

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 Chen⁶, Ritchlynn Aranha³, Nathan P. Croft³, Divya Duscharla³, Chen Li^{3,7}, Rochelle Ayala³, Jonathan Cebon^{1,2}, Anthony W. Purcell^{3*}, Ralf B. Schittenhelm^{3,8*}, Andreas Behren^{1,2*}

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

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

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

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

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

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

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

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

⁹These authors contributed equally

*corresponding authors

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

2 anthony.purcell@monash.edu

3

4 **Running title:** Defining the immunogenic components of the melanoma

5 immunopeptidome.

6

7 **Keywords:** Immunopeptidome, Immunoproteasome, transpeptidation, post-
8 translational splicing, Melanoma, Antigen processing, IFN γ , Inflammation, HLA,
9 Immunotherapy, DIA/SWATH.

10

11 **Summary**

12 Antigen-recognition by CD8⁺ T cells is governed largely by the pool of peptide
13 antigens presented on the cell surface in the context of MHC class I complexes.
14 Recent studies have shown not only a high degree of plasticity in the
15 immunopeptidome, but also that a considerable fraction of all presented peptides are
16 generated through proteasome-mediated splicing of non-contiguous regions of
17 proteins to form novel peptide antigens. Here we used high-resolution mass-
18 spectrometry combined with new bioinformatic approaches to characterize the
19 immunopeptidome of melanoma cells in the presence or absence of interferon- γ . In
20 total, we identified 30,120 peptides and demonstrate that interferon- γ induces marked
21 changes in the peptidome (with an overlap of only 49.3% between conditions as
22 revealed by data independent acquisition mass spectrometry). Moreover, around 6%
23 (1,821) of the peptides were identified as *cis*-spliced peptides with 712 derived from
24 known melanoma-associated antigens. Several of these peptides were shown to be
25 immunogenic in unrelated melanoma patients. These observations highlight the

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

4

5 **Funding support:** This project was funded in part by Ludwig Cancer Research,
6 Melanoma Research Alliance (MRA), the Victorian Cancer Agency supported
7 Melbourne Melanoma Project (MMP), Australian National Health and Medical
8 Research Council (NHMRC) Project Grants 1007381 and 1165490 (to AWP), and the
9 Victorian State Government Operational Infrastructure Support Program. JC was
10 supported by Australian NHMRC Practitioner Fellowship 487905. AWP is supported
11 by NHMRC Principal Research Fellowship 1137739. AB is the recipient of a
12 Fellowship from the Victorian Government Department of Health and Human
13 Services acting through the Victorian Cancer Agency.

14

15 **Conflict of Interest Statement:** The authors declare no conflict of interest.

16

1 **Introduction:**

2 Antigen recognition by cytotoxic T cells and subsequent tumour cell destruction is the
3 key component underlying cancer immunotherapy strategies. Its importance has been
4 widely demonstrated, and loss-of-function of elements in the antigen processing and
5 presentation pathways has been shown to confer therapeutic resistance[1]. Correlative
6 findings point to neo-antigens arising from tumour mutations as an important source
7 of immunogenic antigens in the context of melanoma and other cancers[2].
8 Nonetheless, tumours with low mutational burdens can respond to checkpoint
9 inhibitor therapy, and the presence of a high tumour-mutational load does not
10 necessarily correspond to the efficacy of treatment[3]. HLA-presented peptides (*p*-
11 HLA-I) arising from the mutant proteins are mostly heterogeneously expressed and
12 additionally determined by the patient-specific HLA-subtypes, making predictions
13 about their presentation and immunogenicity unreliable. While a number of recent
14 studies have reported the utility of mass spectrometry combined with exome
15 sequencing in identifying HLA-presented peptides derived from mutated proteins[4-
16 6], the analysis of the contribution of mutational neo-antigens to the overall tumour
17 immunogenicity remains complicated and unresolved.

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

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

responses to pools of IFN γ 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 μ g/ml Streptomycin and 10% heat-inactivated fetal calf serum (all Invitrogen). For induction of immunoproteasome catalytic subunits cells were incubated with 100 ng/ml IFN γ (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

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

3

4 *Isolation of peptides bound to HLA class I molecules*

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

12

13 *Fractionation of HLA-bound peptides by reversed-phase high-performance liquid* 14 *chromatography (RP-HPLC)*

15 The HLA-peptide eluates were loaded onto a 4.6 mm internal diameter x 50 mm
16 monolithic C18 RP-HPLC column (Chromolith Speed Rod; Merck) at a flow rate of 1
17 ml/min using an EttanLC HPLC system (GE Healthcare) with buffer A (0.1%
18 trifluoroacetic acid (TFA)) and buffer B (80% ACN / 0.1% TFA) as mobile phases.
19 The bound peptides were separated from the class I heavy chains and β 2m molecules
20 using increasing concentration of buffer B. Peptide-containing fractions (500 μ l) were
21 collected, vacuum concentrated to ~5 μ l and reconstituted to 15 μ l with 0.1 % formic
22 acid (FA). Indexed retention time (iRT) peptides[27] were spiked in for retention time
23 alignment.

24

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

2 Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate
3 3000 RS autosampler, the samples were loaded via an Acclaim PepMap 100 trap
4 column (100 µm x 2 cm, nanoViper, C18, 5 µm, 100Å; Thermo Scientific) onto an
5 Acclaim PepMap RSLC analytical column (75 µm x 50 cm, nanoViper, C18, 2 µm,
6 100Å; Thermo Scientific). The peptides were separated by increasing concentrations
7 of 80% ACN / 0.1% FA at a flow of 250 nl/min for 65 min and analyzed with a
8 QExactive Plus mass spectrometer (Thermo Scientific). In each cycle, a full ms1 scan
9 (resolution: 70.000; AGC target: 3e6; maximum IT: 120 ms; scan range: 375-1800
10 m/z) preceded up to 12 subsequent ms2 scans (resolution: 17.500; AGC target: 1e5;
11 maximum IT: 120 ms; isolation window: 1.8 m/z; scan range: 200-2000 m/z; NCE:
12 27). To minimize repeated sequencing of the same peptides, dynamic exclusion was
13 set to 15 sec and the 'exclude isotopes' option was activated.

14

15 *Quantification of HLA bound-peptides using data-independent acquisition mass* 16 *spectrometry (DIA-MS)*

17 The identical instrument setup as described above (Dionex UltiMate 3000 LC system
18 coupled to a QExactive Plus mass spectrometer) was used to quantify HLA-bound
19 peptides using data-independent acquisition (DIA). In each cycle, 28 sequential DIA
20 windows with an isolation width of 25 m/z between 300 - 1000 Da were acquired
21 (resolution: 17.500; AGC target: 2e5; maximum IT: auto; NCE: 27) following a full
22 ms1 scan (resolution: 70.000; AGC target: 3e6; maximum IT: 55 ms; scan range: 300-
23 1000 m/z). A 90 min gradient of increasing concentrations of 80% ACN / 0.1% FA
24 was used to separate the peptides for the DIA acquisition.

25

1 *DDA data analysis*

2 Linear and cis-spliced peptide sequences were identified as described previously[14].

3 In brief, the acquired .raw files were searched with PEAKS Studio 8.5

4 (Bioinformatics Solutions) against the human UniProtKB/TrEMBL

5 UniProtKB/SwissProt database (v2017_10). The parent mass error tolerance was set

6 to 10 ppm and the fragment mass error tolerance to 0.02 Da. Oxidation of M,

7 deamidation of N & Q and phosphorylation of S, T & Y were set as variable

8 modifications and a FDR cutoff of 1% was applied. High confidence *de-novo* peptide

9 sequences without any linear peptide match in the provided database were further

10 interrogated with the “Hybrid finder” algorithm[28] and the identified *cis*-spliced

11 peptide sequences added back to the original UniProtKB/SwissProt database and all

12 data researched using PEAKS DB . Linear and spliced peptides in this search were

13 extracted at 1% FDR to create the final list of identified peptides.

