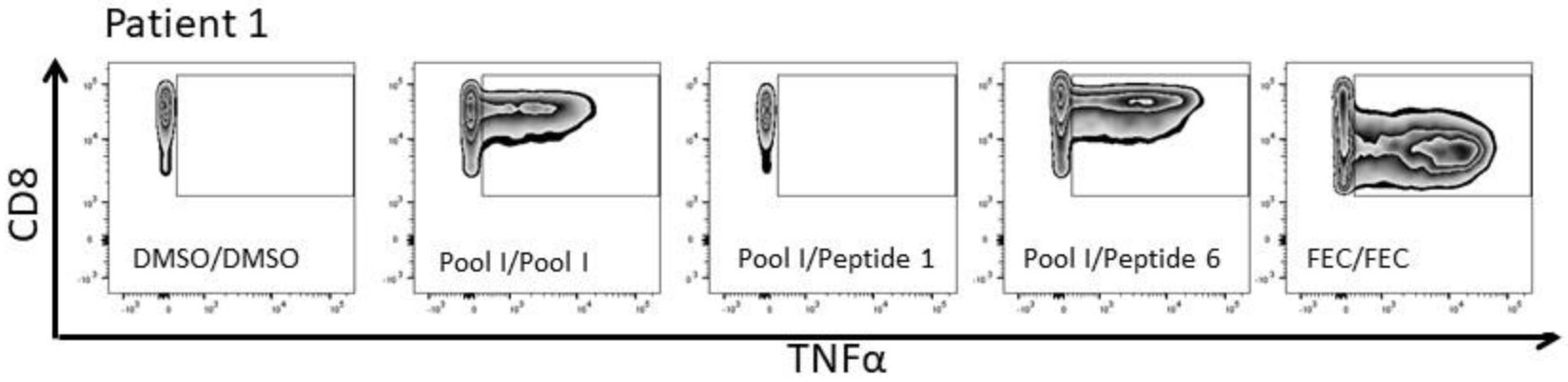
Pool I	Pool II	Pool III
FIMDKHFSV	FIIHPLLL	VLDEVVVH
FLWEILERL	VALEHVVRV	VTDFLSHL
KLLILELHV (1829)*	LLSLLIPAL (1841)*	LLALRILSL
LIASFLDKV	LLSLLLPAI (1842)*	LLAIRLISI
AIMTAVVKI	ILSLILPAL (1843)*	LLALRLLSL
LILGLLTKV (1832)*	LLSLLLPAL	LLLPLHEVL
VLTDILHTL	LAIQLKTLL	LLLEALEQL (1860)*
KLTSLNIKV	EEVPAAESRKY	LVPPPPPLL
MLTEKQHLL	VADLQRTL	