14

15 *DIA data analysis*

16 Spectronaut 11 Pulsar (Biognosys) was used to generate a spectral library, which

17 contained information of all linear and *cis*-spliced HLA class I peptides identified in

18 this study, as well as to quantify peptides within the all DIA dataset.

19

20 *T cell stimulation assay*

21 To assess T cell responses selected peptides were synthesised (Mimotopes, VIC,

22 Australia). PBMC from healthy donors (Australian Red Cross, VIC, Australia), or

23 melanoma patients (Austin Health HREC approved protocol HREC H2006/02633)

24 were purified by density centrifugation over Ficoll Hi-Paque. Cells were cultured in

25 TCRPMI consisting of RPMI 1640, 2mM Glutamax, 100 IU/ml Penicillin, 100 µg/ml

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

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

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

21

22 *HLA-A2 stabilisation assays*

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

1 flasks for 2 – 3 days before the assay. T2 cells (2×10^5 cells/well) were cultured for 16
2 h at 37 °C in 5% CO₂ in 200 µL RF-10 in 96-well U-bottomed plates in presence or
3 absence of 10 µg/ml of synthetic peptides. Peptides from Melan-A (modified aa26-35
4 ELA.....and aa 60-72) served as a positive or negative controls respectively. All
5 peptides were tested in triplicate.

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

11

1 **Results:**

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

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

13 We identified a total of 30,120 peptides (derived from 12,025 source proteins)
14 presented across all HLA class I allotypes expressed by the LM-MEL-44 cells at a
15 false discovery rate (FDR) of 1% (Supplementary Table 1). Approximately 6% of
16 these peptides (1,821 peptides) were assigned as *cis*-spliced in origin (Fig 1b,
17 Supplementary Fig 1a, Supplementary Table 1), being derived from non-contiguous
18 sequences of the same protein. While *trans*-splicing has been described to contribute
19 to the cellular immunopeptidome as well[14], we have here focused on *cis*-spliced
20 peptides only due to the availability of appropriate bioinformatics workflows. This
21 proportion of peptides of *cis*-spliced origin is in agreement with our previous
22 study[14] and others [30, 31]. As expected for HLA class I epitopes, the majority of
23 peptides (27,321 peptides; ~90%) were between 8-12 amino acids in length with no
24 apparent difference between linear and *cis*-spliced sequences (Supplementary Figure
25 1b). Using the NetMHCpan binding algorithm[32], 91.2% of these 8-12mers were

1 predicted to bind to at least one of the HLA class I alleles expressed on the surface of
2 the LM-MEL-44 cells (A*02:01,B*60/*44:02,C*03:04/*05:01)[33] suggesting that
3 the majority of the identified peptide sequences can be considered genuine HLA class
4 I ligands (Supplementary Table 2). The percentage of predicted binders was lower for
5 *cis*-spliced peptides compared to linear peptides (68.5% vs. 92.9%), which can be
6 attributed to the fact that NetMHCpan and other binding algorithms have been
7 developed and based on consensus-binding motifs of linear epitopes, which can differ
8 slightly from consensus-binding motifs of *cis*-spliced peptides[13, 14]. Indeed, similar
9 marginal differences between the consensus-binding motifs of linear and *cis*-spliced
10 epitopes are also evident in this dataset, which are more pronounced at the *N*-terminal
11 anchor positions P2 / P3 compared to the *C*-terminal PΩ position (Figure 1c).
12 Taken together, we identified more than 30,000 predominantly novel HLA class I
13 ligands presented on the surface of the LM-MEL-44 melanoma cells including nearly
14 2,000 *cis*-spliced peptides, which have not been described before. Of note, none of the
15 mutational neoantigens predicted based on exome sequencing data of LM-MEL-
16 44[34] were identified in our dataset (Supplementary table 3); however this cell line
17 has a relatively low mutational load.
18
19 *IFNγ*-treatment leads to significant changes in the MHC class I presented peptide
20 pool. Considering the well-described clinical relevance of so called “hot” versus
21 “cold” tumour microenvironments and previous work demonstrating the influence of
22 cytokine exposure on antigen-presentation pathways[10], we wanted to examine on a
23 global scale the impact of IFNγ exposure on the immunopeptidome. An initial
24 qualitative analysis revealed that only 49.3% of the 30,120 peptides were identified
25 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 γ -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 γ . To determine their degree of variation, we calculated their log₂ fold changes (+IFN γ /- IFN γ) 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 γ 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.

24

1 *Identification of novel cancer specific peptides in the melanoma immunopeptidome.*

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

13

14 *CD8⁺ T lymphocytes frequently recognised novel linear melanoma-specific epitopes.*

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

1 melanoma patient 2. We found that CD8⁺ T lymphocyte responses to the tested
 2 peptides were more frequently seen in melanoma patients (Figure 4a (individual) and
 3 4b (pooled donors)). Novel peptides derived from 15 of the melanoma antigens
 4 identified in our screen stimulated specific CD8⁺ T lymphocyte responses (over 2%
 5 TNFα⁺ cells) in three or more donors, demonstrating novel, functional, melanoma T
 6 cell epitopes (highlighted in Supplementary Table 6, Representative examples, Figure
 7 4c). The strongest responses were induced by the peptides derived from SART1
 8 (U4/U6.U5 tri-snRNP-associated protein 1) and PGK1 (Phosphoglycerate kinase 1),
 9 both of which stimulated responses in 2-7% of T lymphocytes from 4 melanoma
 10 patients. When the same selection of peptides were pooled in groups of those up/down
 11 regulated, or unchanged following IFNγ treatment no appreciable difference in
 12 functional immunogenicity in melanoma patients was observed between groups.
 13 Interestingly however, T lymphocyte responses to pools of IFNγ upregulated peptides
 14 were not seen in healthy donors. One pool in each group was made on the basis of
 15 higher *in silico*-predicted immunogenicity (www.iedb.org[40], Figure 4a,b, asterisks).
 16 However, these groups did not display enhanced ability to activate CD8⁺ T
 17 lymphocytes in either melanoma patients or healthy donors.

18

19 *Melanoma patients expressing immunoproteasome genes have a survival advantage.*

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

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

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

18

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

21 The potential implications of the presence of spliced peptides for all facets of
22 immunity have sparked intense discussions in the last 2 years[14, 42-44]. In cancer,
23 their presence would dramatically widen the repertoire of potentially targetable
24 epitopes and may allow for many more tumour-specific antigens (including
25 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 TNF α 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-

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

3

4 **Discussion:**

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

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

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

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

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

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

3 The identification of spliced peptides as tumour antigens in cancer was first described
4 in 2004 in both the FGF5 protein in renal cancer[45] and the gp100 protein in
5 melanoma[46], and since then only a further 4 *cis*-spliced peptides have been
6 described in cancer[42]. To date their identification has been hampered by lack of
7 feasible identification methods. In this study, we have used high-resolution mass
8 spectrometry to identify more than 30,000 endogenous HLA class I peptide ligands.
9 Approximately 2,000 (6%) of these peptides represent *cis*-spliced peptides
10 demonstrating that *cis*-spliced peptides are commonly generated in cancer cells, which
11 broadens the repertoire of potential targets in cancer immunotherapy. Consistent with
12 our observations a recent study by Liepe *et al*[13] identified 750 and 486 *cis*-spliced
13 peptides in colon and breast cancer cell lines respectively – however their
14 immunogenicity was not described. Of note, 38 of our 2,000 *cis*-spliced peptides (and
15 712 of all identified peptides) were derived from melanoma-associated antigens, and
16 with the exception of a few linear epitopes, none of these epitopes have been
17 described before. Interestingly, of those that had been described, most were identified
18 by mass spectrometry based immunopeptidome screening studies[38, 39]. Although
19 others have identified HLA-binding *cis*-spliced epitopes by bioinformatic approaches
20 [11, 61], few have addressed the functional immunogenicity of the identified epitopes
21 – *i.e.* can a T lymphocyte recognise and respond to it? Importantly, we demonstrated
22 functional immunogenicity of 6 (23%) of the *cis*-spliced epitopes, which represents a
23 significant advance in the field since it doubles the number of known immunogenic
24 cancer *cis*-spliced peptides and validates a platform to identify these going forward.

25

References:

1. Cai, L., et al., *Defective HLA class I antigen processing machinery in cancer*. Cancer Immunol Immunother, 2018. **67**(6): p. 999-1009.
2. Parmiani, G., et al., *Unique human tumor antigens: immunobiology and use in clinical trials*. J Immunol, 2007. **178**(4): p. 1975-9.
3. Goodman, A.M., et al., *Tumor Mutational Burden as an Independent Predictor of Response to Immunotherapy in Diverse Cancers*. Mol Cancer Ther, 2017. **16**(11): p. 2598-2608.
4. Schumacher, T.N. and R.D. Schreiber, *Neoantigens in cancer immunotherapy*. Science, 2015. **348**(6230): p. 69-74.
5. Ott, P.A., et al., *An immunogenic personal neoantigen vaccine for patients with melanoma*. Nature, 2017. **547**(7662): p. 217-221.
6. Yadav, M., et al., *Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing*. Nature, 2014. **515**(7528): p. 572-6.
7. Vigneron, N. and B.J. Van den Eynde, *Proteasome subtypes and regulators in the processing of antigenic peptides presented by class I molecules of the major histocompatibility complex*. Biomolecules, 2014. **4**(4): p. 994-1025.
8. Dahlmann, B., *Mammalian proteasome subtypes: Their diversity in structure and function*. Arch Biochem Biophys, 2016. **591**: p. 132-40.
9. Yang, Y., et al., *Proteasomes are regulated by interferon gamma: implications for antigen processing*. Proc Natl Acad Sci U S A, 1992. **89**(11): p. 4928-32.
10. Javitt, A., et al., *Pro-inflammatory cytokines alter the immunopeptidome landscape by modulation of HLA-B expression*. Frontiers in Immunology, 2019. **10**(141).
11. Chong, C., et al., *High-throughput and Sensitive Immunopeptidomics Platform Reveals Profound Interferongamma-Mediated Remodeling of the Human Leukocyte Antigen (HLA) Ligandome*. Mol Cell Proteomics, 2018. **17**(3): p. 533-548.
12. Woods, K., et al., *Mismatch in epitope specificities between IFNgamma inflamed and uninflamed conditions leads to escape from T lymphocyte killing in melanoma*. J Immunother Cancer, 2016. **4**: p. 10.
13. Liepe, J., et al., *A large fraction of HLA class I ligands are proteasome-generated spliced peptides*. Science, 2016. **354**(6310): p. 354-358.
14. Faridi, P., et al., *A subset of HLA-I peptides are not genomically templated: Evidence for cis- and trans-spliced peptide ligands*. Sci Immunol, 2018. **3**(28).
15. Faridi, P., A.W. Purcell, and N.P. Croft, *In Immunopeptidomics We Need a Sniper Instead of a Shotgun*. Proteomics, 2018. **18**(12): p. e1700464.
16. Liepe, J., et al., *Mapping the MHC Class I-Spliced Immunopeptidome of Cancer Cells*. Cancer Immunol Res, 2019. **7**(1): p. 62-76.
17. Anaka, M., et al., *Stem cell media culture of melanoma results in the induction of a nonrepresentative neural expression profile*. Stem Cells, 2012. **30**(2): p. 336-43.
18. Behren, A., et al., *The Ludwig institute for cancer research Melbourne melanoma cell line panel*. Pigment Cell Melanoma Res, 2013. **26**(4): p. 597-600.
19. Wong, S.Q., et al., *Whole exome sequencing identifies a recurrent RQCD1 P131L mutation in cutaneous melanoma*. Oncotarget, 2015. **6**(2): p. 1115-27.
20. Li, H. and R. Durbin, *Fast and accurate short read alignment with Burrows-Wheeler transform*. Bioinformatics, 2009. **25**(14): p. 1754-60.

- 1 21. McKenna, A., et al., *The Genome Analysis Toolkit: a MapReduce framework*
2 *for analyzing next-generation DNA sequencing data*. Genome Res, 2010.
3 **20**(9): p. 1297-303.
- 4 22. DePristo, M.A., et al., *A framework for variation discovery and genotyping*
5 *using next-generation DNA sequencing data*. Nat Genet, 2011. **43**(5): p. 491-8.
- 6 23. Cibulskis, K., et al., *Sensitive detection of somatic point mutations in impure*
7 *and heterogeneous cancer samples*. Nat Biotechnol, 2013. **31**(3): p. 213-9.
- 8 24. Illing, P.T., et al., *Immune self-reactivity triggered by drug-modified HLA-*
9 *peptide repertoire*. Nature, 2012. **486**(7404): p. 554-8.
- 10 25. Croft, N.P., et al., *Kinetics of antigen expression and epitope presentation*
11 *during virus infection*. PLoS Pathog, 2013. **9**(1): p. e1003129.
- 12 26. Schittenhelm, R.B., et al., *Revisiting the arthritogenic peptide theory:*
13 *quantitative not qualitative changes in the peptide repertoire of HLA-B27*
14 *allotypes*. Arthritis Rheumatol, 2015. **67**(3): p. 702-13.
- 15 27. Escher, C., et al., *Using iRT, a normalized retention time for more targeted*
16 *measurement of peptides*. Proteomics, 2012. **12**(8): p. 1111-21.
- 17 28. Faridi, P., et al., *A subset of HLA-I peptides are not genomically templated:*
18 *Evidence for cis- and trans-spliced peptide ligands*. Science Immunology,
19 2018. **3**(28).
- 20 29. Prevosto, C., et al., *Allele-Independent Turnover of Human Leukocyte Antigen*
21 *(HLA) Class Ia Molecules*. PLoS One, 2016. **11**(8): p. e0161011.
- 22 30. Mylonas, R., et al., *Estimating the Contribution of Proteasomal Spliced*
23 *Peptides to the HLA-I Ligandome*. Mol Cell Proteomics, 2018. **17**(12): p.
24 2347-2357.
- 25 31. Rolfs, Z., et al., *Global Identification of Post-Translationally Spliced Peptides*
26 *with Neo-Fusion*. J Proteome Res, 2018.
- 27 32. Karosiene, E., et al., *NetMHCcons: a consensus method for the major*
28 *histocompatibility complex class I predictions*. Immunogenetics, 2012. **64**(3):
29 p. 177-86.
- 30 33. Ebert, L.M., et al., *The regulatory T cell-associated transcription factor*
31 *FoxP3 is expressed by tumor cells*. Cancer Res, 2008. **68**(8): p. 3001-9.
- 32 34. Alexandrov, L.B., et al., *Signatures of mutational processes in human cancer*.
33 Nature, 2013. **500**(7463): p. 415-21.
- 34 35. Pitcovski, J., et al., *Melanoma antigens and related immunological markers*.
35 Crit Rev Oncol Hematol, 2017. **115**: p. 36-49.
- 36 36. Simpson, A.J., et al., *Cancer/testis antigens, gametogenesis and cancer*. Nat
37 Rev Cancer, 2005. **5**(8): p. 615-25.
- 38 37. Vigneron, N., et al., *Database of T cell-defined human tumor antigens: the*
39 *2013 update*. Cancer Immun, 2013. **13**: p. 15.
- 40 38. Ritz, D., et al., *Data-Independent Acquisition of HLA Class I Peptidomes on*
41 *the Q Exactive Mass Spectrometer Platform*. Proteomics, 2017. **17**(19).
- 42 39. Gloger, A., et al., *Mass spectrometric analysis of the HLA class I peptidome of*
43 *melanoma cell lines as a promising tool for the identification of putative*
44 *tumor-associated HLA epitopes*. Cancer Immunol Immunother, 2016. **65**(11):
45 p. 1377-1393.
- 46 40. Vita, R., et al., *The Immune Epitope Database (IEDB): 2018 update*. Nucleic
47 Acids Res, 2019. **47**(D1): p. D339-D343.
- 48 41. Anaya, J., *OncoLnc: linking TCGA survival data to mRNAs, miRNAs, and*
49 *lncRNAs*. PeerJ CompSci, 2016. **2**(e67).