*denotes immunogenicity based on $CD8^+/TNF\alpha^+$ in at least 1 assay

C

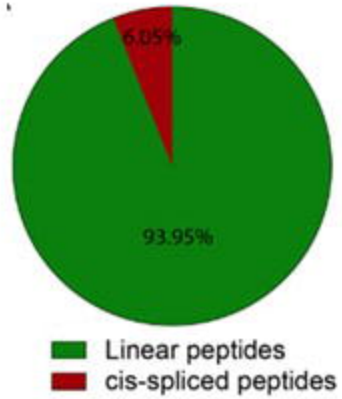


B

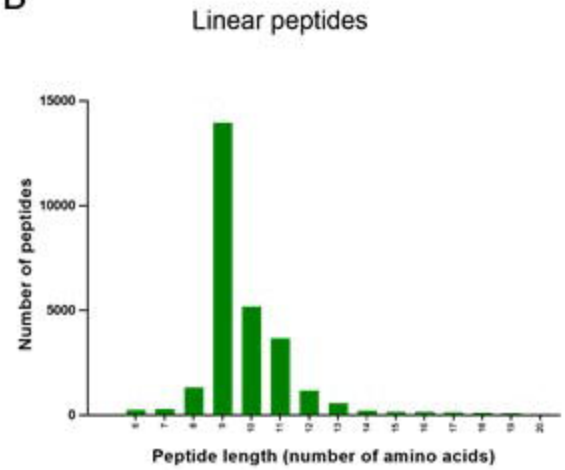


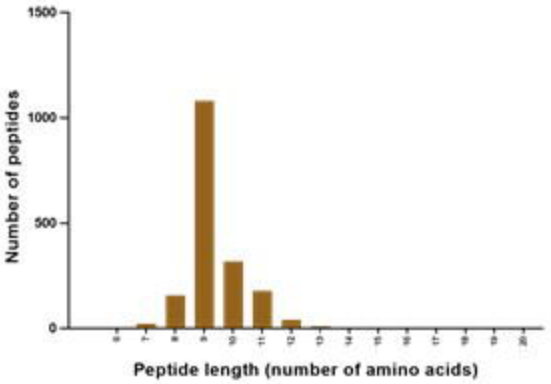
SUPPLEMENTARY FIGURE 1

A



B





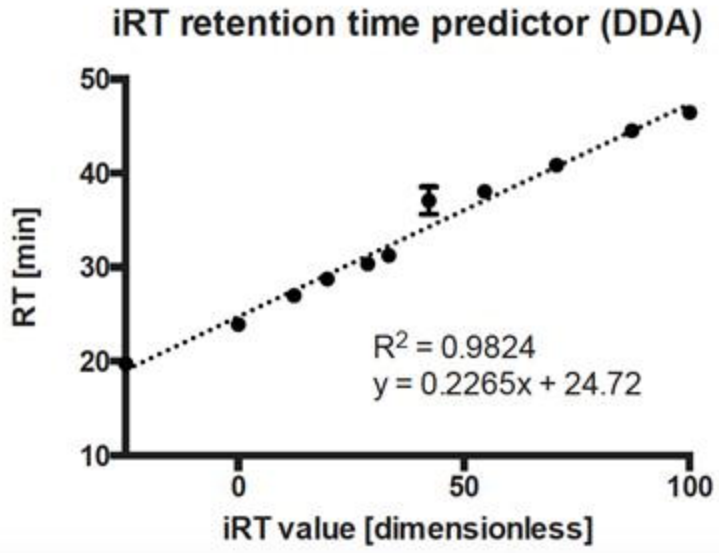
SUPPLEMENTARY FIGURE 2

A

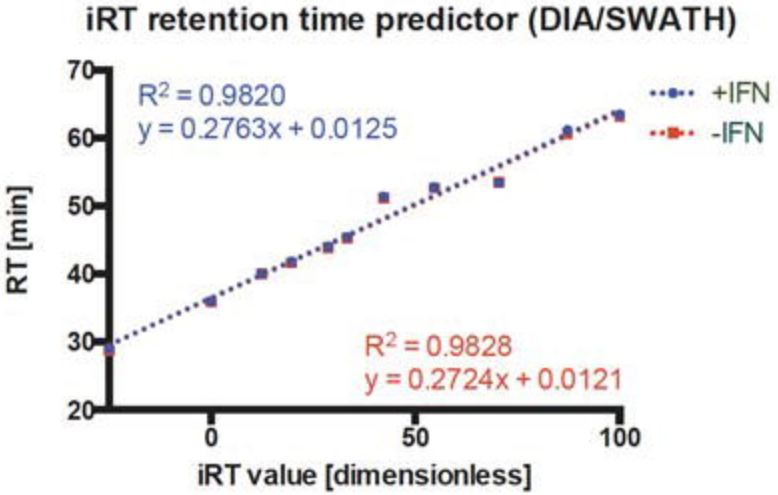
iRT retention time predictor

Peptide	Sequence	iRT value[1]	RT [min]	StDev
iRT-A	LGGNEQVTR	-24.92	19.76	0.11
iRT-B	GAGSSEPVTGLDAK	0.00	23.91	0.13
iRT-C	VEATFGVDESNAK	12.39	26.99	0.05
iRT-D	YILAGVENSK	19.79	28.76	0.05
iRT-E	TPVISGGPYEYR	28.71	30.36	0.05
iRT-F	TPVITGAPYEYR	33.38	31.25	0.03
iRT-G	DGLDAASYYAPVR	42.26	37.09	1.47
iRT-H	ADVTPADFSEWSK	54.62	38.04	0.2
iRT-I	GTFIIDPGGVIR	70.52	40.85	0.0
iRT-K	GTFIIDPAAVIR	87.23	44.51	0.0
iRT-L	LFLQFGAQGSPFLK	100.00	46.46	0.0