- 1 42. Vigneron, N., et al., *Peptide splicing by the proteasome*. J Biol Chem, 2017.
2 **292**(51): p. 21170-21179.
- 3 43. Platteel, A.C.M., et al., *An Unexpected Major Role for Proteasome-Catalyzed*
4 *Peptide Splicing in Generation of T Cell Epitopes: Is There Relevance for*
5 *Vaccine Development?* Front Immunol, 2017. **8**: p. 1441.
- 6 44. Liepe, J., H. Ovaa, and M. Mishto, *Why do proteases mess up with antigen*
7 *presentation by re-shuffling antigen sequences?* Curr Opin Immunol, 2018.
8 **52**: p. 81-86.
- 9 45. Hanada, K., J.W. Yewdell, and J.C. Yang, *Immune recognition of a human*
10 *renal cancer antigen through post-translational protein splicing*. Nature,
11 2004. **427**(6971): p. 252-6.
- 12 46. Vigneron, N., et al., *An antigenic peptide produced by peptide splicing in the*
13 *proteasome*. Science, 2004. **304**(5670): p. 587-90.
- 14 47. Warren, E.H., et al., *An antigen produced by splicing of noncontiguous*
15 *peptides in the reverse order*. Science, 2006. **313**(5792): p. 1444-7.
- 16 48. Dalet, A., et al., *An antigenic peptide produced by reverse splicing and double*
17 *asparagine deamidation*. Proc Natl Acad Sci U S A, 2011. **108**(29): p. E323-
18 31.
- 19 49. Michaux, A., et al., *A spliced antigenic peptide comprising a single spliced*
20 *amino acid is produced in the proteasome by reverse splicing of a longer*
21 *peptide fragment followed by trimming*. J Immunol, 2014. **192**(4): p. 1962-71.
- 22 50. Ebstein, F., et al., *Exposure to Melan-A/MART-126-35 tumor epitope specific*
23 *CD8(+)T cells reveals immune escape by affecting the ubiquitin-proteasome*
24 *system (UPS)*. Sci Rep, 2016. **6**: p. 25208.
- 25 51. Kawakami, Y., et al., *Identification of the immunodominant peptides of the*
26 *MART-1 human melanoma antigen recognized by the majority of HLA-A2-*
27 *restricted tumor infiltrating lymphocytes*. J Exp Med, 1994. **180**(1): p. 347-52.
- 28 52. Gillet, L.C., et al., *Targeted data extraction of the MS/MS spectra generated*
29 *by data-independent acquisition: a new concept for consistent and accurate*
30 *proteome analysis*. Mol Cell Proteomics, 2012. **11**(6): p. O111 016717.
- 31 53. Tate, S., et al., *Label-free quantitative proteomics trends for protein-protein*
32 *interactions*. J Proteomics, 2013. **81**: p. 91-101.
- 33 54. Croft, N.P., A.W. Purcell, and D.C. Tscharke, *Quantifying epitope*
34 *presentation using mass spectrometry*. Mol Immunol, 2015. **68**(2 Pt A): p. 77-
35 80.
- 36 55. Schittenhelm, R.B., et al., *Human Leukocyte Antigen (HLA) B27 Allotype-*
37 *Specific Binding and Candidate Arthritogenic Peptides Revealed through*
38 *Heuristic Clustering of Data-independent Acquisition Mass Spectrometry*
39 *(DIA-MS) Data*. Mol Cell Proteomics, 2016. **15**(6): p. 1867-76.
- 40 56. Caron, E., et al., *An open-source computational and data resource to analyze*
41 *digital maps of immunopeptidomes*. Elife, 2015. **4**.
- 42 57. Faridi, P., R. Aebersold, and E. Caron, *A first dataset toward a standardized*
43 *community-driven global mapping of the human immunopeptidome*. Data
44 Brief, 2016. **7**: p. 201-5.
- 45 58. Ritz, D., et al., *Membranal and Blood-Soluble HLA Class II Peptidome*
46 *Analyses Using Data-Dependent and Independent Acquisition*. Proteomics,
47 2018. **18**(12): p. e1700246.
- 48 59. Basham, T.Y., et al., *Interferon increases HLA synthesis in melanoma cells:*
49 *interferon-resistant and -sensitive cell lines*. Proc Natl Acad Sci U S A, 1982.
50 **79**(10): p. 3265-9.

- 1 60. Tumeh, P.C., et al., *PD-1 blockade induces responses by inhibiting adaptive*
2 *immune resistance*. Nature, 2014. **515**(7528): p. 568-71.
- 3 61. Liepe, J., et al., *The 20S proteasome splicing activity discovered by SpliceMet*.
4 PLoS Comput Biol, 2010. **6**(6): p. e1000830.
- 5

1 **Figure legends:**

2 *Figure 1: Experimental Workflow & consensus-binding motifs*

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

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

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

24

25 *Figure 2: Exposure to IFN γ largely alters the presented immunopeptidome*

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

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

5

6 *Figure 3: Identified cancer-specific peptides*

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

13

14 *Figure 4: Immunogenicity of identified melanoma-associated epitopes*

15 Selected melanoma-associated peptides were pooled and incubated at 10 μ M final
16 concentration with PBMC from healthy donors (n=4) or melanoma patients (n=6) for
17 10 days in presence of IL-2. All patients were HLA-A2⁺, with the exception of
18 Melanoma patient 6, which was a HLA-A2⁻ control. On day 10, CD8⁺ T lymphocytes
19 were re-stimulated with the individual peptides, or pools of those peptides which were
20 up/down regulated or unchanged with IFN γ as indicated, in presence of brefeldin A.
21 Cells were labeled with fluorescent antibodies for surface (CD3, CD8) and
22 intracellular (TNF α) proteins, and analysed by flow cytometry. Data show the
23 percentage of TNF α ⁺ CD8⁺ T lymphocytes in response to each peptide combined (A)
24 or as individual values (B). Representative plots from melanoma patient 2 are shown
25 for negative (DMSO) and positive (FEC) controls, and 2 positive and 1 negative

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

3

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

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

9

10 *Figure 6: Immunogenicity of cis-spliced peptide pools*

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

16

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

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

- 1 HLA-A2 epitope served as negative and positive control respectively.* denotes
- 2 immunogenic peptides.
- 3
- 4

1 **Supplementary Figures:**

2

3 *Supplementary Figure 1: Peptide length*

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

7

8 *Supplementary Figure 2: iRT retention time predictor*

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

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

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

18

19 *Supplementary Figure 3: Multiple charge states*

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

24

25

1 *Supplementary Figure 4*

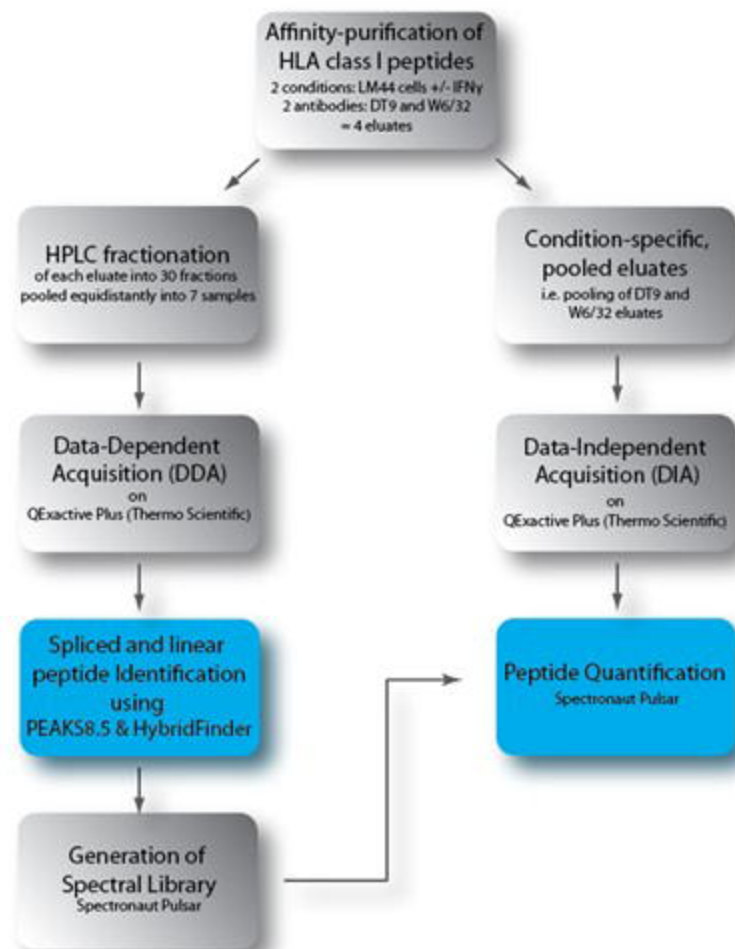
2 Using the TCGA-SKCM and FM-AD datasets looking at nevi and melanomas, the top
3 and bottom quartiles of samples expressing IP or cP subunits, and CD3g were
4 identified, and the top quartile of the CD3g expressing samples were removed. The
5 remainder of the top and bottom quartiles of samples expressing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

7

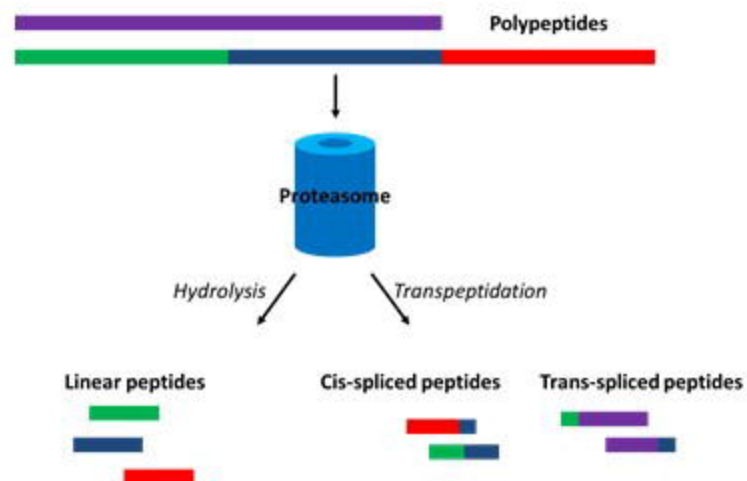
- 1 **Supplementary Tables:**
- 2 **Table 1: All linear and cis-spliced peptides identified**
- 3 **Table 2: NetMHCpan predicted class I binders from LM-MEL-44 HLAs**
- 4 **Table 3: mutated peptides based on LM-MEL-44 sequencing data**
- 5 **Table 4: DIA-MS quantified 9799 peptides**
- 6 **Table 5: 96 MAAs**
- 7 **Table 6: linear peptides tested in immunogenicity assay**
- 8
- 9

FIGURE 1

A



B



C

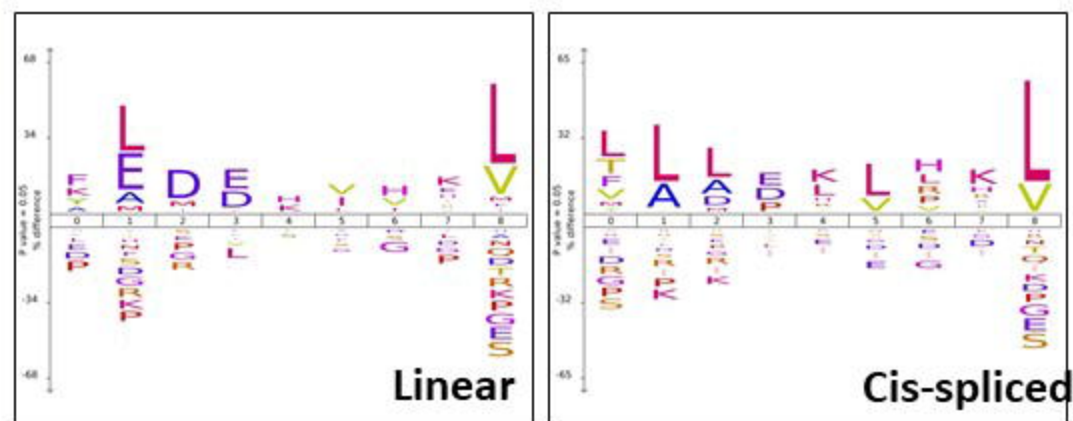
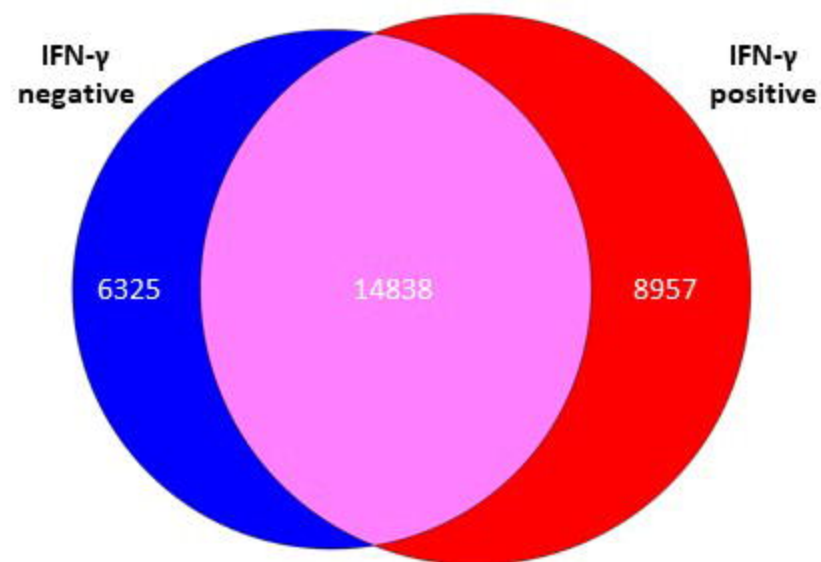
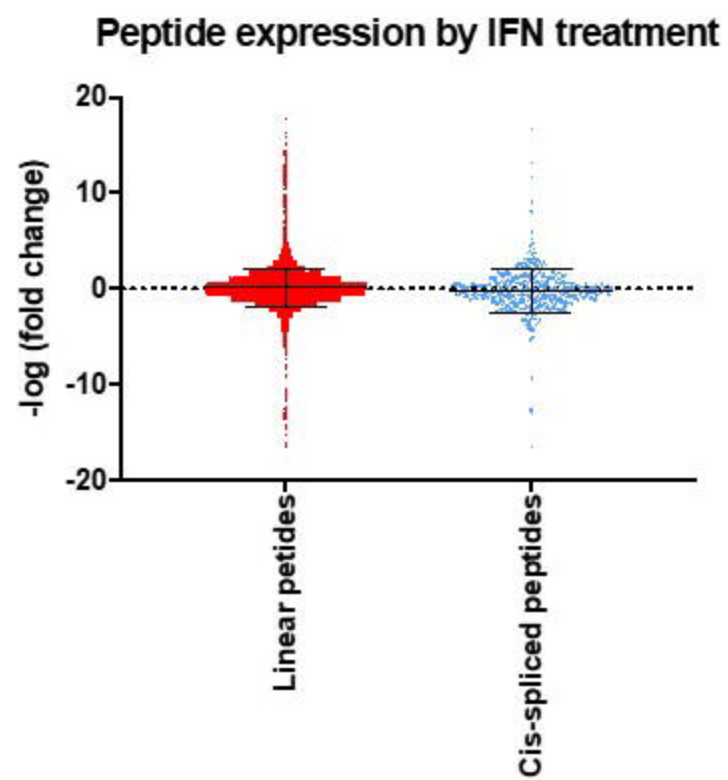