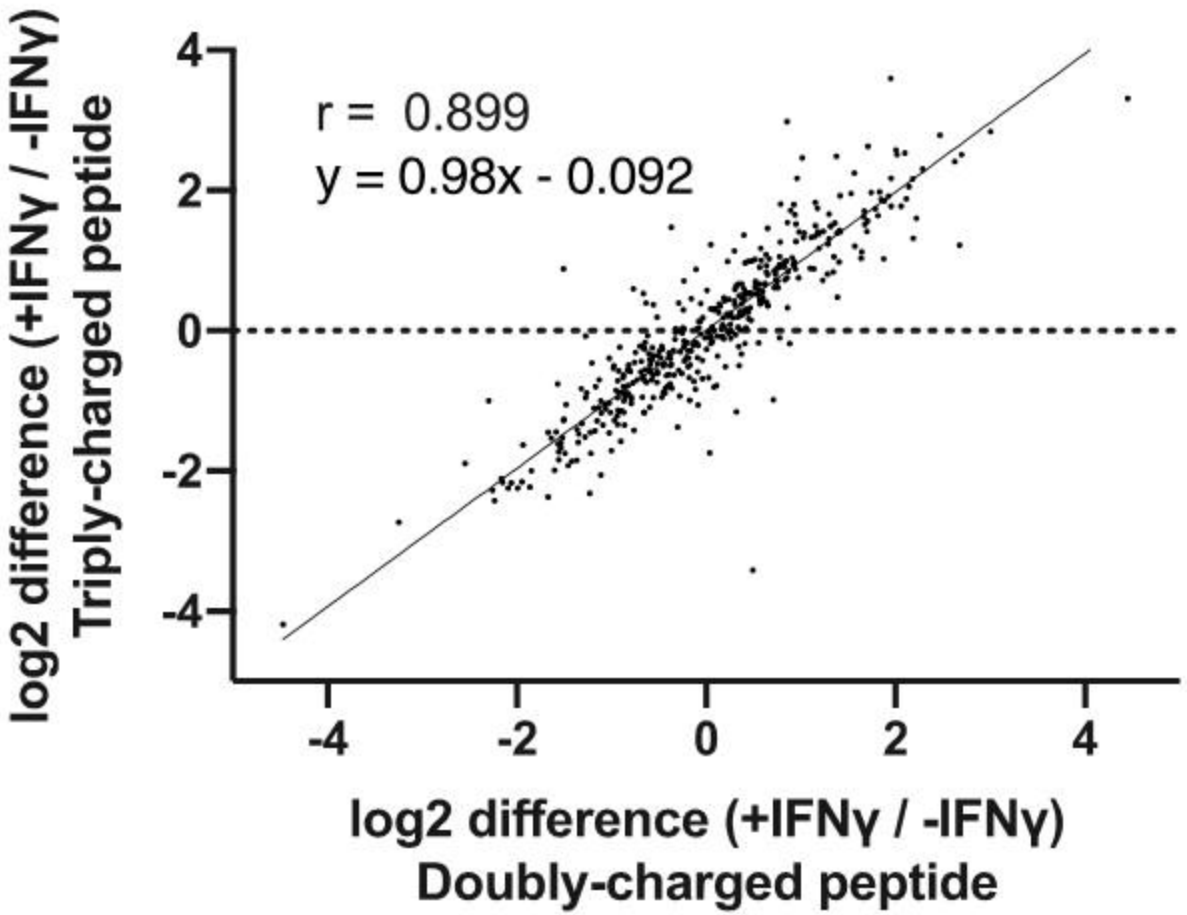
B



C



Supplementary Figure 3



Supplementary Figure 4