FIGURE 2

A



B



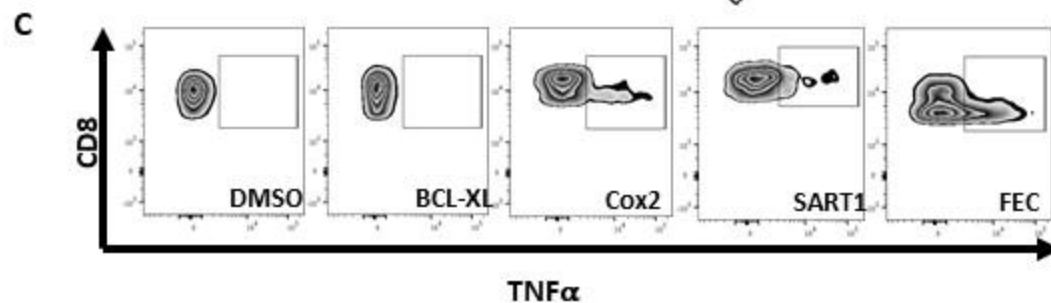
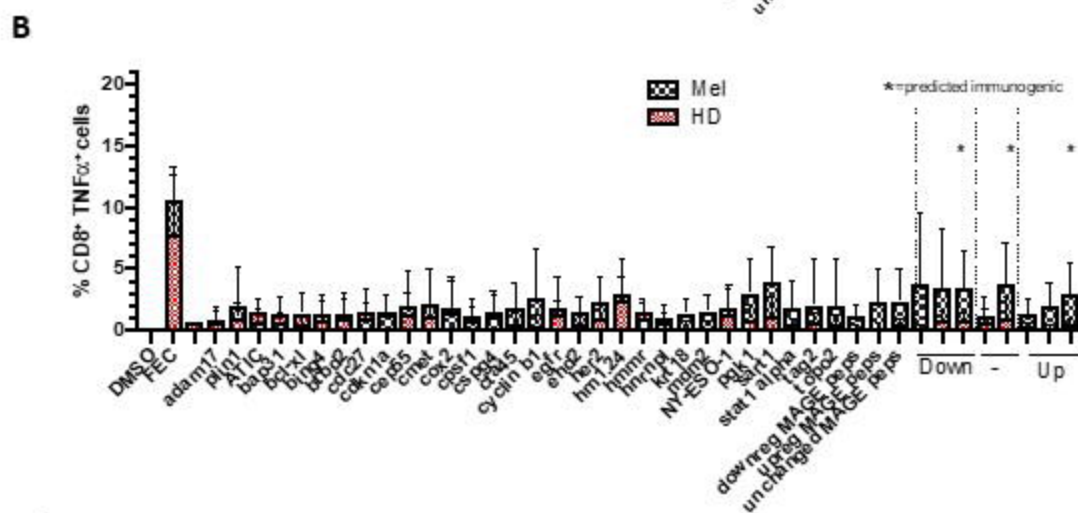
[illegible]

Figure 5

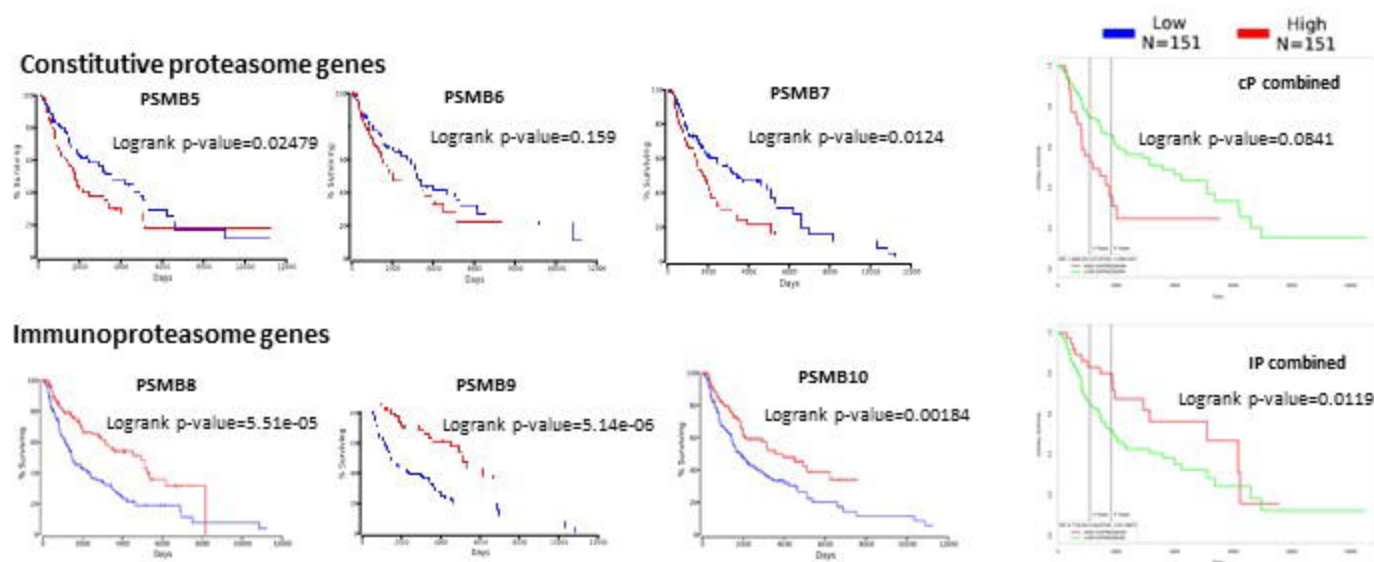
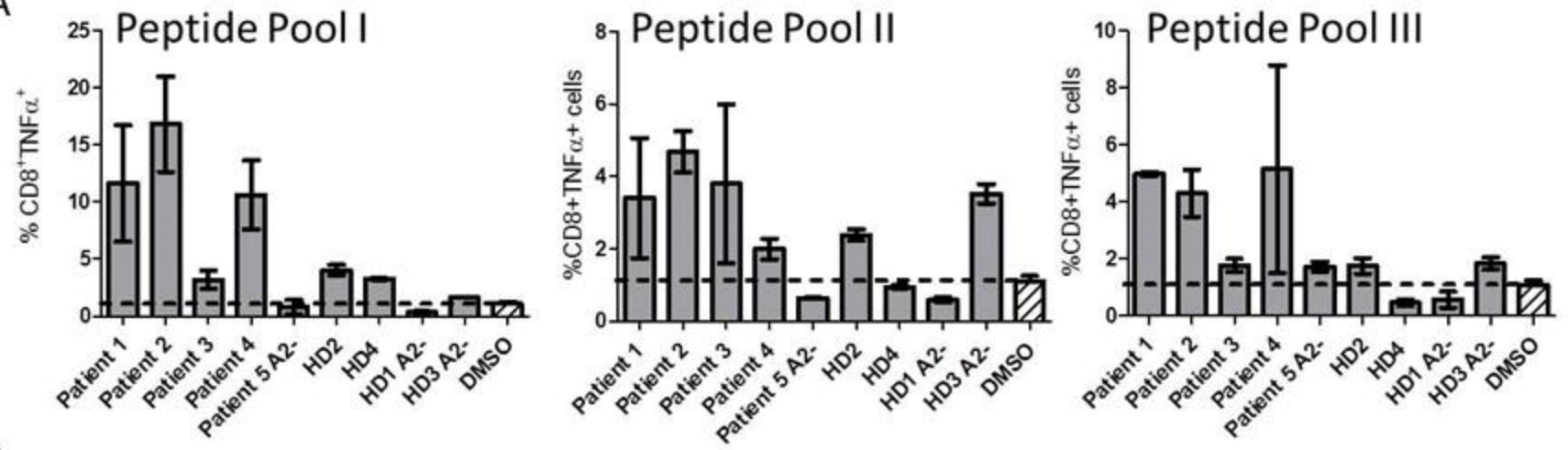


Figure 6

A



B

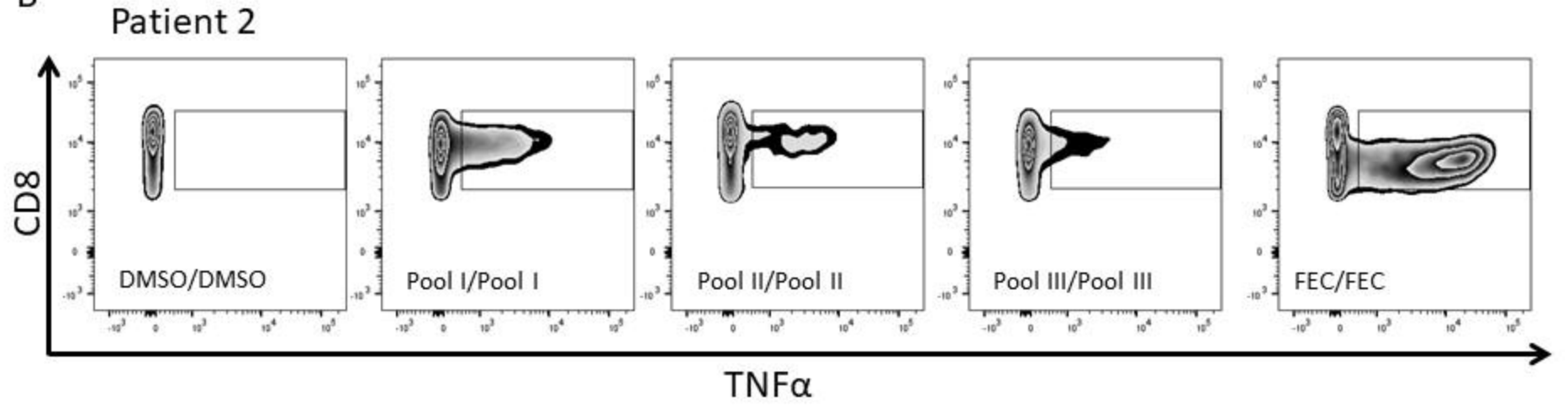


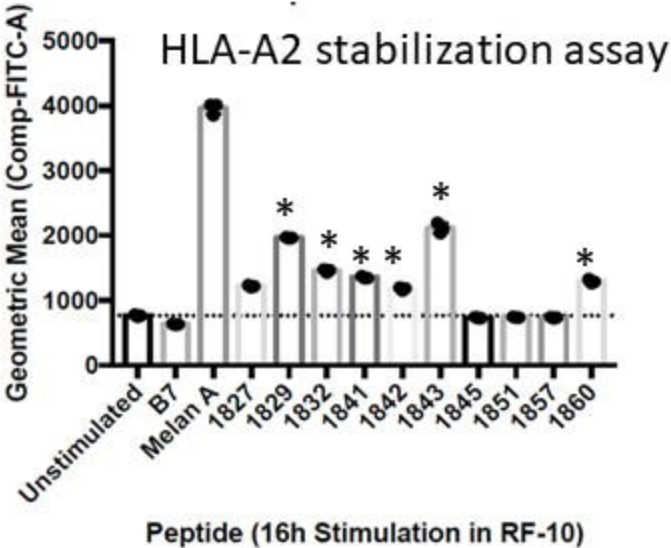
Figure 7

A

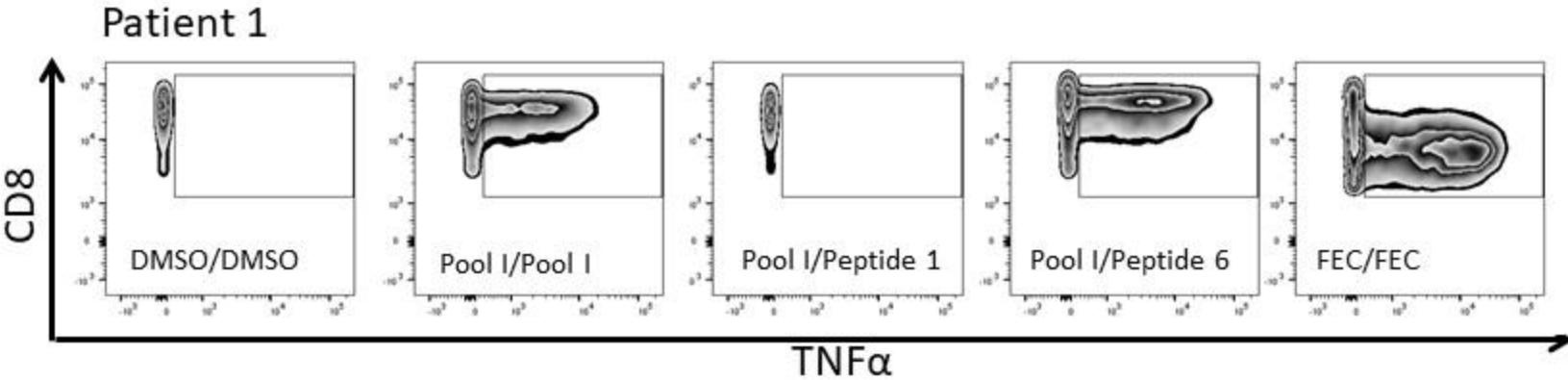
Pool I	Pool II	Pool III
FIMDKHFSV	FIHPLLL	VLDEVVVH
FLWEILERL	VALEHVVRV	VTDFLSHL
KLLILELHV (1829)*	LLSLLPAL (1841)*	LLALRILSL
LIASFLDKV	LLSLLPAL (1842)*	LLAIRLISI
AIMTAVVKI	ILSLIPAL (1843)*	LLALRLLSL
LILGLLTKV (1832)*	LLSLLPAL	LLLPLHEVL
VLTDILHTL	LAIQLKTL	LLLEALEQL (1860)*
KLTSLNIV	EEVPAAESRKY	LVPPPPPLL
MLTEKQHLL	VADLQRTL	

*denotes immunogenicity based on CD8⁺/TNF α ⁺ in at least 1 assay

C

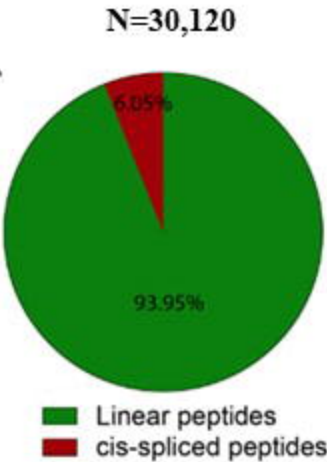


B



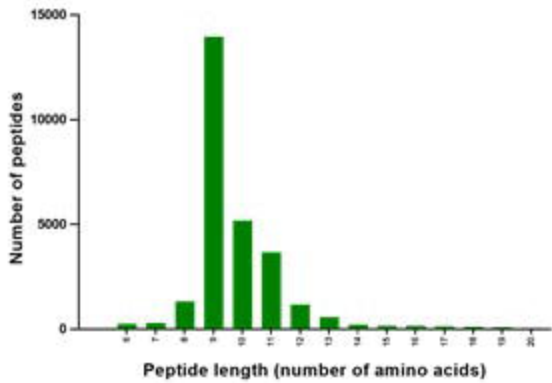
SUPPLEMENTARY FIGURE 1

A

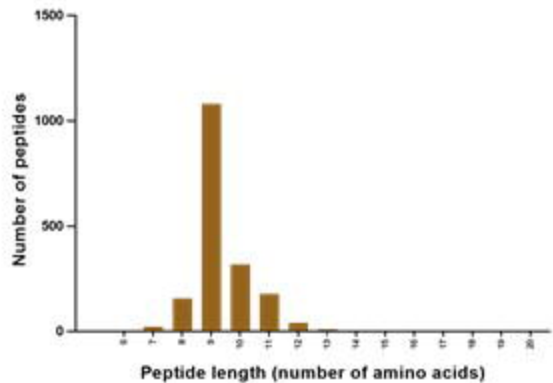


B

Linear peptides



Spliced peptides

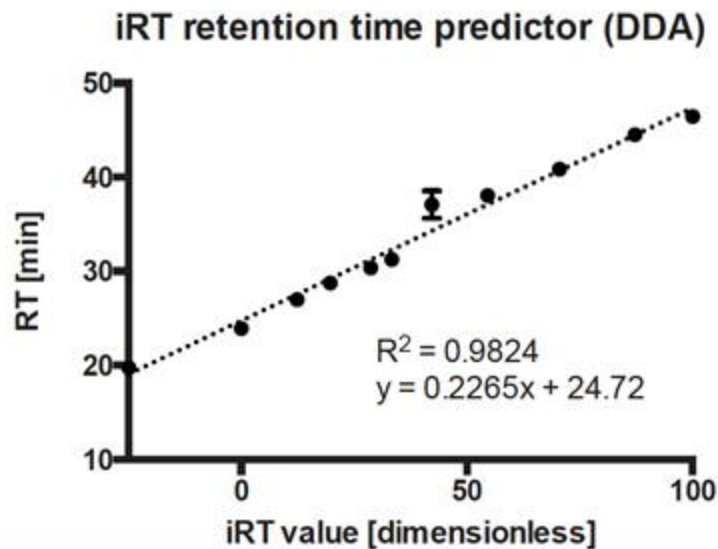


SUPPLEMENTARY FIGURE 2

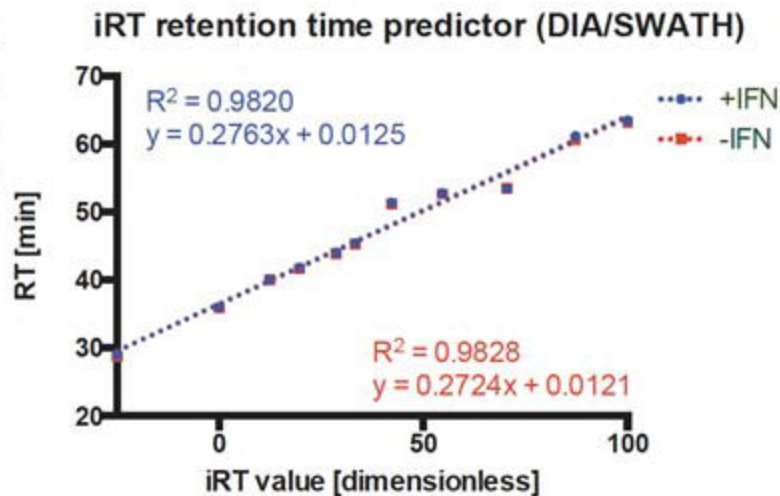
A

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	VEATFGVDESNK	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	DGLDAASYAPVR	42.26	37.09	1.47
iRT-H	ADVTPADDFSEWSK	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

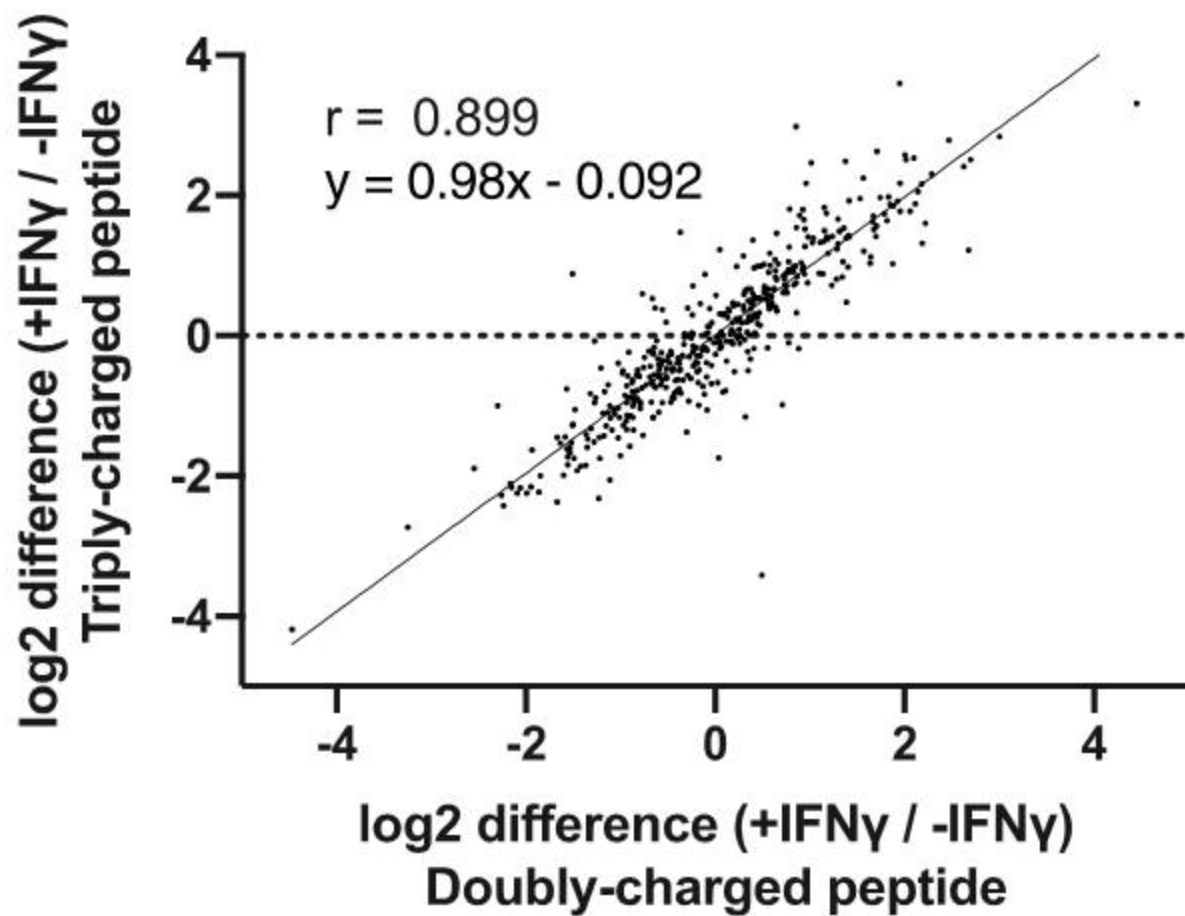
B



C

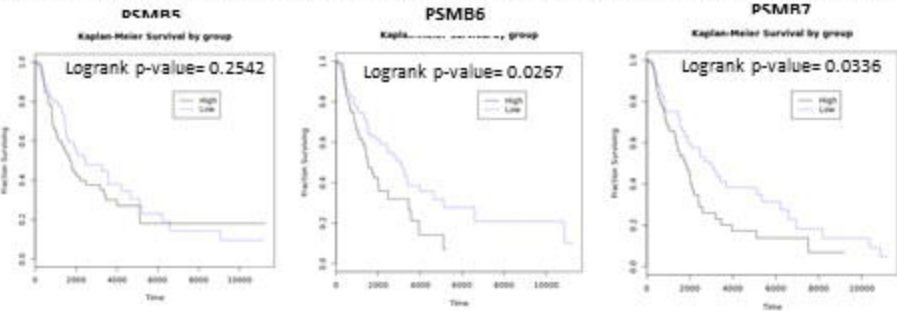


Supplementary Figure 3



Supplementary Figure 4

Constitutive proteasome genes – top quartile of CD3 expressing samples removed



Immunoproteasome genes – top quartile of CD3 expressing samples removed

